

JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS

Volume 32, No. 3, May - June, 2018

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EDITORIAL

DICLOFENAC EPOLAMINE TOPICAL PATCH FOR THE TREATMENT OF PAIN

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Received January 19, 2018 – Accepted April 11, 2018

Topical nonsteroidal anti-inflammatory drugs produce local pain relief while avoiding systemic adverse events, thanks to minimal systemic absorption. This review evaluates the effectiveness and safety of a topical diclofenac preparation, diclofenac epolamine (DHEP) patch 1.3% or diclofenac epolamine patch with heparin as excipient (DHEP+H) in treating mild-to-moderate pain. DHEP patch was associated with significant pain relief and improved function in numerous pain conditions, from minor soft tissue injuries to osteoarthritis and myofascial pain syndromes. Tolerability was good-to-excellent in all studies, with no serious adverse events. DHEP+H further improved efficacy without affecting tolerability. This patch is effective and safe for localized mild-to-moderate somatic pain.

Nonsteroidal anti-inflammatory drugs (NSAIDs), the most frequently used compounds worldwide for pain treatment, exert their anti-inflammatory action by decreasing prostaglandin production through inhibition of cyclooxygenase (COX) enzymes and modulating release and uptake of arachidonic acid. Oral nonselective and selective COX-2 inhibitor NSAIDs are highly effective in numerous inflammatory pain conditions, but bear a high risk of adverse events (AEs), mainly gastrointestinal (GI), renovascular and/or cardiovascular, which is particularly problematic for elderly people and patients with comorbidities. In addition, long-term oral NSAID therapy also raises concerns about hepatic function abnormalities (1). Diclofenac, a commonly-employed NSAID inhibiting both COX-1 and COX-2 isoenzymes, is effective in numerous pain conditions with oral or parenteral administration but, as is the case with all NSAIDs, can produce significant

AEs, including elevations of aminotransferases (approximately 15% of the cases) (1).

Topical NSAID formulations, including topical diclofenac, have therefore been developed to produce clinically significant pain relief while avoiding systemic AEs and drug-drug interactions (DDIs), thanks to high local concentration and minimal systemic drug absorption (e.g., incidence of hepatic enzyme elevation three times lower with topical than with oral diclofenac). Their efficacy and safety have been demonstrated in several mild-to-moderate pain conditions (2, 3). IBSA Institut Biochimique SA (Lugano, Switzerland) produced and patented a diclofenac epolamine topical gel before developing the 1.3% diclofenac epolamine (DHEP) topical patch (3). The DHEP patch was approved in Europe in 1993 and in the United States (US) in 2007 (Flector[®] Patch) for acute pain from minor strains, sprains, and contusions. DHEP

Key words: nonsteroidal anti-inflammatory drugs, diclofenac epolamine patch, mild-to-moderate pain, inflammation, soft tissue injury

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0393-974X (2018)

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patch is marketed in the European Union under several registered brand names, including Flector Tissugel®, Flector® EP Tissugel, Flector® cerotto medicato, Flector® EP Plaster, Flector® EP náplast', Dicloplast®, Dicloream® cerotto medicato, Flector® EP tapasz. More recently, a patch of diclofenac epolamine with heparin as excipient (DHEP+H) has also been developed. This paper reviews the studies conducted on DHEP patch and DHEP+H patch in numerous pain conditions since their appearance on the market, with particular emphasis on recent data.

DICLOFENAC EPOLAMINE TOPICAL PATCH

The DHEP 10x14 cm patch consists of an adhesive inner layer (poultice) containing 180 mg of 1.3% diclofenac epolamine in a polymeric hydrogel, covered by an outer layer of non-woven polyester felt backing aimed at preventing hydrogel drying and at hydrating the skin under the patch during perspiration. The whole patch is covered by a polypropylene film-release liner to be removed before use. Applied to intact skin, the hydrogel promotes a gradual, sustained and constant drug release into the tissue for up to 12-24 hours (3, 4). Unlike creams/gels, requiring multiple applications, the patch delivers a defined dose to a predetermined skin area over 12–24 hours (conventional dose: once or twice/daily), improving patient compliance (5). It provides local analgesic, anti-inflammatory, and anti-edematous effects, with very limited systemic absorption (4, 5).

Pharmacologic properties

Diclofenac in its acidic form is a hydrophobic molecule, but its epolamine salt, diclofenac epolamine (also known as diclofenac hydroxyethylpyrrolidine; 2-[(2,6-dichlorophenyl)amino]-benzene acetic acid, *N*-(2-hydroxyethyl-(pyrrolidine) salt) is highly soluble both in water and in non-polar solvents. Epolamine is thus a drug carrier without intrinsic pharmacological activity, used to form a diclofenac salt and improve hydrophilicity (3). Diclofenac plasma concentrations are significantly lower after DHEP patch application than oral administration of diclofenac sodium enteric-coated tablets, with

a lack of systemic effects (4). After DHEP patch application, q12h for 4 consecutive days, diclofenac plasma levels were well below those reached after single-dose oral diclofenac 50 mg. Maximal drug concentration (C_{max}) at steady state with DHEP patch was 1.7 ± 0.9 ng/mL, vs 1214 ± 750 ng/mL after oral diclofenac (6). The area under the plasma concentration curve (AUC) at 0–12 hours (AUC_{0-12}) was 15.2 ± 6.9 ng•h/mL with DHEP patch at steady state, vs 1754 ± 1060 ng•h/mL with oral diclofenac. Systemic exposure at steady state after DHEP patch was over 99% lower than after a single 50 mg oral diclofenac dose, indicating absence of systemic effects. The dose-adjusted relative percent bioavailability of diclofenac after patch treatment vs diclofenac was $0.7\% \pm 0.4\%$ (6). Bioequivalence of topical patch and gel formulations of diclofenac epolamine is shown by the AUC_{0-12} values in human volunteers (5). Diclofenac plasma concentrations with DHEP patch 1.3% were comparable to those achieved with equivalent concentration of diclofenac epolamine 1% topical gel, and plasma half-life ($t_{1/2}$) was also similar (approximately 26 hours). However, diclofenac absorption was more prolonged from the patch compared with topical gel (t_{max} 5.4 vs 3.1 hours). The local action of DHEP patch is demonstrated by the onset of significant clinical pain relief vs placebo 3 hours after application, when no diclofenac is predicted to be in plasma (7) (first plasma diclofenac appearance: 4.5 hours after single DHEP patch application for 12 hours) (4). The high local tissue penetration of diclofenac from DHEP was also shown in knee osteoarthritis (4) and in subjects without spontaneous pain (8). In the latter, measurement of pain thresholds to electrical (ETs) and periarticular pain thresholds (PPTs) to pressure stimulation in somatic tissues showed selective hypoalgesic effects in muscles produced by DHEP, i.e., thresholds were raised to an extent proportional to that of tissue pain sensitivity in basal conditions (i.e., higher effects in hyperalgesia) (8). DHEP patch also does not interfere with concurrent pharmacologic treatment in patients with comorbidities, an aspect of great importance as DDIs between coadministered medications are easily neglected by clinicians. DDIs resulting from pharmacokinetic and pharmacodynamic interactions

have been reported when oral/parenteral NSAIDs are coadministered with commonly used medications such as antihypertensives, antithrombotics, antidepressants, and corticosteroids, but this risk is negligible with plaster use (1).

Efficacy

Minor soft tissue injuries / musculoskeletal inflammatory pain

In minor traumatic sport and overload injuries significant decreases in spontaneous pain from baseline and vs placebo were shown with DHEP from day 1, with functional improvement, improvements in freedom of movement, resolution of pain and tenderness, and favorable patient- and investigator-rated Global Assessment of efficacy. Tolerability was good/excellent, the most common AEs being mild local skin reactions at the application site, similar with DHEP patch and placebo (3-5, 8-13), probably related to vehicle rather than active ingredient. Incidence and severity of GI AEs was similar in DHEP and placebo groups (<2% of patients) (14).

In mild-to-moderate pain from ankle sprain occurring within the previous 48 hours, DHEP patch once-daily for 7 days reduced pain on movement (VAS scale, at 3 hours with sustained reduction at days 3 and 7), pain at rest, on passive stretch and palpation and ankle swelling and improved the possibility of single foot leaning (7). Treatment was judged more effective than placebo at days 3 and 7 and tolerability was rated as excellent by most patients and investigators, AEs being primarily mild-to-moderate local skin reactions. After DHEP patch, the onset of significant pain reduction occurs early (1.27 hours), a crucial aspect in acute pain treatment (15). A pooled analysis on data from 1,185 patients with minor soft tissue injuries confirmed the overall analgesic effect of DHEP patch, particularly in shoulder, lower leg, elbow and back, and its favorable safety and tolerability profile (3).

DHEP patch twice-daily for 7 days in minor soft tissue injuries produced a higher change in pain on movement (VAS) vs baseline than placebo; evident from day 1, differences became progressively greater throughout treatment. Both DHEP patch and placebo were well tolerated, the few AEs were

mostly application site reactions (16).

Pooled data analysis from 5 studies on 1,165 patients with acute minor soft tissue injuries and inflammatory pathologies showed greater improvements from baseline to day 14 in pain (VAS) from injuries of the back, elbow, shoulder, and foot with DHEP (twice-daily for 14 days) compared with placebo (17). DHEP patch tolerability ratings by patients and investigators were comparable to placebo, except for leg pain (investigator-rated DHEP patch tolerability better than placebo). The most common AEs were pruritus and other application site reactions (17).

In patients with various inflammatory conditions both DHEP patch 1.3% (twice-daily) and diclofenac diethylammonium emulgel 1.16% (4 times daily) produced rapid pain decrease (18). However, significantly more patients and physicians reported superior efficacy with DHEP patch than with diclofenac diethylammonium emulgel (constant diclofenac release from the patch vs 4 applications/day with gel). Both treatments were well tolerated; 2.6% of the patients showed transient mild pruritus or erythema.

Twice-daily DHEP patch was significantly more effective than placebo in decreasing pain on motion, functional impairment and local inflammation in inflammatory peri- and extra-articular rheumatological diseases and produced almost complete resolution of swelling from inflamed tendons with the 2-week treatment, with good tolerability (19).

DHEP patch every 12 hours for 7 or 14 days was effective and well tolerated in acute back pain from nonradicular back strain (20).

Osteoarthritis

Topical NSAIDs are recommended by the American College of Rheumatology for the initial management of knee OA (21).

A pooled analysis of two randomized, double-blind clinical studies involving 235 patients with symptomatic knee osteoarthritis comparing DHEP patch to placebo showed a mean pain decrease of 59.5% with DHEP patch vs 29.9% with placebo, with an estimated treatment effect in favor of DHEP patch

equivalent to a reduction of 21.9% in the Lequesne's index and 30% in pain intensity. DHEP patch also reduced periarticular hyperalgesia (22).

In bilateral knee osteoarthritis of comparable extent, 24-hour DHEP application (two patches/day) on one knee *vs* placebo on the contralateral knee significantly reduced pain at rest and on movement and periarticular hyperalgesia *vs* baseline (increasing PPTs) (23). In a longer-term trial (72-hour treatment) DHEP patch, but not placebo, showed prolonged effects by significantly decreasing pain at rest and on movement and periarticular hyperalgesia (increased PPTs and electrical subcutis and muscle pain thresholds) progressively during treatment and up to 96 hours after its suspension, without AEs (23).

Myofascial trigger points in headache

Myofascial trigger points (TrPs) are sites of extreme tenderness in muscles or their fascia, localized within taut bands of muscle fibers which are tender on compression and refer pain at a distance (target area). Mainly caused by microtraumatic events, they are an area of focal contracture and local release of inflammatory mediators. When active, they produce regional musculoskeletal pains termed myofascial pain syndromes. TrPs can also act as peripheral pain generators in other pain conditions, such as headache (24). Migraine attacks become more frequent and intense when cervical TrPs have targets coinciding with migraine sites. Local treatment of these TrPs with intramuscular anesthetic injection (gold standard procedure) in migraineurs not only improves myofascial symptoms and desensitizes soft tissues at target level (increasing pain thresholds), but also decreases migraine symptoms (number/intensity of attacks, rescue medication intake) (24), probably due to decreased nociceptive input from the TrP towards central neurons transmitting migraine pain. As TrP injection is an invasive procedure, a study with DHEP patch as TrP treatment in migraineurs with concurrent TrPs evaluated whether this approach would have similar effects on headache symptoms as the injection, but with higher acceptability to the patients (25). Migraineurs (≥ 6 attacks/month) with cervical TrPs whose targets coincided with migraine sites (unilateral frontal and/or temporal) received a quarter DHEP

patch [7x5 cm] over the most active TrP, twice-daily every 3 days for 60 days, or no TrP treatment. All had intolerance/scarcely responsiveness to classic migraine treatments, necessitating alternative therapies. Skin, subcutis, and muscle electrical thresholds (ETs) in trigger and target were significantly lower in patients than healthy subjects at baseline. In treated but not untreated patients, all thresholds significantly increased after 60 days, maintained until day 180. The reduction of attack number and increase in muscle threshold in the target were directly and linearly correlated. No side effects or discomfort were reported. DHEP patch is thus useful in treating migraine with the described characteristics; it could be particularly indicated in cases where standard migraine therapies cannot be employed, but also as an adjunct to classic treatments to enhance therapeutic outcomes in all migraineurs with comorbid TrPs.

Safety

A comprehensive analysis published in 2008 on clinical trial data on DHEP patch showed that AEs occurred in about 10% of patients overall (3). These were all minor, mostly local skin reactions (usually pruritus). GI reactions occurred in <1.5% of patients, none involving ulceration and bleeding. In contrast to oral diclofenac, no elevation of liver enzymes was reported. Later clinical trials have confirmed these favorable data.

DICLOFENAC EPOLAMINE PATCH + HEPARIN

A new medicated plaster contains 180mg diclofenac epolamine and heparin sodium 5600IU as excipient, acting as a permeability enhancer, increasing release and skin penetration of the drug (IBSA Internal Data – Pharmaceutical Development).

DHEP+H patch proved more effective than placebo in acute ankle sprains with edema, with greater reduction of joint swelling and pain within 3 hours of first application, without significant AEs (26). In mild-to-moderate muscle contusions and sprains complicated by hematoma, both DHEP+H and DHEP patches for at least 12 hours/day for 10 days produced greater reduction of pain on movement

than placebo and were well tolerated (27). However, the time to complete hematoma dissolution was shorter and the success rate in achieving dissolution higher with DHEP+H vs both DHEP and placebo.

Both DHEP and DHEP+H patches (once-daily for 7 days) produced greater pain relief than placebo in mild-to-moderate ankle sprain. However, reductions from baseline of pain on movement after 3 days and of edema on day 7 were greater with DHEP+H than with DHEP patch. AEs for active treatments were similar to placebo (28).

In mild-to-moderate muscle contusions both DHEP+H and DHEP patches daily (for ≥ 20 hours) for 14 days resolved pain more rapidly than placebo (with DHEP+H superior to DHEP). Reduction in pain on movement after 3 days was higher with DHEP+H patch than DHEP. Both active treatments showed a placebo-like safety profile (29).

DHEP+H patch was also studied in conditions where spontaneous pain is absent, such as asymptomatic muscle hyperalgesia resulting from previous traumas/microtraumas (30). In a prospective, double-blind, four-arm trial, 104 subjects (84 women, 20 men, 42.2 ± 13.3 years) with muscle hyperalgesia in one thigh were randomized to one of four groups ($n=26$ in each) for the following patch treatments: DHEP+H, DHEP, heparin, or placebo for 7 days (patches replaced every 24 hours). Pressure and electrical pain thresholds of vastus lateralis and overlying subcutis and skin, and structure/thickness of subcutis and muscle on ultrasound were measured before treatment (day 1), and at days 4 and 8. Thresholds did not change significantly with placebo and heparin (apart from a slight increase in subcutis), but all significantly increased with DHEP and DHEP+H patches. Muscle ETs increased significantly more with DHEP+H than with DHEP, heparin, and placebo, while no edema/thickness changes in muscle and subcutis were detected on ultrasound. Mild-to-moderate local skin reactions occurred in three subjects: erythema alone (heparin, $n=1$), erythema with pruritus (heparin, $n=1$), and erythema with a burning sensation (placebo, $n=1$). All spontaneously resolved after patch removal. No systemic/serious AEs occurred in any group (30). DHEP+H patch thus reduces deep

somatic hyperalgesia significantly more than DHEP patch alone in subjects without spontaneous pain, independent of the anti-edematous action of heparin, indicating that heparin addition to diclofenac in a patch could be useful for managing pain conditions even in the absence of objective signs of injury/edema/hematoma.

CONCLUSIONS

The evidence for DHEP patch use in treating mild-to-moderate acute somatic pain from strains, sprains, and contusions is robust. In addition, DHEP patch also demonstrates potential in other pain conditions, including osteoarthritis, tendinopathies, rheumatologic and myofascial pain syndromes and, indirectly, migraine associated with cervical myofascial trigger points. The twice-daily application (a few days–14 days) is advantageous compared to other topical NSAID preparations requiring multiple daily application, which contributes to better patient compliance. Pain relief occurs early (within the first hour) and persists after removal of the last patch. The addition of heparin as excipient to DHEP patch seems to further enhance efficacy, particularly, but not exclusively, in cases of edema.

AEs from DHEP patch are infrequent and minor, mostly local reactions. The limited systemic absorption of diclofenac from the patch and the lack of interference with concurrent drugs contribute to the formulation's good tolerability profile. The patch may be preferable to the oral formulation, especially in cases of high risk of systemic side effects from NSAIDs, including the elderly or patients receiving polytherapy for comorbidities.

Though ideally suited for the treatment of local conditions as described, patch therapy is less appropriate in settings where more generalized, systemic/anti-inflammatory/ analgesic effects are required. Careful clinical selection of cases to be treated is therefore essential to ensure the optimal use of this topical therapy.

ACKNOWLEDGEMENTS

IBSA Institute Biochimique made open access

possible through an unconditional grant. No other funding was received for the writing of this review. We thank IBSA Institut Biochimique for kindly providing papers to be analyzed for the present review, upon our request. We are grateful to Mrs Lesley Skeens for the English revision of the manuscript. We thank Ray Hill for additional editing and journal styling before submission on behalf of Health Publishing & Services Srl. IBSA Institut Biochimique SA funded this assistance.

DISCLOSURE: MAG has received funding from Epitech Group, Helsinn Healthcare and IBSA Institute Biochimique for research unrelated to the topic of the present paper, and honoraria from Helsinn Healthcare, IBSA Institute Biochimique, and royalties from IASP Press. GA has received honoraria from Epitech Group and Helsinn Healthcare. DL and RC report no conflict of interest in this work.

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EDITORIAL

TIME TO LOOK PAST TNF AND THALIDOMIDE FOR CACHEXIA - COULD MAST CELLS AND FLAVONOIDS BE THE ANSWER?L. KING¹, I. TSILIONI² and T.C. THEOHARIDES^{2,3,4}

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Received May 17, 2018 - Accepted June 10, 2018

Cachexia is a wasting condition associated with late stages of many chronic illnesses and may be present in up to 80% of patients with advanced cancers. Cachexia is a metabolic derangement resulting in a disturbance to the homeostasis of muscle breakdown and synthesis, favoring catabolism and muscle loss. Despite making strides in treating cancer itself, there have been no major advances in the treatment of cachexia pharmacologically or nutritionally. Clinical trials using anti-TNF biologics and thalidomide have largely failed. A new approach may be to focus on other possible waste-inducing mediators, possibly derived from mast cells, and the beneficial action of select natural flavonoids.

The Greek physician Galenos (Galen) first described Marasmus (1) to denote cachexia (from the Greek, kakos=bad and exis=condition), a wasting condition associated with extreme muscle atrophy and fatigue present in late stages of many chronic illnesses including patients with heart failure, chronic pulmonary disease (COPD), chronic kidney disease, AIDS, and particularly cancer. Cachexia may be present in up to 80% of patients with advanced cancers, especially pancreatic, gastric, lung, esophageal, colorectal, and head and neck cancers, and may be responsible for one-third of cancer deaths (2). At its core, cachexia is a metabolic derangement resulting in a disturbance of the muscle homeostasis, favoring catabolism and muscle loss (3). Despite making strides in treating cancer itself, there have been no major advances in the treatment

of cachexia pharmacologically or nutritionally.

The lack of a unified definition of cancer cachexia has hampered progress. An international consensus report described cancer cachexia as a “multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment” (4). Cachexia can be divided into three categories: pre-cachexia, cachexia, and refractory cachexia. For cancer cachexia diagnosis, one of several criteria may be used: (a) 5% of unintentional weight loss over the previous 6 months, (b) more than 2% weight loss in individuals with a BMI of less than 20 kg/m²; or (c) more than 2% weight loss in patients with evident sarcopenia.

Regardless of weight, BMI and fat mass,

Key words: TNF, thalidomide, cachexia, mast cells, flavonoids, inflammation

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0393-974X (2018)

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Table I. *Cachexia interventions from the last 10 years.*

Monotherapy	Possible Mechanism of Action
Targeted nutrition therapy ω -3 Fatty Acid	Increase levels of leukotriene and prostaglandin while suppressing the production of inflammatory cytokines
Nutrition supplement with ω -3 fatty acid, micronutrients, and probiotics	Anti-inflammatory
Essential amino acid supplement	Dietary essential amino acids have anabolic properties
L-Carnitine	Deficiency in cancer cachexia; Modulate the inflammatory response
Ghrelin	Appetite stimulation
Anamorelin	Ghrelin mimetic
Cyproheptadine hydrochloride	Appetite stimulation
Selumetinib	Anti-IL-6
Etanercept	TNF inhibitor
Ruxolitinib	Janus kinase (JAK)1/JAK2 inhibitor
MABp1	IL-1 α antibody
Carvedilol	α_1 -/ β_1 -/ β_2 -receptor antagonist
Megestrol Acetate	Appetite Stimulant
Combined Therapy	
Megestrol Acetate $\pm$ Carnitine + Celecoxib	Appetite stimulation; Carnitine supplementation for deficiency in cachexia; Anti-inflammatory
Megestrol Acetate + Thalidomide	Appetite stimulation; Anti-inflammatory, immunomodulation
Megestrol Acetate + Formoterol Fumarate	Appetite stimulation; β_2 -agonist with skeletal muscle selectivity, anabolic, anti-catabolic
Megestrol Acetate + Meloxicam	Appetite stimulation; anti-inflammation

decreased lean body mass, as seen in cachexia, is an independent risk factor for mortality in cancer. This can be ascribed to delays in treatment related to increased treatment toxicity and decreased treatment efficacy (5).

In a review of PubMed using the search term

“cachexia” and refining the results to human clinical trials within the last 10 years, 140 articles were retrieved and the abstracts of 84 were reviewed. Table I summarizes those studies demonstrating moderately improved outcomes with the designated interventions. These studies are not limited to

Table II. *Potential cachexins*

Adiponectin
Adenomedullin
Atrogins
Fat-mobilizing factor
GHRH
IGF
IL-1 β
IL-6
IL-33
Leptin
Myostatin
TNF
UCP2
Zinc- α 2-glycoprotein

GHRH=growth releasing hormone

IGF=insulin-like growth factor

patients with cancer cachexia, but also participants who were cachectic or at high risk for cachexia in other disease states. The outcomes measured range from functional improvement and increased weight/lean body mass and include a range of ages. Thematic targets include: appetite stimulation, stimulation of anabolism and anti-inflammation.

Thalidomide

Many different approaches have been attempted for the treatment of cachexia, (6, 7) but they have all failed. In 1995, the FDA approved thalidomide for the compassionate use in AIDS-associated wasting (8). While thalidomide may have anti-TNF properties, its mechanism of action in cachexia is not known. The history of thalidomide was marred by the teratogenic effects of the S(-) enantiomer leading to thousands of children with mild to severe malformations. In pre-clinical trials of the drug, mice were used to demonstrate thalidomide's safety and efficacy. However, for still unknown reasons, mice are less sensitive to the teratogenic effects of

thalidomide compared to humans and other species of pre-clinical animal models (9).

Only four clinical trials were published beginning in 2003 until 2012 using thalidomide as a mono-therapeutic intervention for cachexia (10-13). In the most recent trial, the researchers looked at thalidomide's "role in cancer-related anorexia symptoms", but with no specific cancer inclusion criteria (patients with some cancers have a higher risk of developing and quickly progressing in cachexia) and no outcomes were designated as primary or secondary endpoints (10). Moreover, there is no power calculation, and no distinct endpoint. The duration of thalidomide intervention was only 14 days, based on a previous study in which cachexia-related symptoms improved in 10 days, (14) and they used a bioelectrical impedance analysis (BIA) for body composition instead of the most accepted CT, MRI, DEXA, or mid-arm muscle area for improved accuracy (4). Within the thalidomide group, results showed significant decreases in both fat mass and fat-free mass, but no significant decrease in weight from baseline to day 15 as compared to the placebo group. Strangely, there were significant decreases in TNF and IL-8 in both the thalidomide and placebo groups, and there were additionally significant decreases in IL-6 and IL-IRA in the placebo group. An additional shortcoming of this study is the decrease in sample size from baseline to day 15 and day 29 in both groups (n=14, 9, 7; respectively) (14).

Two other thalidomide clinical trials for cancer cachexia (11, 12) and two studies in AIDS-related wasting (15, 16) showed statistically significant but minimal gains in weight. Other studies of thalidomide for both AIDS- and cancer-related cachexia repeatedly showed no significant changes in blood TNF levels between thalidomide and placebo (11, 15, 16).

Another study using the phosphodiesterase inhibitor, pentoxifylline, which also has some anti-TNF actions, showed no significant difference in weight or arm circumference compared to controls (17). While the inflammatory cytokine TNF (cachexin) is often implicated in cachexia, systemic inflammation is not always overtly present in cachexia (4).

Mast cells and flavonoids

A number of papers described the involvement of possible cachexia mediators (Table II), many of which can be secreted from mast cells, immune cells that reside in vascularized tissues with multiple diverse functions, most notably the development of allergic reactions (18). Stimulated mast cells secrete granule-stored molecules, including histamine, heparin, tryptase and TNF (19). Mast cells also secrete *de-novo* synthesized pro-inflammatory mediators, such as IL-6, IL-31, TNF (19). Mast cell mediators, such as adrenomedullin, (20) TNF and leptin (21) could participate in the pathogenesis of cancer (22-24) and cachexia.

Naturally-occurring flavonoids, quercetin and luteolin, have potent anti-oxidant, anti-inflammatory and anticancer actions (25-26). Luteolin inhibits human mast cells (27), and cancer (28). Flavonoids have also been reported to reduce cachexia in animal models (29).

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EDITORIAL

NEW CONCEPTS IN NEUROINFLAMMATION: MAST CELLS PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINE MEDIATORS

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Received May 19, 2018 - Accepted June 6, 2018

The activation of brain nociceptors and neurons may lead to neurogenic inflammation, an event that involves immune cells including mast cells (MCs). Microglia are similar to macrophages and secrete pro-inflammatory IL-1 family members and TNF. TNF is rapidly released (first 10 minutes from MC granules) and is subsequently secreted along with other pro-inflammatory cytokines with a new synthesis after several hours. MC-derived TNF is a very powerful pro-inflammatory cytokine which mediates sensitization of the meningeal nociceptors. Here, we report the involvement of MCs in neuroinflammation, the role of inflammatory cytokine IL-1 family members, and of TNF, as well as the potential inhibition of IL-37.

In the CNS, microglia and astrocytes play a protective and curative function against pathogens by producing cytokines and chemokines that promote the recruitment of circulating immune cells (1). Microglia belong to the family of myeloid cells, and represent the macrophage of the brain and are resident in the parenchyma of the CNS. In innate immunity, when microglia are activated, they show morphological changes and generate pro-inflammatory compounds. Their activation is regulated by the chemokine receptor gene CX3CR1 which represents a distress signal (2).

Innate immune responses in the brain occur during neuroinflammatory phenomena provoking vasodilation, resulting in a pathological state of brain tissue. Moreover, microglia secrete several cytokines, including IL-23 and IL-1, which in turn act on the production of GM-CSF, contributing to the inflammatory state (3).

Mast cells (MCs) participate in tissue sensitization which can be caused by the neurogenic inflammation of the meninges in cooperation with the arteries and venous sinuses, which are responsive to trigeminal pain (4). Neurotransmitters play a crucial role in

Key words: neuroinflammation, mast cells, neurons, glia, cytokines

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0393-974X (2018)

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inflammation, and cross-talk between neurons and immune cells, including MCs, may contribute to an inflammatory response.

In the CNS, the augmentation of neurotransmitter substance P (SP), which activates and provokes MC release, interacts with blood vessels, neurons, and immune cells generating neurogenic inflammation by provoking vasodilation, extravasation, and glial cell activation (5).

The nociceptive nerves release secretory vesicles containing neuropeptides such as SP and corticotropin-releasing hormone (CRH) produced by MCs. Several studies have reported that indeed mast cell-derived histamine plays a crucial role in the pathogenesis and management of CNS inflammatory response (6).

MC immune mediators interact, sensitize and activate the nociceptors of the meninges. Therefore, the immune-vascular meningeal interaction is an important mechanism for the sensitization of nociceptors.

Peripheral bacterial infections are recognized by toll-like receptors (TLRs) (located on the plasma membrane or in the endosomal compartment of the cell) and activate innate immune cells that internalize antigenic proteins, process them, place them with MHC class II on their cellular surface and instruct CD4⁺ T cells which are largely driven by IL-4 (2). The activation of TLRs leads to the up-regulation of NF- κ B with an increase of transcription of genes encoding IL-1 family cytokines and TNF. Th1, Th2, and Th17 lymphocyte effector cells, after activation, express a series of cytokines that act on the innate immune cells to fight infections.

Mast cells

MCs are born in the bone marrow from the stem cells and circulate as CD34⁺ mononuclear cells and mature in long-term resident tissues including perivascular location and CNS where they perform their functions *in situ*. They are important “first responder” cells of the innate immune system, contributing to immune response against pathogens (7). MCs possess constitutively c-kit and Fc ϵ RI (about $1 \times 10^{10} \text{ M}^{-1}$) high affinity receptor for IgE. The cross-linking of IgE with Fc ϵ RI causes the

activation of protein tyrosine kinases (Syk and Lyn) which in turn stimulate the activation of mitogen-activated protein kinase (MAPK) cascade. MAPK in MC nucleus provokes inflammatory cytokine gene expression activation including IL-33 and TNF (8).

Activated MCs lead to immediate degranulation, and release stored mediators such as histamine, heparin, tryptase, chymase, kinogenases, carboxypeptidase A3 and TNF. MCs respond to many different stimuli and are usually associated to immediate hypersensitivity and allergic disorders, as well as in certain protective immune responses to microbes and fungi which bind TLRs and lead to the production of *de novo* synthesis of pro-inflammatory cytokines (9). MCs also generate growth factors, leukotrienes, prostaglandins, and NO. MC preformed mediators include histamine, heparin, proteases (tryptase, chimase), hydrolases, cathepsin, carboxypeptidases, peroxidase and TNF (10). There are at least two subpopulations of mast cells: mast cells with tryptase and mast cells with both tryptase and chymase.

MCs, known as important cellular elements in allergic diseases, are also present in the cerebral tissue where they can release IL-1 and TNF mediators that are decisive for the inflammatory state (11). MCs reside close to primary nociceptive neurons, associate with nerves and are able to trigger local inflammation, lower the surrounding pH, sensitizes neurons and activate the release of neuropeptides including SP. The development of MCs occurs under the control of the stem cell factor (SCF) through c-kit which in pathological cases can be inhibited, leading to pathologies of the blood cells (12).

The binding of IgE to the Fc ϵ RI receptor causes receptor aggregation with activation of the cytokine/chemokine pathway and secretion of histamine and other chemical mediators (13).

The MCs present in the CNS are fixed, are activated through the Fc ϵ RI receptor in inflammatory diseases, produce tryptase and histamine, and are implicated in inflammatory diseases, but can also contribute to the protection of brain tissue. Therefore, histamine is an important mediator of MCs with sensitizing effects on nociceptors (14).

It has been observed that the TNF released by

the MCs, dura mater and pia mater, is involved in the regulation of the brain blood barrier and in the infiltration of immune cells such as T cells and myeloid cells in the brain tissue (15). Chemokine production by MCs exerts chemo attraction on other immune cells. Their cross-talk in the CNS causes an immune protection effect, but they can also cause neuroinflammation (16).

The blood vessels carry immune cells which pass through the meninges, enter the brain and modulate immunocytes trafficking in the CNS. The effect of immune cells, including MCs resident in the meninges can play a critical role in development of inflammation in many diseases (17).

MCs are found in the meninges and brain parenchyma where they participate in recruiting other immune cells and the activation of macrophages in several diseases. However, the role of meningeal MCs in modulating brain pathology is not clear.

Antigen binds to FcεRI receptors on the surface of MCs which release vasoactive mediators, chemotactic factors and cytokines that promotes leukocyte infiltration and exacerbate tissue inflammation. MC-derived cytokines/chemokines contribute to the cellular component of the late-phase response and increase inflammatory reaction

with the contribution of IL-4 generation (18). The production of IL-4 by MCs promote the development of Th2 cells that also release IL-4 (19).

TNF and neuroinflammation

Activated MCs participate in the innate and adaptive response, and function as “sentinels” during such host responses. MCs contain preformed TNF in their granules, as has been observed in experiments made with genetically MC-deficient Kit^{W/Wv} mice and the corresponding control (Kit^{+/+}) mice (19).

MCs constitutively express preformed TNF which can be quickly secreted (within 10 minutes), or they can exhibit enhanced gene expression (within hours) upon activation (7). The action of TNF is carried out through the binding to its receptor subtype TNF receptor 1 (TNFR1) expressed on the cells of the somatic and vascular nociceptors. These effects lead to the generation and release of inflammatory mediators produced by immune cells, including MCs. Therefore, TNF is a highly inflammatory cytokine produced by MCs that plays an important role in the pathogenesis of inflammatory disorders including neurogenic inflammation.

TNF quickly secreted by MC cytoplasmic granules acts on induction of adhesion molecules such

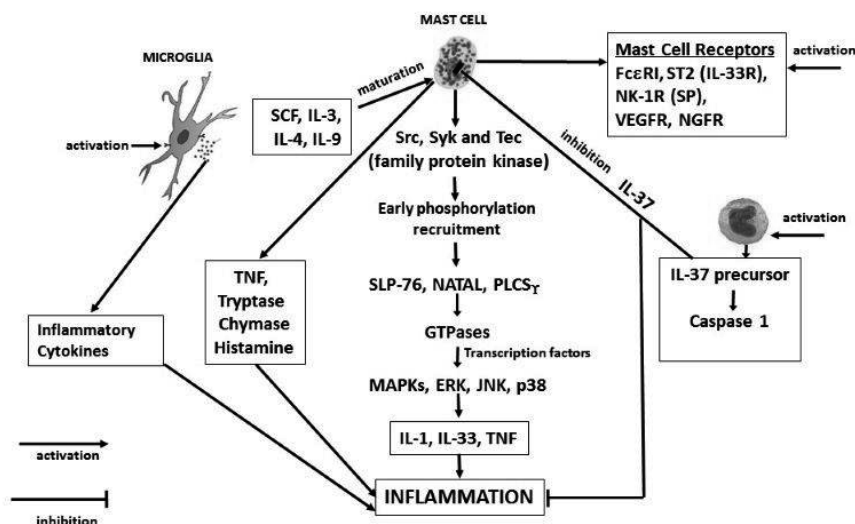


Fig. 1. Activation of microglia and mast cells in the central nervous system leads to neuroinflammation, an effect that can be potentially inhibited by IL-37 released by activated macrophages.

as ICAM-1, VCAM-1 and ELAM-1 contributing to the various inflammatory process (18).

Neuroinflammation is mediated by inflammatory and neurotoxic cytokines such as pro-inflammatory IL-1 family members (IL-1, IL-18, and IL-33), and TNF generated by immune cells. Therefore, neuroinflammation is mediated by pro-inflammatory cytokines, including TNF, generated by activated microglia, neurons, astrocytes and MCs (20) (Fig. 1). Pro-inflammatory cells that cross the blood-brain barrier release cytokines/chemokines and TNF, and activate the adhesion factors. TNF and inflammatory cytokines activate microglia and astrocytes, causing tissue inflammation. In addition, TNF can be released into the pia mater after an inflammatory event with accumulation and damage of the brain histamine (21).

Therefore, the inhibition of neuroinflammation by down-regulation of the immune cells, can have beneficial consequences, on the other hand it may have detrimental effects on infection brain tissue immune response.

IL-1

IL-1 is an old multifunctional and major pathogenic cytokine mediator of which the pro-inflammatory effects have been well studied (22). After appropriate stimulation, MCs, like many other immune and non-immune cells (macrophages, lymphocytes, endothelial cells, etc.), can synthesize and release IL-1 (23).

This cytokine stimulates IL-6 in MCs which is implicated in the traffic of immune cells to the CNS. In addition, IL-6 mediates inflammation by stimulating arachidonic acid products including PGE₂ released from MCs and vascular endothelial cells of the CNS (24).

Microglia, after treatment with IL-33, produce and secrete various pro-inflammatory cytokines including TNF, IL-1, IL-12, and IL-23. Microglia and astrocytes express the ST2 receptor IL-33 which increases the production of different cytokines and chemokines. Furthermore, IL-33 increases phagocytosis in microglia, proving to be a pro-inflammatory cytokine mediator in cerebral pathophysiology (25).

IL-1 is a highly inflammatory protein

which, when blocked, can be the therapeutic key of many inflammatory disorders including neuroinflammation.

Since the IL-1 anti-inflammatory drugs used to date do not provide any therapeutic effects, we propose that IL-37 could be a new promoter anti-inflammatory cytokine.

IL-33

IL-33, mainly involved in type 2 immunity and inflammation, is mostly produced by macrophages and is an important cytokine called "alarmin" since it is released by the cells after mechanical, chemical, physical or biological damage (26). ST2 IL-33 receptor is expressed on MCs that after activation produce a series of cytokines and chemokines, but it is also expressed by the precursors of MCs, such as CD34⁺ cells, favoring cell maturation.

It has been reported that IL-33 exacerbates antigen-induced inflammation in mice by activating MCs and it also mediates IgE-induced anaphylaxis in mice. In addition, IL-33 induces the generation and secretion of IL-6 from mouse bone marrow-derived cultured MCs, and the CXC chemokine IL-8 from human umbilical cord blood-derived cultured MCs.

IL-33 is one of the newest pro-inflammatory members of the IL-1 family involved in the regulation of innate and adaptive immune responses. IL-33 causes the release of pro-inflammatory mediators on MCs and it increases the effects of IgE and nerve growth factor on MC cell lines *in vitro*.

IL-33 exerts its biological effects through ST2 and IL-1αP receptors that act at the intracellular level on the MAP kinase and NF-κB cascade. The IL-33 ST2 receptors are expressed in the cerebral tissue and are involved in the exacerbation of psoriasis.

IL-33 stimulates the production of cytokine TH2 and monocytes and is involved in many pathophysiological conditions (25). IL-33 is generated in the CNS by endothelial cells and astrocytes that, together with the microglia, express the ST2 receptor. The microglia of the CNS generate different cytokines and chemokines and NO that mediate inflammation. It has been reported that IL-33 stimulates microglia and increases phagocytosis (25). The inhibition of IL-33 with IL-37 in the CNS

could improve the inflammatory and functional state of brain tissue.

IL-37

IL-37 is an intracellular regulator which modulates the signaling events initiated by FcεRI in MCs. In addition, this anti-inflammatory cytokine may act on receptor stimulation such as TLR-9 and TLR-3 and c-kit, but it is not a receptor antagonist (27). IL-37 down-regulates pro-inflammatory signals STAT transcription factors, p53, and NF-κB, affecting pro-inflammatory IL-1 family members (28). In fact, IL-37 inhibits activated macrophages that secrete TNF, IL-1, IL-18 and IL-33 which are also generated by MCs and are important in immunoregulation and inflammation.

In allergic inflammation mediated by Th2 cells and MCs, IL-33 can be inhibited by IL-37. This inhibitory cytokine decreases the expression, production and function of IL-1 proinflammatory cytokine family members. Silencing of IL-37 genes in human monocytes provokes an increase in inflammatory response (29).

However, blocking IL-1 family members may lead to an undesirable inhibitory effect of innate immunity and consequently adaptive immune responses, exposing the brain and the body in general to external insults.

The use of IL-37 in neuroinflammation could potentially help to improve the course of many neurological diseases mediated by inflammatory cytokine IL-1 family members. This is an emerging and attractive field, but many IL-37-mediated reactions are still unclear.

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EFFECTS OF SOYBEAN ISOFLAVONES ON Wnt/ β -CATENIN AND THE TGF- β 1 SIGNALING PATHWAY IN RENAL TISSUE OF TYPE 2 DIABETIC RATS

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Received December 8, 2017 – Accepted March 12, 2018

To observe the effect of Soyisoflavones (SI) on the expression of Wnt/ β -catenin signaling pathway elements, transforming growth factor- β (THGF- β) and its related proteins in the renal interstitia of diabetic nephropathic (DN) rats, 48 DN rats were randomly divided into 4 groups: DN model group (group DN), soybean isoflavone treatment group (group DA), DN model group + losartan treatment group (group DL), DN model group + soybean isoflavones combined with losartan treatment group (group SL). Each group comprised 12 rats. Twelve healthy Wistar rats were selected as normal controls (group N). After 12 weeks of continuous administration of soybean isoflavone or losartan or those two combined, the body weight of rats was recorded and serum urea nitrogen (BUN) and creatinine (Scr) were measured. The expression of Wnt4, β -catenin, and TGF- β ₁ proteins, as well as mRNA, in the renal interstitium were detected by immunohistochemistry and real-time quantitative PCR (FQ-PCR). In all the groups, Wnt4, β -catenin and TGF- β ₁ protein were only expressed in renal interstitial and renal tubular epithelial cells. There was no significant difference between group DA and group DL ($P > 0.05$). FQ-PCR results showed that Wnt4, β -catenin and TGF- β ₁ mRNA were consistent with the expression of these proteins in the renal tissue of each group. Soy isoflavones can reduce 24-h urinary protein quantification, alleviate renal interstitial pathological damage, and regulate the expression of Wnt4, β -catenin and TGF- β ₁ in the renal interstitium. This suggests that soybean isoflavones could delay the process of renal interstitial fibrosis in DN rats by decreasing the expression of Wnt4, β -catenin and TGF- β ₁ in the renal interstitium, thus demonstrating that soybean isoflavones plus losartan have the best protective effects against diabetes-induced renal fibrosis.

Diabetic nephropathy (DN) is one of the most common chronic complications of diabetes (1-3). Renal interstitial fibrosis is one of the major pathological manifestations of its late stage. In recent years, studies have found that the severity of

tubulointerstitial involvement is more closely related to the progression of renal function and prognosis in diabetic nephropathy than is the degree of glomerulosclerosis (4). It is the leading cause of DN in patients with diabetes. Therefore, its occurrence,

Key words: diabetic nephropathy, soybean isoflavones, losartan, Wnt4/ β -catenin signaling pathway, TGF- β ₁

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0393-974X (2018)

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mechanism, prevention and control measures have received ever increasing interest (5).

Transforming growth factor- β_1 (TGF- β_1) is a commonly recognized key factor in mediating glomerulosclerosis and tubulointerstitial fibrosis (6). In the hyperglycemic state, the signal pathway regulates renal cell proliferation and differentiation through receptor signaling and increases the protein content of the extracellular matrix (ECM), thereby promoting tubulointerstitial fibrosis (7-9). In recent years, a large number of clinical and animal studies have found that Wnt/ β -catenin signaling pathways play an important role in pathophysiological processes of the heart, liver, lung, kidney and other organs (10-11). Studies have shown that this pathway is increased in streptozotocin (STZ)-induced DN rats, as well as in unilateral ureteral obstruction rats (UUO). This further suggests that overexpression of the Wnt/ β -catenin signaling pathway elements may be involved in the process of renal interstitial fibrosis in diabetic nephropathy (12). It is well known that intracellular signal transduction pathways are not independent. They are intertwined with each other to form a network of mutual influence and interaction, and they jointly promote the progression of various diseases. TGF- β_1 can regulate the activation of the Wnt/ β -catenin signaling pathway either directly or by affecting the production of Wnt ligands. Baarsma et al. silenced β -catenin expression by siRNA, and the expression of TGF- β_1 was decreased in lung tissue. After the use of a TGF- β_1 inducer, however, expression of β -catenin was elevated in the interstitial lungs of individuals both with and without chronic obstructive pulmonary disease. This suggests that there may be crosstalk between the Wnt/ β -catenin and TGF- β_1 signaling pathways, and that both pathways may contribute to the development of organ fibrosis through their interaction (13-15).

Soybean isoflavones have multi-target characteristics, can reduce urinary protein and the blood creatinine, in addition to having other clinical effects via resisting oxidation, improving the glomerular filtration barrier and regulating immunity. They offer a certain degree of protective effects on the kidney. However, at present, there is no literature reporting on whether soy isoflavones can delay the

progression of DN renal interstitial fibrosis (15-17).

The aim of this study was to investigate the expression of Wnt/ β -catenin signaling pathway elements and the expression of TGF- β_1 in the renal interstitium of DN rats, along with the effect of soybean isoflavones on their expression. Their possible anti-fibrotic mechanism was explored to provide a more complete theoretical and experimental basis for their application in clinical DN therapy.

MATERIALS AND METHODS

Study animals

Sixty healthy Wistar rats were selected. They were 6-8 weeks old and weighed 180~200 g and were kept 6 per cage, at a temperature of $20\pm 2^\circ\text{C}$, and relative humidity of $55\pm 2\%$. The animals were provided by the Experimental Animal Center of Shanxi Medical University.

Reagents

Streptozotocin, Sigma; Citrate, Shanghai biochemical reagent second factory; Chloral hydrate, Shanghai biochemical reagent second factory; Soy isoflavones (extracted from Glycine max), Crude drug (Inner Mongolia), water decoction concentration is 0.7g/mL; Losartan potassium tablets, American Merck & Company; Rabbit against rat Wnt4 polyclonal antibody, American SantaCruz Corporation; Rabbit anti-rat β -catenin polyclonal antibody, American SantaCruz Corporation; Rabbit anti-rat TGF- β_1 polyclonal antibody, American SantaCruz Corporation; Immunohistochemistry kit, Wuhan Boshi Biotechnology Co., Ltd; Urine protein quantitative kit, Nanjing built Technology Co., Ltd; DAB color solution, Beijing Zhongyu Jinqiao Biotechnology Co., Ltd; RNA extraction kit, QIAGEN; Reverse transcription reagent, Lithuanian MBI; Dntp, Promega.

Instruments and equipment

Blood glucose meter and its supporting test paper (Johnson & Johnson); Metabolic cage (Changchun Institute of Regenerative Medicine); -20°C low temperature refrigerator (Japan SANYO company); -80°C low temperature refrigerator (Thermo Forma, USA); Optical microscope (Japan Olympus Corporation); Dehydration machine (Germany Leica LEICA-ASP300);

Embedding machine(Germany Leica LEICA-EG1150H + C); Slicer(Germany Leica LEICA-RM255); Pathology and image analysis system(JD-801 image analysis system); Electronic analytical balance(Sartorius, USA); Low temperature high speed centrifuge(Beckma, USA); CE1020 UV spectrophotometer(British CECIL company); Temperate box, JC-PW (Shanghai Longfei Instrument Co., Ltd.); Automatic multi-functional biochemical analysis equipment (Japan HITACH company); DNA Engine Option TM real time fluorescence quantitative PCR(MJ Research, USA).

Experimental grouping and DN rat model preparation

A diabetic rat model was prepared by intraperitoneal injection of 60mg/kg streptozotocin (STZ). STZ was dissolved in a freshly prepared 0.1mmol/L citrate buffer (pH 4.2) to yield a 2% STZ solution. After 72 h, the DN model group was measured for blood glucose and urine. If the following criteria were met, the model was considered to be valid: fasting blood glucose ≥ 16.7 mmol/L. The urine sugar results of model mouse were 3+~4+, showing that the diabetic rat model is valid. After 4 weeks, urinary protein excretion was measured and the DN model was successfully established with 24-h urinary protein ≥ 30 mg (n = 48). Forty-eight DN rats were randomly divided into 4 groups: DN model group (group DN), soybean isoflavones treatment group (group DA), DN model group + losartan treatment group (group DL), DN model group + soybean isoflavones combined with losartan treatment group (group SL). Each group comprised 12 rats. At the same time, 12 healthy Wistar rats were selected as the normal control group (group N). For group DA, 3 mL (equivalent to 2.1 g crude drug) of soy isoflavones decoction were given by gavage daily. Group DL was orally administered 3 mL (30 mg/kg⁻¹/d⁻¹) losartan daily. For the SL group, 3 mL of the soy isoflavones and losartan mixture were given via gavage daily. The DN and N groups were given the same volume of distilled water daily, continuously administered for 12 weeks. All rats were free to eat and drink during the experiment. To avoid the effects of exogenous drugs, all rats were treated without insulin or hypoglycemic agents.

Detection indicator

The general conditions of mental status, hair color, activity, water intake and urine volume of the rats were

observed daily. The mental status of rats was evaluated based on their behavior, including change of behavior, listlessness, paralysis, circling or head tilt. On the third day after intraperitoneal injection of STZ or solvent, the body weights of the rats were measured and at the end of the second week, fourth week, sixth week, eighth week, tenth week, and twelfth week, respectively.

Blood sugar

On the third day after intraperitoneal injection of STZ or solvent at the end of each week, the rats' tails were cut to determine blood sugar levels (in order to determine the successful date of modeling), and rats that did not meet the criteria were discarded.

24-h urinary protein quantification

On the third day after intraperitoneal injection of STZ or solvent the rats were placed in a washed metal metabolic cage (Shanxi Medical University Animal Experimental Center) at the end of the second week, fourth week, sixth week, eighth week, tenth week, and twelfth week, respectively (again, in order to determine the successful date of modeling). 24-h urine was collected. During the 24 h, the rats were fasted, but they were able to drink water. Urine volumes were recorded. After adequate mixing, approximately 4-6 mL of urine was taken. The supernatant was removed after centrifugation in a clean and dry tube. The biuret method was used to determine 24-h urinary protein.

BUN and Scr

After the confirmation of the rats' urine sugar, they received intraperitoneal injection of STZ or solvent. At the end of the 12th week from the start of the experiment, the rats were sacrificed. Before euthanasia, approximately 4 mL of blood were extracted from the rats' hearts, and kept at room temperature for 1 h. After storing for 6 h in a refrigerator at 4°C, the serum was centrifuged at 2000r/min. 10 min., and then frozen at -20°C. Scr and BUN were analyzed using an automatic multifunctional biochemical analyzer.

Renal interstitial Masson staining method

The rat kidney tissue was fixed in saturated picric acid and embedded in paraffin. Routine sections were 4 μ m; Conventional dewaxing to water, distilled water 3 min;

Celestite blue stained 7 min 30 s, tap water rinse 5 min; Hematoxylin staining 6 min., tap water rinse 5 min; 1% Pearcro and 2% acid magenta were fully mixed at a ratio of 1:2. The kidney tissue was covered with mixture for 16 min, excess dye was discarded, and the surrounding dye was dried; 1% phosphomolybdic acid 45s, excess dye was discarded, and the surrounding dye was dried; 0.5% glacial acetic acid 30s, excess dye was discarded, and the surrounding dye was dried; Brilliant Green was added for 8 min, and the excess dye discarded; 1% phosphomolybdic acid 45s, excess dye on the surface of the slice was discarded, and the surrounding dye was dried; 0.5% glacial acetic acid 30s, after drying, for observation under microscope.

The results showed: under light microscope, the nucleus was blue, the cytoplasm and red blood cells were red, and the collagen fibers were green. Quantitative analysis of interstitial fibrosis utilized ImageProPlus multimedia color pathological image analysis software. The relative area of renal interstitium was calculated according to the method described in the literature.

Immunohistochemistry

After confirmation of the rats' urine sugar, the animals received intraperitoneal injection of STZ or solvent. At the end of the 12th week from the start of the experiment, blood samples were taken from the rats. The left kidney was rapidly removed and the renal capsule was stripped. At the hilum, the kidney was cut longitudinally, saturated with picric acid solution, paraffin-embedded with routine section. The expression of Wnt4, β -catenin and TGF- β_1 in the renal interstitium of DN rats was detected by SP method.

Immunohistochemical assay: 1 min under high-temperature and high-pressure conditions using citrate buffer for Wnt4 antigen repair (β -catenin and TGF- β_1 using EDTA solution high-temperature-and-pressure repair 1 min), ice water cooling about 20 min, then returned to room temperature. 0.01mol/LPBS liquid wash tablets 4 min $\times$ 3 times. 1:300 Wnt4 polyclonal antibody of Rabbit anti rat and 1:100 β -catenin and 1:150 TGF- β_1 were added dropwise with 75 μ L to the sample. At the same time, PBS was added instead of the primary antibody as a negative control, and then put it in a 4°C refrigerator overnight. After 14 h, the slice was stored at 4°C and the PBS solution washed for 4 min $\times$ 3 times. Reagent B was

added to completely cover the kidney tissue, and it was incubated at 37°C for 18 min. 0.01mol/LPBS liquid wash tablets were used for 4 min $\times$ 3 times. A freshly prepared DAB solution (1: 1000) was used for color development (protected from light). Under the microscope, the degree of color control was observed (time was generally about 3 min). When the tissue was clear, brown and yellow, the final color was halted with tap water. Hematoxylin was used to re-bleach for 1 min 40s, and hydrochloric acid alcohol was used to differentiate for 2 s. It was then sealed with neutral resin. Under the microscope, the positive coloring featured brownish-yellow particles.

Image semi-quantitative analysis: Renal tubulointerstitial without glomeruli and arteries were observed under a 400x microscope. Image density and positive area percentages of positive targets were analyzed by Image-Pro Plus Version 6.0 image analysis software. The average optical density (IOD/area) represented the expression indicator.

Real time fluorescence quantitative PCR assay

After confirmation of the rats' urine sugar, the animals received intraperitoneal injection of STZ or solvent. At the end of the 12th week from the start of the experiment, after the blood samples were taken, the left kidney was quickly separated, and the renal capsule was stripped, then stored at -80°C.

The complete RNA extraction process was as follows: the rats were euthanized on an ice bed, 50 mg of renal interstitium was taken, and 1 mL Trizol was added to prepare for the ultrasonic homogenate. After adding 200 μ L of chloroform, the mixture was vigorously shaken on a vortex mixer for 30 s and centrifuged at 12,000 g at 4°C for 5 min. The supernatant was transferred to another EP tube and 200 μ L of isopropanol was added. The mixture was allowed to stand at room temperature for 5 min to mix well, and was then centrifuged at 12,000 g for 5 min. The supernatant was discarded and the precipitated RNA was washed twice with 1 mL 70% ethanol and centrifuged at 12,000 g for 2 min at 4°C. The RNA pellet was obtained at the bottom of the EP tube and dissolved in 50 μ L of 0.1% DEPC water as a reverse transcription template.

For the process of RNA reverse transcription into a cDNA, total RNA 5 μ g was incubated at 65°C for 5 min after rapid centrifugation, placed on ice, and the following reaction system was added: 11 μ L of RNA (0.09 μ g/ μ L),

1 μ L random primers (0.2 μ g/mL), 5 $\times$ 4 μ L Buffer, 3 μ L dNTP(10mmol/L), 1 μ L of RNase inhibitor (20 U/ μ L) and 1 μ L of reverse transcriptase (20 U/ μ L), and Nuclease-Free was added to obtain a total volume of 20 μ L. The system was incubated at 25°C for 10 min, 42°C for 1 h, and finally at 72°C for 15 min.

Real-time fluorescence quantitative PCR process was as follows: PCR was carried out under the catalysis of Taq DNA polymerase using the cDNA obtained by reverse transcription. The Wnt4 upstream primers were 5'-CTGGAGAAGTGTGATGTGA-3', the downstream primers were 5'-GGACTGTGAGAAGGCTACGC-3', the product length was 108bp. The β -catenin's upstream primers were 5'-GGCAGAGAGCTCAGGTATGG-3', the downstream primers were 5'-GAGCTTGCTTTCCTGATTGC-3', the product length was 202bp. The TGF- β_1 upstream primer was 5'-TGAGTGGCTGTCTTTTGACG-3', downstream primer was 5'-ACTGAAGCGAAAGCCCTGTA-3', the product length was 82bp. The β -actin upstream primers were 5'-GTCAGGTCATCACTATCGGCAAT-3', the downstream primers were 5'-AGAGGTCTTTACGGATGTCAACGT-3', the product length was 147bp. Primers were made by Shanghai Health and Biological Engineering Co., Ltd. The target gene and the internal parameters expanded and grew in the same tube.

The PCR amplification reaction system was as follows: 2 μ L cDNA; 0.5 μ L upstream primers (15 uM); 0.5 μ L downstream primer (15 uM); 10 μ L Fast Start Universal SYBR Green Master (ROX); 7 μ L water without DNase and RNase. The reaction system totaled 20 μ L.

The conditions were as follows: 94°C pre-denaturation for 10 min, Tag enzyme activated; 94°C for 15 s, 60°C for 60 s for a total of 45 cycles.

Statistical analysis

All statistical analyses were performed by using SPSS13.0 statistical software. All data are expressed as mean $\pm$ standard deviation. The difference between the two groups was analyzed by *t*-test. Data analysis was performed by multivariate analysis. Correlation analysis was performed by the Pearson method and real-time fluorescence quantitative PCR. Relative quantitative analysis of gene expression was performed using Excel software. $P < 0.05$ for the difference was statistically significant.

RESULTS

Water intake and urine volume of rats in group N were normal; their mental status was good and their activity quick. Moreover, during the whole experiment, they did not change significantly. After the success of the diabetic model, the rats in the experimental group had increased food and water intake, and urine volume increased with weight loss. At the fourth week, the above symptoms became more severe: urine output increased significantly, and the malodor was more intense. At the eighth week, the rats in the experimental group had rough and dull hair color, poor activity, diminished water intake and less weight loss. At the twelfth week, the rats in the DN group were rough and dull in color, demonstrated poor activity, were apathetic and lethargic. Their diet was significantly reduced, and their weight was significantly diminished. The rats' abdomens were in some cases bulging, and the toe and tail showed edema, ulceration or necrosis. The mental status, activity and diet of group DA, group DL and group SL were better than those of group DN, and the ketone odor of their urine was slightly reduced.

24-h urinary protein quantification

At the weekend of week 2, week 4 and week 6, compared with group N, 24-h urinary protein was increased in groups DN, DA, DL and SL, but the difference was not statistically significant ($P > 0.05$). Follow-up observation found that with the progress of the disease, there was a progressive increase in the trend. At the end of week 8 and week 12, 24-h urinary protein in group DN rats increased significantly compared to group N ($P < 0.05$). After 12 weeks of treatment with soybean isoflavones and losartan, the levels of 24-h urinary protein in groups DA, DL, and SL were significantly decreased compared with those in group DN, and the difference was statistically significant ($P < 0.05$). Multiple comparisons were performed in the three drug intervention groups. The quantitative decrease of 24-h urinary protein in groups SL, DA and DL was more obvious, and the difference was statistically significant ($P < 0.05$). However, there was no significant difference between group DA

and group DL ($P>0.05$). Changes in 24-h urinary protein for each group are shown in Fig. 1.

BUN and Scr

At the end of 12 weeks, serum creatinine and urea nitrogen were significantly increased in groups DN, DA, DL and SL compared to group N. The difference was statistically significant ($P<0.05$). After 12 weeks of treatment with soy isoflavones and/or losartan, the levels of serum BUN and Scr in groups DA, DL and SL were significantly lower than those in group DN. The difference was statistically significant ($P<0.05$). Multiple comparisons were performed among the three drug intervention groups. The decrease of serum BUN and Scr in groups SL, DA and DL were more obvious, indicating that the difference

was statistically significant ($P<0.05$). There was, however, no significant difference between group DA and group DL ($P>0.05$). Comparison of 24-h urinary protein, serum Scr and BUN in each group is shown in Table I.

Masson staining

Masson staining showed that the rats' renal interstitial collagen relative areas (the ratio of Masson-stained renal interstitial green area to total green area of visual field, %) were 0.72 ± 0.29 , 47.30 ± 2.42 , 33.14 ± 3.31 , 34.52 ± 3.29 , and 26.92 ± 1.68 , respectively, at the end of the 12 weeks. There was only a small amount of green dye in the interstitial area of group N. Compared with group N, the relative areas of renal interstitial collagen

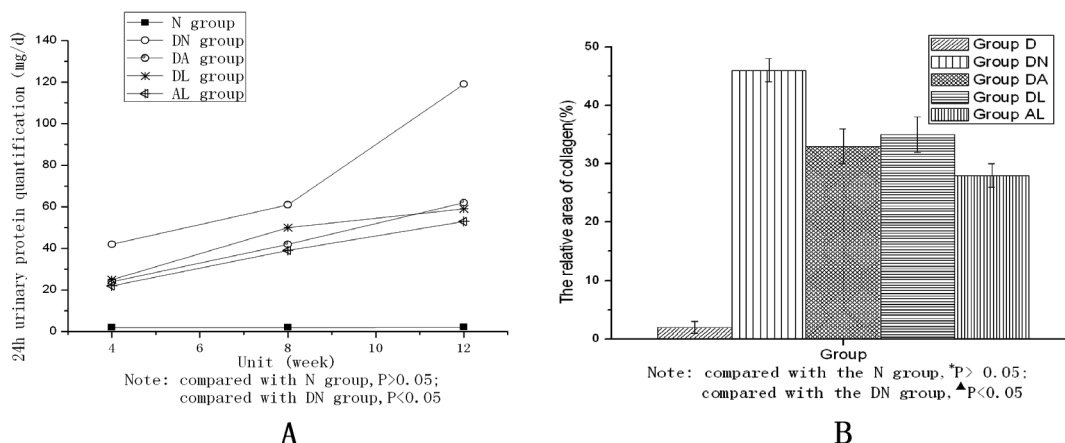


Fig. 1. Changes in 24-h urinary protein and relative area of renal collagen in each group. **A)** Changes in 24-h urinary protein. **B)** The relative area of renal collagen.

Table I. Comparison of 24-h urinary protein, serum Scr and BUN in each group.

Group	n	Scr(mmol/L)	BUN(μ mol/L)	PRO(g/L)
Group N	12	$32.69\pm6.58^\Delta$	$4.34\pm0.87^\Delta$	$5.92\pm1.06^\Delta$
Group DA	11	$82.33\pm6.50^{*\Delta}$	$12.79\pm3.16^{*\Delta}$	$32.67\pm7.03^{*\Delta}$
Group DL	12	$75.52\pm7.40^{*\Delta}$	$12.28\pm3.68^{*\Delta}$	$28.48\pm7.17^{*\Delta}$
Group SL	11	$72.86\pm4.65^{*\Delta}$	$7.01\pm1.58^{*\Delta}$	$19.03\pm4.45^{*\Delta}$
Group DN	10	$116.43\pm6.56^{*\Delta}$	$14.38\pm3.08^*$	$35.05\pm4.44^*$

($X\pm S$). Compared with group N, $^*P>0.05$; compared with group DN, $^\Delta P<0.05$.

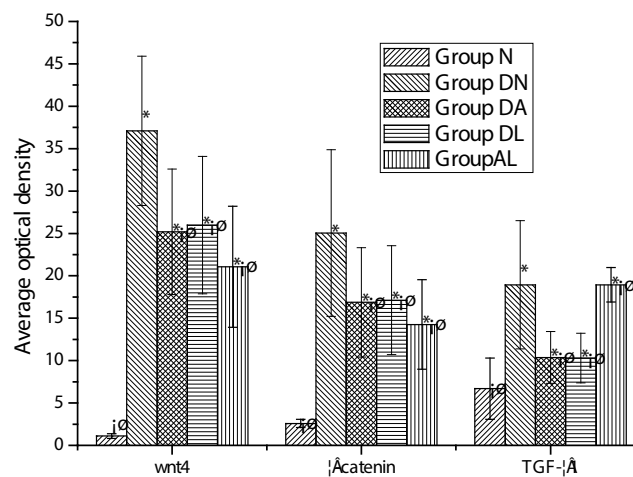
in groups DN, DA, DL and SL were significantly increased, and the difference was statistically significant ($P < 0.05$). The relative areas of renal interstitial collagen in groups DA, DL and SL were significantly decreased after 12 weeks of treatment with soybean isoflavones and/or losartan. The difference was statistically significant ($P < 0.05$). Multiple comparisons of the 5 groups together were

performed among the three drug intervention groups. The relative area of interstitial collagen in groups SL, DA and DL decreased more conspicuously. The difference was statistically significant ($P < 0.05$). There was, however, no significant difference between group DA and group DL ($P > 0.05$). The relative area of collagen in the renal interstitia of rats in each group is shown in Fig. 1.

Table II. Expression of *Wnt4*, β -catenin and *TGF- β_1* protein in each group.

Group	n	Wnt4	β -catenin	TGF- β_1
renal interstitia				
Group N	12	1.11 $\pm$ 0.28 [▲]	2.59 $\pm$ 0.50 [▲]	6.70 $\pm$ 3.65 [▲]
Group DA	11	25.20 $\pm$ 7.47* [▲]	16.87 $\pm$ 3.16* [▲]	10.36 $\pm$ 3.08* [▲]
Group DL	11	25.92 $\pm$ 8.12* [▲]	17.14 $\pm$ 6.44* [▲]	10.32 $\pm$ 2.89* [▲]
Group SL	10	21.09 $\pm$ 7.15* [▲]	14.28 $\pm$ 5.31* [▲]	9.10 $\pm$ 2.01* [▲]
Group DN	10	37.12 $\pm$ 8.06*	25.08 $\pm$ 9.79*	18.97 $\pm$ 7.52*
renal tissue				
N group	12	18.95 $\pm$ 0.89 [▲]	13.38 $\pm$ 3.28 [▲]	23.42 $\pm$ 1.95 [▲]
DA group	12	39.77 $\pm$ 0.96* [▲]	36.69 $\pm$ 4.81* [▲]	45.64 $\pm$ 3.24* [▲]
DL group	12	38.29 $\pm$ 2.98* [▲]	35.41 $\pm$ 3.92* [▲]	43.67 $\pm$ 2.74* [▲]
SL group	12	29.86 $\pm$ 2.12* [▲]	20.21 $\pm$ 1.35* [▲]	40.88 $\pm$ 2.75* [▲]
DN group	12	45.15 $\pm$ 1.32*	64.16 $\pm$ 4.65*	77.83 $\pm$ 5.42*

Compared with the group N, * $P > 0.05$; compared with group DN, [▲] $P < 0.05$.



Note: Compared with the N group, * $P > 0.05$;
compared with the DN group, ⁱ $P < 0.05$

Fig. 2. Expression of *Wnt4*, β -catenin and *TGF- β_1* proteins in the renal masses of each group.

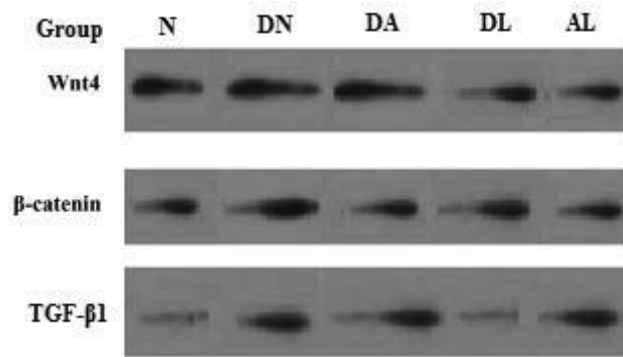


Fig. 3. Western blot results of Wnt4, β -catenin and TGF- β 1 protein.

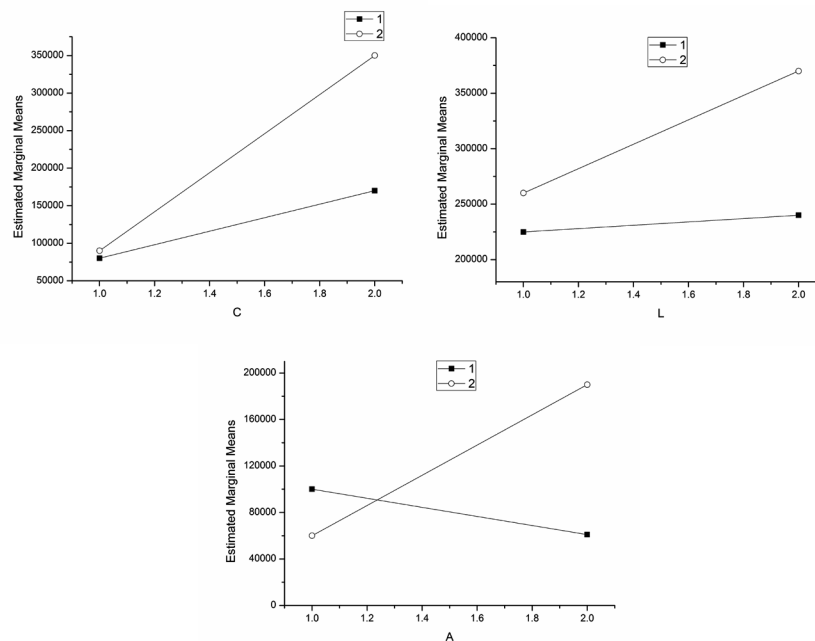


Fig. 4. Soybean isoflavones and losartan inhibit Wnt4, β -catenin and TGF- β 1 protein expression.

Expression of Wnt4, β -catenin and TGF- β 1 proteins in the renal interstitium of rats in each group

Wnt4 in group N was weakly expressed in the cytoplasm of renal tubular epithelium and renal interstitial cells. Compared with the normal control group, the expression of Wnt4 in groups DN, DA, DL and SL was enhanced in the cytoplasm of renal tubular epithelium and renal interstitial cells and the difference was statistically significant ($P > 0.05$).

β -catenin in group N was weakly expressed on the basal side of renal tubular epithelial cells.

Compared with the normal control group, expression of β -catenin in renal tubular epithelial cells was significantly enhanced in groups DN, DA, DL and SL, and the difference was statistically significant ($P > 0.05$).

Multiple comparisons were performed among the three drug intervention groups. The expression of Wnt4, β -catenin and TGF- β 1 in groups SL, DA and DL was more obvious in the renal interstitium and the difference was statistically significant ($P < 0.05$). There was, however, no significant difference

between groups DA and DL ($P > 0.05$). The expression of Wnt4, β -catenin and TGF- β_1 proteins in the renal interstitia of each group is shown in Table II and in Fig. 2. The Western blot results are shown in Fig. 3.

Analysis of the intervening factors of soybean isoflavones and losartan

The results of factorial analysis showed that there was a joint effect between soybean isoflavones and losartan intervening in the inhibition of Wnt4, β -catenin and TGF- β_1 protein expression in renal interstitial cells of DN rats (FWnt4=4.241, PWnt4=0.046<0.05; F β -catenin=4.177, P β -catenin=0.046<0.05; FTGF- β_1 =79.923, PTGF- β_1 =0.003<0.05). Particularly, the inhibitory effect of soybean isoflavones and losartan on the expression of Wnt4, β -catenin and TGF- β_1 proteins in renal masses of DN rats was the most conspicuous. The interaction of effects of soybean isoflavones and losartan on the expression of Wnt4, β -catenin and TGF- β_1 proteins are shown in Fig. 4.

Correlation between expression of TGF- β_1 and Wnt4 and β -catenin in renal tubulointerstitium of DN rats

The expression of TGF- β_1 in the renal interstitia of DN rats was positively correlated with the expression of Wnt4 in the renal interstitia of DN rats ($r = 0.676$, $P < 0.05$). The expression of TGF- β_1 in the renal interstitia of DN rats was positively correlated with the expression of β -catenin in the renal interstitia of DN rats ($r = 0.817$, $P < 0.05$).

The expression of Wnt4, β -catenin and TGF- β_1 mRNA in the renal tissue of rats in each group

At the end of 12 weeks, Wnt4, β -catenin and TGF- β_1 mRNA were only expressed in group N. Compared with group N, the expression of Wnt4, β -catenin and TGF- β_1 mRNA in groups DN, DA, DL and SL were significantly higher than those in group N and the difference was statistically significant ($P < 0.05$). Compared with group DN, the expression in groups DA, DL and SL were significantly decreased ($P < 0.05$). Moreover, there was no significant difference between group DA and group DL ($P > 0.05$). Compared with the groups, AL, DA, DL, the difference in expression was significant ($P < 0.05$).

The expression of Wnt4, β -catenin and TGF- β_1 mRNA proteins in the renal tissue of rats in each group is shown in Table II.

DISCUSSION

In this study, Wistar rats were used as subjects. Through a diabetes model induced by a single intraperitoneal injection of streptozotocin, islet β -cell function was completely compromised and insulin secretion lost, resulting in insulin-dependent (type 1) diabetes. In the light of the literature data and previous studies on the effects of various doses for modeling, 60 mg/kg was chosen (13). The model success rate was 100% and the mortality of the modeled rats was 6.25%. The modeling method was considered to be reproducible and operable. Study shows that at the end of 8 weeks, 24-hour urine was collected for the measurement of the albumin concentration: blood samples were collected for serum biochemistry and the rats were sacrificed for kidney measurement of oxidative stress and histomorphological features (14). Baarsma et al. used siRNA to silence the expression of the β -catenin protein, and the expression of TGF- β_1 protein and nucleic acid in lung tissue was subsequently decreased (15). This suggests that soy isoflavones and losartan can reduce urinary protein, and can thus play a protective role against stimulation of diabetes-induced tubulointerstitial lesions.

In conclusion, the expression of Wnt/ β -catenin signaling pathway elements and TGF- β_1 were increased in the process of diabetic renal interstitial fibrosis, and the expression of Wnt/ β -catenin was positively correlated. This provides new theoretical and experimental evidence for their clinical application. The combination of soy isoflavones and losartan can produce interactive effects and enhance each other's anti-fibrosis properties, helping to provide guidance for the clinical treatment of DN. The exact compound contribution to the effect in the soybean isoflavones requires further research.

ACKNOWLEDGEMENTS

This work was supported by the Scientific

Research Project of Heilongjiang Health and Family Planning Commission (No.2017-316); The Natural Science Foundation of Heilongjiang Province, China (No.H2015078).

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THE ROLE FOR CYCLIC GLYCINE-PROLINE, A BIOLOGICAL REGULATOR OF INSULIN-LIKE GROWTH FACTOR-1 IN PREGNANCY-RELATED OBESITY AND WEIGHT CHANGES

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Received October 16, 2017 – Accepted March 26, 2018

Cyclic Glycine-Proline (cGP) regulates the homeostasis of insulin-like growth factor (IGF)-1 function and cGP/IGF-1 ratio determines IGF-1 bioactivity *in vitro* and *in vivo*. Plasma IGF-1 represents largely inactive IGF-1 and weakly associated with human obesity and hypertension. We evaluated the regulatory role for cGP in pregnancy-related obesity and hypertension, and in obesity status between pregnancy and postpartum. Women were recruited in their first pregnancy. A cross-sectional study compared plasma concentration of cGP, IGF-1 and IGF binding protein (IGFBP)-3 in women with obesity and/or hypertension to normal controls 6-year postpartum using UPLC-MS and ELISA. A longitudinal study compared the changes of these peptides from 15-week gestation to 6-year post-partum in the women who remained normal weight, remained obese or changed to obese or to normal respectively. Study 1 is a cross-sectional study. The obese group had lower IGF-1 ($p = 0.001$), higher cGP/IGF-1 ratio ($p = 0.0055$) and the hypertensive group had lower IGFBP-3 ($p = 0.046$) and cGP ($p = 0.043$) than the controls. Study 2 is a longitudinal study. Women with weight loss had increased cGP/IGF-1 ratio ($p = 0.0026$) and decreased IGFBP-3 ($p = 0.0001$) compared with women whose weight remained normal. Women with weight gain had lower IGFBP-3 ($p < 0.0001$) only. Women who remained obese had increased cGP/IGF-1 ratio ($p = 0.006$) only. Increase in cGP/IGF-1 ratio is associated with obesity, but not hypertension. Changes of IGFBP-3 and/or cGP/IGF-1 ratio are associated with weight changes. The data suggest the role for cGP in obesity through autocrine regulation of IGF-1.

Key words: cGP, biological regulator, homeostasis of IGF-1, obesity, hypertension and autocrine regulation

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0393-974X (2018)

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Obesity is a complex metabolic disorder characterised by an increase of adipose mass. Clinically it is defined as a BMI of $\geq 30 \text{ kg/m}^2$, however this definition fails to take into account the differences in muscle mass and relative amounts of visceral and peripheral fat in gender. Visceral fat or abdominal obesity are independent markers of cardiovascular morbidity and mortality. The association of obesity with dyslipidemia, hyperglycemia and hypertension collectively defined as metabolic syndrome identifies those at higher risk of cardiovascular disease (1). The additional biological changes for predicting changes of obesity status are desirable.

Insulin-like growth factor-1 (IGF-1), a growth peptide plays an important role in energy metabolism (2, 3). IGF-1 deficiency is associated with metabolic dysfunction (2, 3) including obesity (4) and hypertension (5). Epidemiological research shows that plasma IGF-1 in the upper normal range is associated with reduced blood pressure, lipids and increased insulin sensitivity (6). The changes of circulating IGF-1 and IGF binding protein-3 (IGFBP-3) have been used for indicating the degree of IGF-1 deficiency in metabolic disorders (2, 4, 5, 7, 8), however reported inconsistently (6, 7, 9). These changes may not represent bioactive IGF-1 accurately.

The majority of IGF-1 circulates in blood in a form bound to IGFBPs, of which $>75\%$ is IGFBP-3 (2). The binding to IGFBPs protects IGF-1 from being metabolised and also from activating IGF-1 receptors (2). Thus IGFBPs regulate IGF-1 function through either reducing or increasing IGF-1 bioavailability, characterised as autocrine regulation for stabilising homeostasis of IGF-1 function (2). However current assays measure the total plasma IGF-1 which is largely inactive. This is not just a technical issue as the window of opportunity for detecting free IGF-1 is narrow because free IGF-1 is either rapidly metabolised or internalised after activating receptors. Despite the disadvantages of measuring total IGF-1, IGF-1/IGFBP-3 ratio has been used for indicating 'free', bioactive IGF-1, which may only partially represent IGF-1 function (10).

N-terminal tripeptides of IGF-1 enzymatically cleave (11, 12) from free IGF-1 (13-15), and then

form cyclic Glycine-Proline (cGP) through peptide cyclisation (16). As a major binding site to IGFBPs (15) the N-terminal peptides retain the ability to interact with IGFBP-3 (17). cGP has been well characterised as neurotrophic peptides (16). The research into the mechanism of cGP demonstrated the role of cGP in maintaining homeostasis of IGF-1 function through altering IGF-1 binding to IGFBP-3 with the higher ratio of cGP/IGF-1 and the greater free, bioactive IGF-1 (17-19). Thus the competitive binding to IGFBP-3 between cGP and IGF-1 determine the autocrine regulation of IGF-1. The concentrations of plasma cGP and IGFBP-3 change relating to plasma IGF-1 during development (18). Compare to their adult counterpart plasma IGF-1 is lower in infant rats (18). In order to increase free, bioactive IGF-1 cGP increases whereas IGFBP-3 decreases in infant rats (18). While the changes of cGP and its relative concentration to IGF-1 may be a better representation of bioactive IGF-1, the plasma concentration of IGF-1 represents growth hormone (GH) regulated liver production of IGF-1, namely endocrine regulation of IGF-1.

Longitudinal studies suggest a transient decline in maternal IGF-1 around 8-16 weeks gestation due to liver resistance to GH associated IGF-1 production in liver (20). The changes in circulating IGF-1 between pregnancy and post-partum may provide a window of opportunity to evaluating the regulatory role of cGP in homeostasis of IGF-1 (18) and its association with the changes of obesity status. To demonstrate the potential involvement of cGP in metabolism, current study for the first time, evaluated the changes of cGP/IGF-1 ratio in human pregnancy associated obesity and/or hypertension and changes of obesity status between early pregnancy and the post-partum. In addition, mathematical modelling techniques have successfully been used to indirectly determine the free concentration of cortisol in clinical subjects (21), and estimates of free IGF-1 (17, 19) and provide important information that is beyond direct biological evaluation.

Experimental design

Study subjects were part of the international

Screening for Pregnancy Endpoints study (SCOPE) in which Auckland women were recruited in their first ongoing pregnancy and post-partum between 2004 and 2008 (Fig.1) (22-24) and the study was approved by the Northern Region Ethics Committee, New Zealand (AKX/02/00/364). All subjects gave informed, signed consent to participate in the study. The subjects were women who attended for assessment and blood collection at 15 weeks' gestation and subsequently at 6 years postpartum, after the first birth. These blood samples were stored at -80°C . We selected 40 EDTA samples collected from non-pregnant women who were (a) obese + normotensive ($n = 10$) or (b) obese + hypertensive ($n = 10$), or (c) non-obese + hypertensive ($n = 10$) or (d) non-obese + normotensive ($n = 10$). In addition, we identified paired samples collected at both 15 weeks' gestation in their first pregnancy and then 6 years later, from normotensive women who were obese or normal weight during their first pregnancy and either remained obese ($n = 10$) or normal weight ($n = 10$) or who changed from obese to normal weight ($n = 10$), or from normal weight to obese ($n = 10$). The samples collected from the normotensive women who were either normal or obese were used for the analysis in both studies.

Plasma extraction of cGP

The assay has been described previously (18). cGP- d_2 , an internal standard (50 μl of 500ng/ml) was added to 100 μl of plasma, and vortex mixed. The solution was transferred to a 1ml Phree phospholipid removal cartridge (cat# 8B-S133-TAK, Phenomenex, Auckland, New Zealand) contained in 4.5-ml tube. 500 μl of 1% formic acid (cat#100264 Merck KGaA Darnstadt, Germany) in acetonitrile (cat# 1.00030 Merck) was added to the cartridge and centrifuged at 1000 g for 5 min at 4°C to enable the collection of the filtrate. The filtrate was dried using a vacuum concentrator (Savant SC250EXP, Thermo Scientific, Asheville, NC, USA), 1.5 mTor for 1 h then 0.7mTor for 45 min, at room temperature. The dried samples were reconstituted in 100 μl of 10% methanol (cat#1.06007 Merck)/water and transferred to a UPLC vial (cat#AR0-39S0-13-C Phenomenex) for quantitation, then centrifuged at 500 rpm for 5

min at 4°C to sediment any remaining particulates. Standards were prepared by spiking cGP into charcoal stripped human plasma (cat#BB220111L SeraCare, Milford MA, USA), quality control samples, with cGP at two different concentrations, were utilised and then subjected to the same extraction procedure as the samples.

UPLC-MS assay

The assay has been described previously (18). Briefly, the chromatography conditions consisted of a Synergy Hydro 2.5 μm column 100 x 2 mm (Cat# 00D-4387-B0, Phenomenex) with an initial mobile phase composition of 10% methanol/90% water flowing at 200 μl per minute with a column temperature of 35°C . Ionization was undertaken using heated electrospray ionization in positive mode with a voltage of 4000V, a sheath gas flow of 30psi, an auxiliary gas flow of 2psi, and a capillary temperature of 250°C . Fragmentation was achieved with argon at 1.2 mTorr as the collision gas and a dissociation voltage of 35V. The mass spectrometer system consisted of an Accela MS pump and autosampler on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer all controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA), it was run in selective reaction monitoring mode with the following two transitions 155.1 $\rightarrow$ 70.2m/z and 157.1 $\rightarrow$ 70.2m/z utilised for cGP and cGP- d_2 respectively. The retention time for both peaks was 3.6 min. Unknown samples were quantitated using the peak area ratio of cGP/cGP- d_2 compared with the standard curve of known concentrations.

ELISA

Plasma concentration of IGF-1 and IGFBP-3 were measured using commercial ELISA kits (Cat# 80572 and 80580 respectively, Crystal Chem, Chicago, IL, USA) according to the manufacturer's instructions. The absorbance values were obtained using a plate reader (BioTek® Synergy™ 2 Multi-detection Microplate Reader, Gen5 software, Winooski, VT, USA) set at 450 nm for the excitation wavelength and at 630 nm for the emission wavelength. Concentrations were reported as ng/ml.

Mathematical model of dynamic changes of IGF-1, cGP and IGFBP-3

The mathematical model of the non-linear dynamics of IGF-1/cGP/IGFBP-3 can be described by a 5 variable model, a simplified version of Model 4 used by Phillips et al. (19). IGF-1 and cGP are assumed to competitively bind to IGFBP3 (17). The rate of change in the concentrations (nM) of unbound IGFBP-3 (q_1), unbound IGF-1 (q_2), bound IGF-1 (q_3), unbound cGP (q_6) and bound cGP (q_8) are

$$\begin{aligned}\frac{dq_1}{dt} &= +k_{-1a}q_3 - k_{+1a}q_2q_1 + k_{-1c}q_8 - k_{+1c}q_6q_1, & q_1(0) &= q_{10} \\ \frac{dq_2}{dt} &= +k_{-1a}q_3 - k_{+1a}q_2q_1, & q_2(0) &= q_{20} \\ \frac{dq_3}{dt} &= -k_{-1a}q_3 + k_{+1a}q_2q_1, & q_3(0) &= q_{30} \\ \frac{dq_6}{dt} &= +k_{-1c}q_8 - k_{+1c}q_6q_1, & q_6(0) &= q_{60} \\ \frac{dq_8}{dt} &= -k_{-1c}q_8 + k_{+1c}q_6q_1, & q_8(0) &= q_{80}\end{aligned}\quad (1)$$

where t is time (min), k_{+1a} is the rate of binding of IGF-1 to IGFBP-3 ($\text{nM}^{-1} \text{min}^{-1}$), k_{-1a} is the rate of

dissociation of IGF-1 from IGFBP-3 (min^{-1}), k_{+1c} is the rate of binding of cGP to IGFBP-3 ($\text{nM}^{-1} \text{min}^{-1}$), k_{-1c} is the rate of dissociation of cGP from IGFBP-3 (min^{-1}), q_{10} is the initial concentration of unbound IGFBP-3 (nM), q_{20} is the initial concentration of unbound IGF-1 (nM), q_{30} is the initial concentration of bound IGF-1 (nM), q_{60} is the initial concentration of unbound cGP (nM), and q_{80} is the initial concentration of bound cGP (nM). We assumed that $k_{+1a} = 0.002 \text{ nM}^{-1} \text{min}^{-1}$ (25, 26), $k_{-1a} = 0.0062 \text{ min}^{-1}$ (25, 26), $k_{+1c} = 0.001 \text{ nM}^{-1} \text{min}^{-1}$, and $k_{-1c} = 0.0062 \text{ min}^{-1}$. The total concentration of IGF is $q_2 + q_3$, the total concentration of cGP is $q_6 + q_8$ and the total concentration of IGFBP-3 is $q_1 + q_3 + q_8$. Differential equations were solved using ode15s in MATLAB (27).

Statistical analysis

For Study 1, two-way ANOVA with multiple comparisons was used for analysing the effects of obesity/non-obese and of normotensive/hypertensive (SAS version 9.6, SAS Institute Inc., Cary, NC, USA). For Study 2, one-way ANOVA was used to analyse the changes between the paired data (SAS

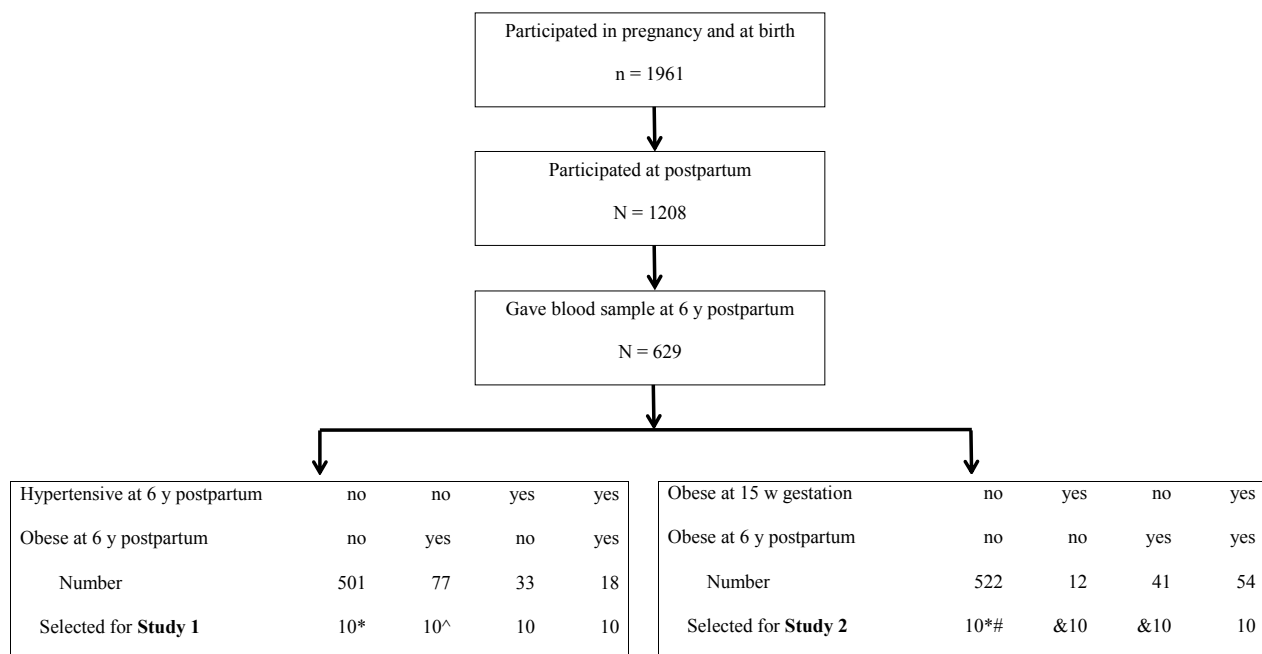


Fig. 1. The study population.

* Same subjects used for the analysis of Study 1 and Study 2

^ Obese at 15 w gestation and 6 y postpartum $n=9$, non-obese at 15 w gestation and at 6 y postpartum $n=1$

Number samples were available for cGP analysis at 15 weeks $n=9$

& Supplementary analysis of Study 1 included another 20 non-pregnant women of Study 2

version 9.6, SAS Institute Inc., Cary, NC, USA) and data are presented as mean $\pm$ SD. Correlation between the parameters was analysed using Pearson tests.

RESULTS

Clinical characteristics of the non-pregnant women in Study 1 selected for either obesity or hypertension are shown in Table I. Women with obesity, defined by BMI > 30 kg/m², also had increased waist circumferences, higher triglycerides and LDL at 6 years post-partum. Those selected for hypertension in the presence of obesity, fulfilled more criteria for metabolic syndrome, as defined by International Diabetes Federation, than those women who were selected for hypertension in the absence of obesity. The characteristics of the women in the second study who were sampled at 15 weeks of pregnancy and again 6 years post-partum were

selected on the basis of discordant obesity status as shown in Table II.

Biochemical Data

Study 1: Cross-sectional changes of IGF-1, cGP and IGFBP-3 in women 6 years postpartum

Compared to normal weight women, the plasma concentrations of IGF-1 were significantly lower in obese women (Fig. 2A, $f[1,36] = 12.72$, $p = 0.001$), and a lower but not statistically significant IGF-1 concentration in hypertensive women ($f[1,36] = 3.54$, $p = 0.068$) was observed. Multiple comparison showed a lower IGF-1 in obese only women when compared to normal control women (Fig. 2A, $p = 0.0014$).

Both cGP (Fig. 2B, $f[1,36] = 4.38$, $p = 0.043$) and IGFBP-3 (Fig. 1C, $f[1,36] = 4.2$, $p = 0.046$) concentrations were moderately lower in hypertensive women compared to the normotensive women. There were no differences in cGP and

Table I. Clinical characteristics of participants in cross-sectional Study 1.

	Non-obese, non-hypertensive (n=10)	Non-obese, hypertensive (n=10)	Obese, non-hypertensive (n=10)	Obese, hypertensive (n=10)
Age (years)	30.1 (3.2)	32.7 (3.3)	32.6 (5.6)	29.4 (5.0)
European	9 (90%)	9 (90%)	9 (90%)	9 (100%)
Waist (cm)	74.4 (3.2)	74.2 (4.1)	108.4 (16.3)	104.9 (10.6)
BMI (kg/m ²)	21.7 (2.0)	22.0 (1.4)	36.7 (6.0)	37 (5.5)
BP (mmHg)				
Systolic	106 (6)	142.5 (7)	123 (8)	137 (7)
Diastolic	64 (5)	78 (10)	72 (7)	80 (11)
Lipids (mmol/L)				
TG	0.7 (0.1)	0.9 (0.3)	1.2 (0.4)	1.3 (0.4)
LDL	2.7 (0.5)	2.5 (0.9)	3.4 (0.5)	3.2 (0.8)
HDL	1.6 (0.5)	1.8 (0.6)	1.6 (0.3)	1.4 (0.3)
Fasting glucose (mmol/L)	4.8 (0.3)	4.8 (0.5)	4.8 (0.6)	5.3 (0.8)
Metabolic syndrome*	0	0	1	6

Values are presented as means

*Metabolic syndrome defined as per IDF criteria of BMI > 30 kg/m² or waist ≥ 80 cm plus any two of the following four features (1) raised triglycerides ≥ 1.7 mmol/L or lipid treatment, (2) reduced HDL < 1.29 mmol/L, (3) raised blood pressure based on systolic BP ≥ 130 or diastolic BP ≥ 85 mmHg, (4) raised fasting glucose ≥ 5.6 mmol/L, or glucose lowering treatment. Values in parentheses denote standard errors.

IGFBP-3 between obese and non-obese groups and no interaction between hypertension and obesity on cGP levels. Multiple comparisons did not show any significant differences between the four groups.

The cGP/IGF-1 ratio was higher in obese groups compared to the non-obese groups (Fig. 2D, $f [1,36] = 8.71$, $p = 0.0055$), there were no differences between hypertension and non-hypertension groups. Multiple comparisons showed a significantly higher ratio of cGP/IGF-1 in the

obese only group compared to the normal control group (Fig. 2D, $p = 0.0185$). The ratio of cGP/IGF-1 was not statistically different between the hypertensive women who were of normal weight and those who were obese.

The IGF-1/IGFBP-3 ratio was significant lower in the obese groups (Fig. 2E, $f [1,36] = 10.77$, $p = 0.0023$) than that of non-obese groups. Obese women, both with and without hypertension, had lower ratios than non-obese/non-hypertension

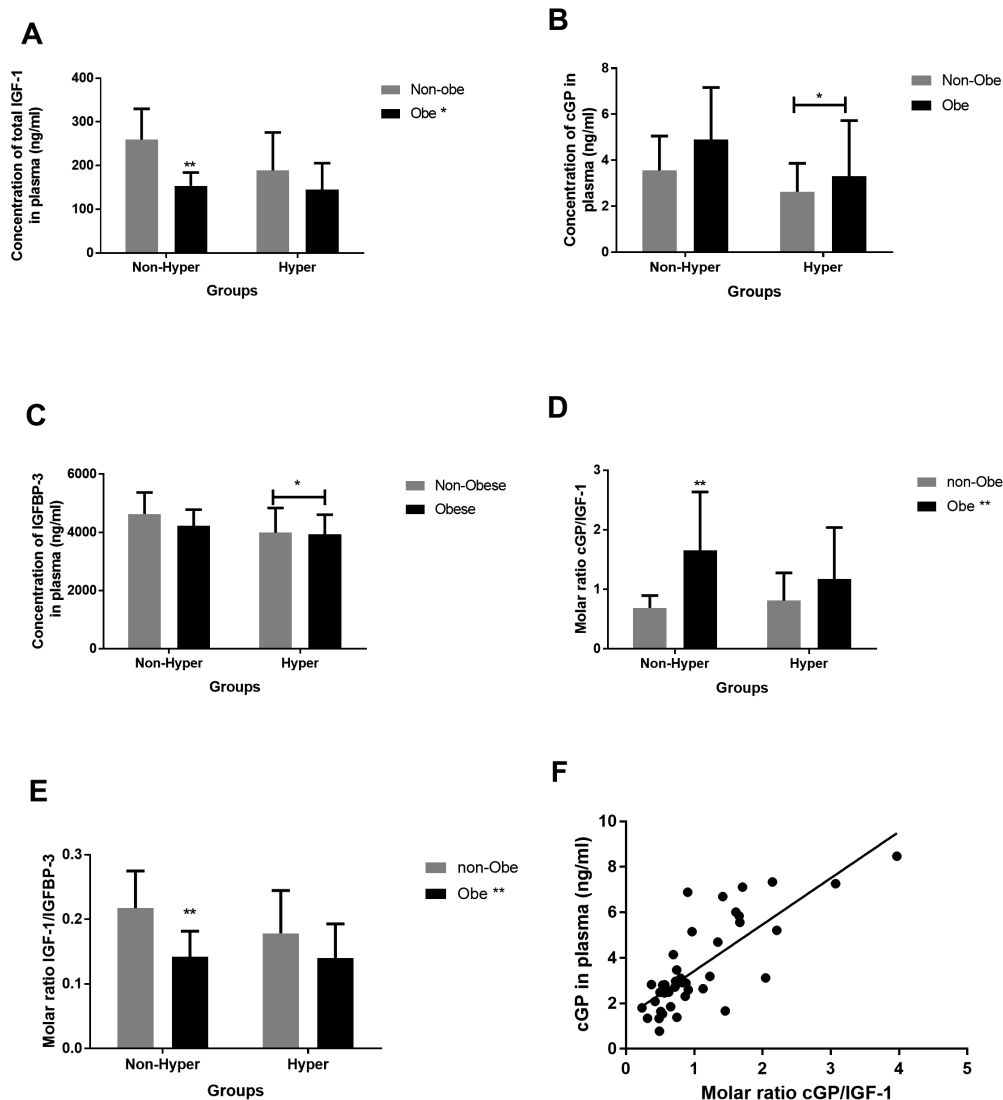


Fig. 2. Plasma concentrations of total IGF-1 (A), cGP (B), IGFBP-3 (C), the molar ratio of cGP/IGF-1 (D) and IGF-1/IGFBP-3 (E) in the women with and without hypertension (hyper) and/or obesity (Obe) and correlation between cGP and cGP/IGF-1 ratio (F). * $p < 0.05$, ** $p < 0.01$ indicate the difference between no-obese and obese; no-hyper and hyper; data presented as mean $\pm$ SD.

women ($p = 0.0093$). There was a significant correlation between cGP concentration and cGP/IGF-1 ratio (Fig. 2F, $r = 0.77$, $p < 0.0001$) in all groups. The design of study 2 meant that there was additional data for another 20 non-pregnant women for the analysis of cross-sectional study.

This showed consistent results for cGP and IGF-1 concentrations (and cGP/IGF-1 ratio) but the IGFBP-3 results no longer showed significant effects of hypertension. In comparison, the molar ratio of IGF-1 to IGFBP-3 continued to show effects of obesity, but not hypertension.

Table II. Clinical characteristics of participants in longitudinal Study 2.

	Obese/obese N=10&	Non obese/non obese (n=10)#	Obese/non obese (n=10)#	Non obese/obese (n=10)&
Age (years)	31.7 (6.1)	30.1 (3.2)	34.1 (3.9)	31.5 (2.6)
European	8 (80%)	10 (100%)	9 (90%)	9 (90%)
Waist (cm)	108.5 (16.2)	74.4 (3.2)	93.9 (7.7)	101.1 (10.3)
BMI at 15 weeks (kg/m ²)	34.6 (2.8)	22.1 (1.4)	30.9 (1.0)	27.5 (1.9)
BMI 6years PP(kg/ m ²)	36.8 (5.8)	21.7 (2.0)	28.2 (1.2)	32.6 (2.4)
BP (mmHg) Systolic Diastolic	122.8 (8) 71 (7)	106 (6) 64 (5)	118 (7) 70 (4)	117 (6.2) 67 (6)
Lipids (mmol/L) TG LDL HDL	1.3 (0.4) 3.4 (0.5) 1.5 (0.3)	0.7 (0.1) 2.7 (0.5) 1.6 (0.5)	1.1 (0.4) 3.4 (0.9) 1.6 (0.5)	1.2 (0.3) 3.4 (0.6) 1.6 (0.8)
Fasting glucose (mmol/L)	4.8 (0.6)	4.8 (0.3)	4.9 (0.4)	5.3 (0.6)
Metabolic syndrome*	1	0	1	2

Values are presented as means &: 20 non-pregnant women from study 2 were used for analysis of Study 1 # Additional 20 non-pregnant women from Study 2 were included the analysis of Study 1 which did not alter the changes of cGP and IGF-1 in both obesity and hypertension, but the change of IGFBP-3 no longer statistically significant in the hypertension group.

*Metabolic syndrome defined as per IDF criteria of BMI $> 30\text{kg/m}^2$ or waist $\geq 80\text{cm}$ plus any two of the following four features (1) raised triglycerides $\geq 1.7\text{mmol/L}$ or lipid treatment, (2) reduced HDL $< 1.29\text{mmol/L}$, (3) raised blood pressure based on systolic BP ≥ 130 or diastolic BP $\geq 85\text{mmHg}$, (4) raised fasting glucose $\geq 5.6\text{mmol/L}$, or glucose lowering treatment. Values in parentheses denote standard errors.

Study 2: Changes between the paired samples at 15 weeks gestation and 6 years postpartum

Compared to the stable normal weight control group (no-no) who showed an increase in IGF-1 over time, the concentrations of plasma IGF-1 were reduced in both groups who were obese at 15 weeks (Fig. 3A, $f[1,36] = 5.16$, $p = 0.0045$; obe-no, $p = 0.004$; obe-obe, $p = 0.018$), but only a marginal reduction in the group with weight gain over time (no-obe).

There was no significant difference in the changes of cGP concentrations between any groups (Fig. 3B). Compared to the control group the concentrations of IGFBP-3 were reduced over time significantly in the groups with weight loss (obe-no, $p = 0.0001$) and weight gain (Fig. 3C, no-obe, $p < 0.0001$), but no difference in the group who remained obese after pregnancy (Fig. 3C, obe-obe).

Compared to the control group, the changes of cGP/

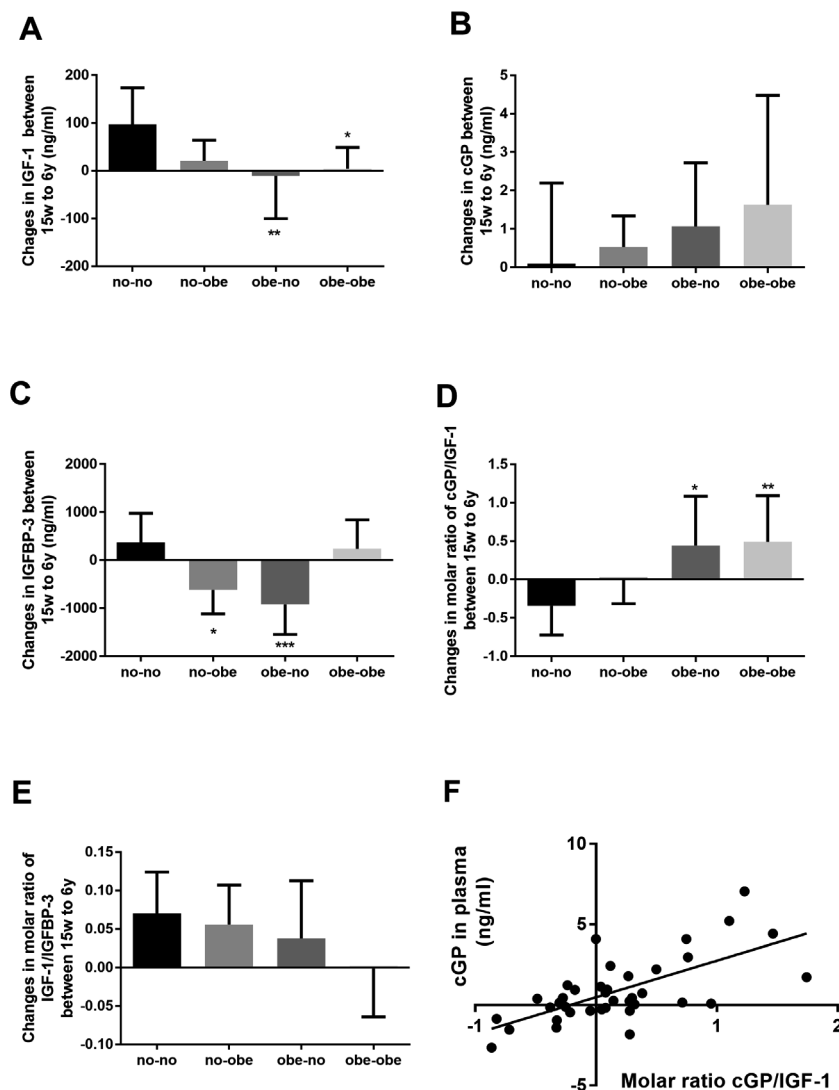


Fig. 3. Changes of plasma concentrations of total IGF-1 (A), cGP (B), IGFBP-3 (C), the molar ratio of cGP/IGF-1 (D) and IGF-1/IGFBP-3 (E) between 15 weeks during and 6 year after pregnancy in the women with weight gain (no-obe), or weight loss (obe-no) or remained to be normal (no-no) or remained to be obese (obe-obe), and the correlation between cGP and cGP/IGF-1 ratio (F). * $p < 0.05$, ** $p < 0.01$ indicate the difference from the control group (no-no), data presented as mean $\pm$ SD.

IGF-1 ratio was increased in the groups that were obese during pregnancy (Fig. 3D, obe-no, $p = 0.0026$, obe-obe, $p = 0.006$). There was no statistical difference in IGF-1/IGFBP-3 ratio between groups (Fig. 3E). The changes of cGP and cGP/IGF-1 ratio of all groups were significantly correlated ($r = 0.68$, $p < 0.001$, Fig. 3F).

Mathematical analysis

The mathematical model was used to estimate the

bound and unbound forms of IGF-1, cGP and IGFBP-3 (17, 19). This allowed a broader range of hypotheses to be tested on the effect of bound/unbound peptides on health outcomes. In study 1: The mathematical model reproduced the biological data of total IGF-1 (bound + free IGF-1), total IGFBP-3 (bound + free IGFBP-3) and cGP (bound + free cGP) and generated estimates of the concentrations of free IGF-1, cGP and IGFBP-3 in the plasma (Figs. 4 and 5).

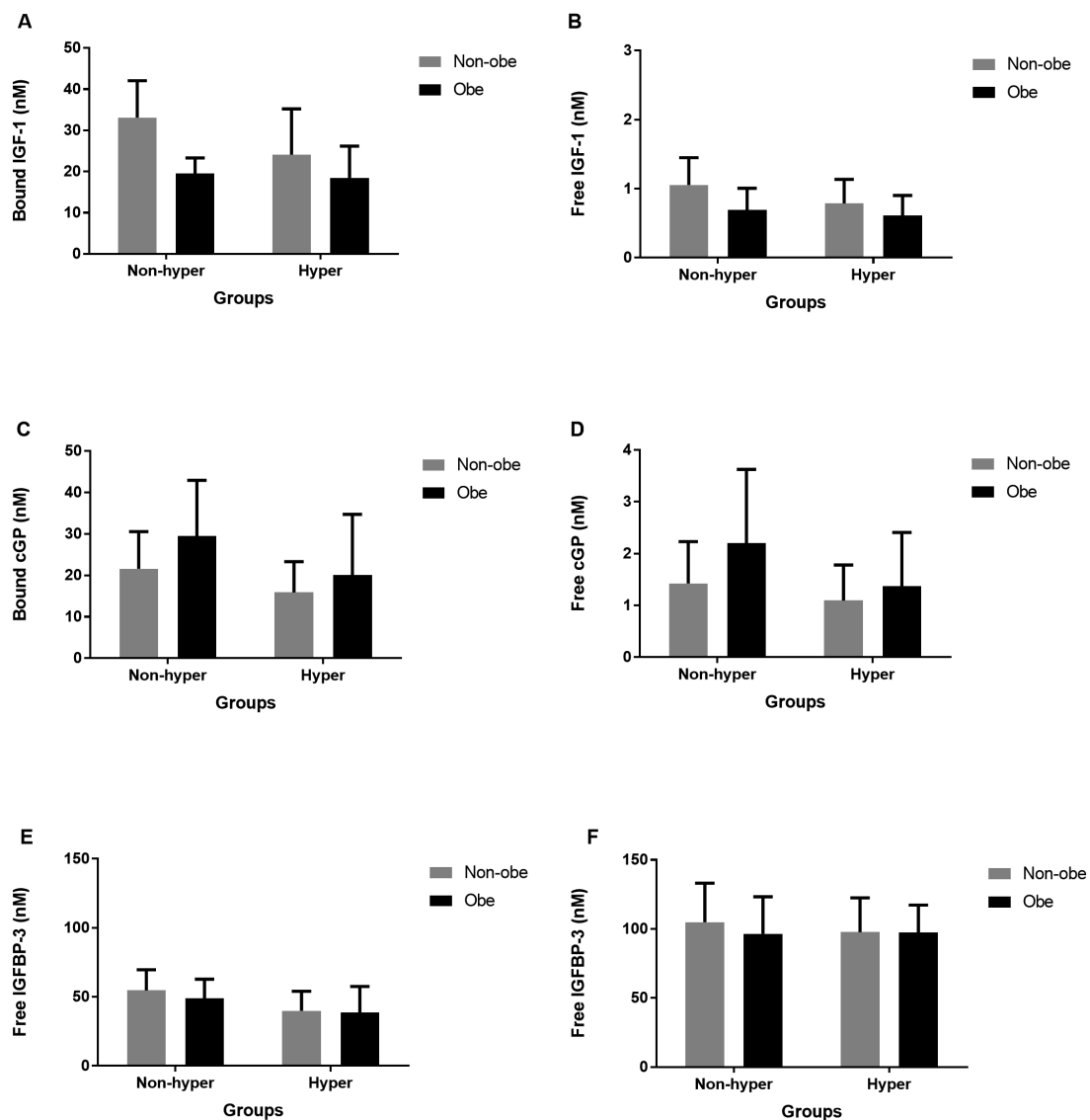


Fig. 4. Bound and free IGF-1 (A, B), bound and free cGP (C, D) and bound and free IGFBP-3 (E, F) which was generated through a mathematical model based on the biological data of Study 1, data presented as mean $\pm$ SD.

The majority of IGF-1 and cGP and only half of the IGFBP-3 were in the bound forms. The free cGP appeared to be higher than that of free IGF-1. The data from study 1 suggested the changes of free and bound peptides were in proportion. In study 2, the changes of bound IGF-1 and cGP, as well as the free IGFBP-3 were representative of biological data. However, the free IGF-1 generated from the mathematic model only represented the biological data of cGP/IGF-1 ratio in the control group and the groups with weight gain and

weight loss, but not the group which remained obese after pregnancy.

DISCUSSION

As a trophic response to low circulating IGF-1(18), cGP/IGF-1 ratio was increased in obese women 6 years post-partum. The data provide a first clinical evidence of the role for cGP in metabolism mediated through autocrine regulation of IGF-1.

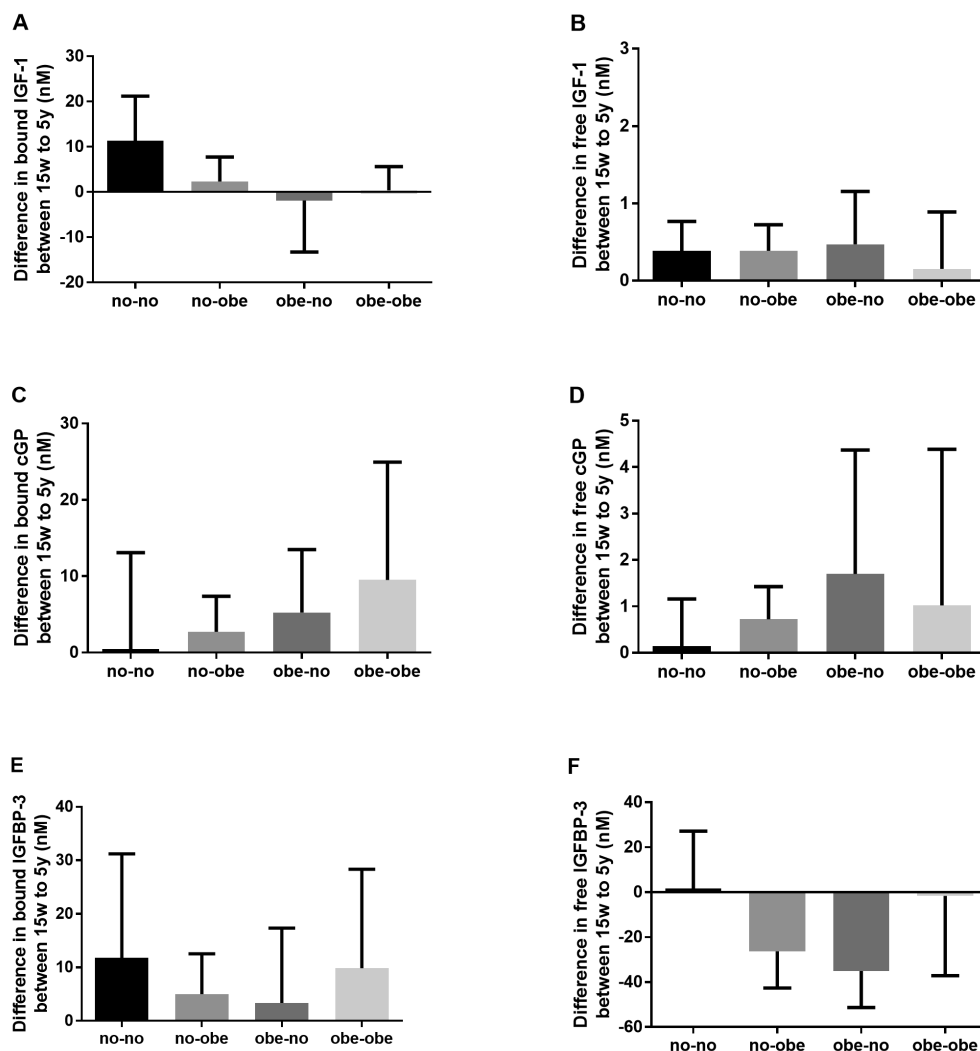


Fig. 5. Bound and free IGF-1 (**A, B**), bound and free cGP (**C, D**) and bound and free IGFBP-3 (**E, F**) which was generated through a mathematical model based on the biological data of Study 2, data presented as mean $\pm$ SD.

By comparing the changes of obese status from 15-week pregnancy to 6 years post-partum, the weight loss over time was associated with both decreased IGFBP-3 and increased cGP/IGF-1 ratio, an effective autocrine regulation to improve IGF-1 function. Women with weight gain over time were associated with a reduced IGFBP-3, but no change in cGP/IGF-1 ratio whereas the women who remained obese had an increased cGP/IGF-1 ratio, but no change in IGFBP-3. Thus, cGP-associated autocrine regulation of IGF-1 plays a role in the changes of obese status.

Low circulating IGF-1 is reported in human obesity (4), however the changes of plasma IGF-1 in hypertensive patients appeared to be more difficult to interpret (5). Consistent with these observations from larger clinical cohorts, we found low plasma IGF-1 in obese groups (Fig. 2A). A trend increase of cGP in the obese group (Fig. 2, A and B) led to an increase in cGP/IGF-1 ratio in obesity (Fig. 2D). We have recently reported that the cGP/IGF-1 ratio determines the bioavailability of IGF-1 with the higher ratio and the more bioavailable IGF-1 *in vitro* (17) and *in vivo* (18). The reversible binding of IGF-1 to IGFBPs acts as autocrine regulation for maintaining the homeostasis of bioavailable IGF-1 (28). As a key binding site of IGF-1 to IGFBPs, cGP regulates IGF-1/IGFBP-3 binding in a concentration-dependent, competitive manner (17-19). As an autocrine response to lower plasma IGF-1, cGP/IGF-1 ratio is higher in infant rats compared to their adolescent and adult counterparts (18).

Even though IGF-1 was not significant lower in hypertensive groups, we found a reduction of IGFBP-3, which could be an autocrine response to improve bioavailability of IGF-1 (Fig. 1C), thus an indirect indication for mild IGF-1 deficiency in hypertension. The hypertensive group also had significant lower cGP, leading to a 'no change' of cGP/IGF-1 ratio in hypertensive groups. The data suggest that cGP-associated autocrine regulation may not be involved in hypertension.

While the results remained the same in obesity, hypertension-associated low IGFBP-3 was no longer significant when the additional 20 post-partum samples from the longitudinal study were added to the analysis of cross-sectional study. Thus, low

IGFBP-3 in hypertensive women could be a chance finding possibly due to the small sample sizes. Even though the data were clear in obesity, larger clinical studies are essential to confirm the current findings.

IGF-1 deficiency has been indicated through circulating IGF-1, which represents largely the bound, inactive IGF-1 due to the small window of opportunity for measuring free IGF-1 (8). While the changes of circulating IGF-1, which associates with endocrine regulation of IGF-1, were similar in obesity and hypertension, cGP/IGF-1 ratio associated autocrine regulation may differentiate IGF-1 regulation and function between obesity and hypertension. With more clear indication of cGP/IGF-1 ratio in obesity, we subsequently analysed whether cGP/IGF-1 ratio would be involved in the change in obesity status over time between pregnancy and post-partum.

The role of GH-IGF system in obesity and metabolic syndrome is complex. GH is principally involved in regulating somatic growth either directly or indirectly by stimulating IGF-1 production, largely in liver, that mediates GH action on peripheral tissues (29). The IGF-1 concentration decreases during the first trimester of pregnancy in women with normal somatotroph functions as the increase of oestrogens during pregnancy suppresses GH regulated IGF-1 induction in the liver, characterised as liver resistance (20), which recovers post-partum. This shift of plasma IGF-1 during pregnancy and post-partum provide an opportunity to evaluate the involvement of cGP in homeostasis of IGF-1 function. The results from Study 2 showed consistent lower plasma IGF-1 in all groups, with IGFBP-3 and cGP largely unchanged during early pregnancy (data not shown). Compared to the data from pregnancy, the concentration of IGF-1 increased 6 years post-partum in the control groups due to the recovery of liver production of IGF-1, but remained low in all obese groups. Thus, we compared the changes between the paired samples collected at 15 weeks' pregnancy and 6 years post-partum.

Compared to the control group who remained normal weight overtime, the changes of IGF-1 were reduced in obese groups, particularly the groups that were obese during early pregnancy (Fig. 3A). The

data suggest that pregnancy-related obesity may associate with the persistence of low GH regulated IGF-1 production postpartum. Low plasma IGFBPs have been reported in women with pregnancy-related obesity (30). Given that binding of IGF-1 to IGFBP-3 reduces bioactive IGF-1, the decreased circulating IGFBP-3 would improve IGF-1 bioavailability. The increased cGP/IGF-1 ratio could further release IGF-1 from IGFBP-3. The group with weight loss showed a reduced IGFBP-3 and an increased cGP/IGF-1 ratio. In contrast, the group with weight gain had reduced IGFBP-3 only and the group who remained obese, without further weight gain, showed an increase in cGP/IGF-1 ratio only. While circulating IGF-1 was low in all obesity groups, collective responses of IGFBP-3 and cGP/IGF-1 ratio appeared to differentiate clinical phenotypes with changing obesity status. Even though the data were significant, a large clinical trial is essential to confirm our findings.

IGF-1 function in the normal control group was largely maintained through endocrine regulation to increase liver production after pregnancy with less involvement with autocrine regulation (17, 18). Similarly, adolescent rats have higher IGF-1 and lower cGP/IGF-1 ratio compared to their infant littermates (18). The changes of IGF-1/IGFBP-3 ratio (Fig. 3E), a commonly used biomarker for free IGF-1, was similar to that of plasma IGF-1 and did not differentiate between the clinical phenotypes with weight changes.

A small number of samples were used in the both cross sectional and longitudinal studies. The significant differences of cGP/IGF-1 ratio and IGFBP-3 from these small studies suggest the changes were sensitive, particularly when the samples from longitudinal study were compared. The sensitivity of these changes were also demonstrated recently in another longitudinal clinical trial that evaluated the regulatory role for cGP in neurological conditions (31). Using 7 paired samples collected before and after a treatment that may relate to the reduced clinical symptoms of anxiety and depression in Parkinson patients (31), we found significant increase of cGP in the cerebrospinal fluid which was correlated with plasma cGP/IGF-1 ratio. Paired

samples from longitudinal studies may eliminate the influence of clinical variations, thus increase the power of the statistics. The sensitive changes of cGP/IGF-1 ratio and IGFBP-3 may develop for clinical use as biomarker for predicting IGF-1 function.

Given the role for GH in regulating liver production of IGF-1 and IGFBP-3, the investigation into liver function and potential GH resistance due to fatty liver is a relevant and important aspect in obese groups (32). Unfortunately, we did not have liver imaging or biochemical data to assess fatty liver status in these participants.

The mathematical model was used to estimate the bound and unbound forms of IGF-1, cGP and IGFBP-3 (17, 19) a task which is beyond biological evaluation. The model was designed based on the concentration-dependent competitive binding between cGP and IGF-1 to the IGFBP-3 with the same affinity and allowed for a broader range of hypotheses to be tested (17, 19). While the bound IGF-1 and bound cGP were the dominant forms, the free IGFBP-3 was double the bound IGFBP-3, suggesting that the biological data were largely representing the bound forms of IGF-1 and cGP and free IGFBP-3 in both studies. The significance of analysing non-linear changes of these peptides is to reveal the relationship between bound peptides and free IGF-1 and dynamic relationships between them. In study 2, for example, the free IGF-1 was lowest in the group that remained obese and free cGP was lowest in the group that remained normal. The bound form of IGFBP-3 was relatively high in both groups where body weights remained unchanged, whereas the bound form of IGFBP-3 was relatively low in both groups with changes of obesity status. Further development of this mathematical model to include the enzymatic activities that breaks down IGFBP-3 and that convert IGF-1 to cGP will provide an additional tool to support large clinical trials.

Our study provides the first clinical confirmation for the role of cGP in energy metabolism through autocrine regulation. The increase in cGP/IGF-1 ratio is a positive response to maintain the homeostasis of IGF-1 function in obesity. The co-changes of cGP/IGF-1 ratio and IGFBP-3 in plasma may indicate the anticipated weight changes, which potentially

could be developed for clinical use as a biomarker if this is further confirmed in larger prospective clinical studies. Given that the pharmacokinetics and dynamics of cGP and its pharmaceutically developed analogues have been well characterised (16), it remains to be tested whether cGP could be developed for treating metabolic disorders.

ACKNOWLEDGEMENTS

The research was founded by the Health Research Council, New Zealand; New Enterprise Research Fund, the Ministry of Business, Innovation and Employment, New Zealand; the Evelyn Bond Fund, Auckland District Health Board Charitable Trust, New Zealand; EM and JT were supported in part by CureKids.

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EXPRESSION OF PROTEOGLYCANS IN TWO TYPES OF AMELOBLASTOMA: NOVEL IMMUNOHISTOCHEMICAL FINDINGS

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Received January 6, 2018 – Accepted March 28, 2018

Alterations in cellular and extracellular matrix components play an important role during tumorigenesis; proteoglycans are included among these components. Ameloblastomas are odontogenic tumors distinguished as invasive and infiltrative neoplasms and are divided into different histological types, the most common of which are the unicystic ameloblastoma and the conventional ameloblastoma. The aim of this study was to identify the presence of two proteoglycans, perlecan and biglycan, in different types of ameloblastoma. Using immunohistochemistry, we determined the presence of both proteins in 28 unicystic ameloblastomas and 23 conventional ameloblastomas. We identified the cytoplasmic and nuclear presence of perlecan and the cytoplasmic presence of biglycan in both types of ameloblastoma. The mean values of immunoexpression were higher in the conventional type compared to the unicystic type. Neither the presence of biglycan in ameloblastomas nor the nuclear presence of perlecan in any odontogenic tumor has previously been reported. The differential immunoexpression of perlecan and biglycan in these types of ameloblastomas suggests their participation in the developmental process of these tumors.

Ameloblastoma (AM) is defined as an epithelial benign odontogenic tumor with aggressive behavior due to its destructive potential, high recurrence rate and risk of malignant transformation (1).

The World Health Organization (2017) recognizes different types of AMs, which are divided based on their histological characteristics into the following types: conventional ameloblastoma (CA), unicystic ameloblastoma (UA) and peripheral ameloblastoma

(2). The most common AMs are the conventional type and the unicystic type. In some Latin-American studies the UA has been described as the most common, in contrast with other regions of the world, where the CA is the most frequent (1).

In addition to the histological differences between these two types of ameloblastoma, certain clinical features also vary, such as patient age at diagnosis, radiographic image findings, and macroscopic tumor

Key words: ameloblastoma, perlecan, biglycan

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0393-974X (2018)

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characteristics. As a consequence, the therapeutic considerations for each type may differ. Furthermore, there are differences at the molecular level; for example, some studies have reported a higher proliferation index in UA than in CA, suggesting that UA displays a more proliferative behavior (3).

During tumorigenesis, cells actively differentiate, resulting in more aggressive cells that are capable of invading the stroma and penetrating into the lymph vessels to allow metastasis (4). This sequence requires various interactions between the cells and their microenvironment; therefore, in order to invade, the cells must develop the ability to mold the extracellular matrix (ECM) through the secretion or activation of certain molecules such as proteoglycans (PGs), growth factors and enzymes (4). When these molecules are produced by tumor cells, they modulate growth and cell differentiation, thereby contributing to biological tumor behavior (5).

PGs represent one of the most important components of the tumor stroma; they are synthesized by stromal and tumor cells (4). PGs are glycoproteins formed by a core protein covalently linked to glycosaminoglycans (GAGs) (6). The primary biological function of different PGs depends on the biochemical and hydrodynamic properties of their GAGs (7).

Perlecan belongs to the family of heparan sulfate proteoglycans (HSPGs), which are major components of the basement membrane and the cell surface. As they are intimately associated with the plasma membrane, HSPGs act as important biological modifiers of growth factors (8). Perlecan is synthesized by fibroblasts, endothelial cells, and basal cells, and it is present in stromal spaces (9); it participates in different cellular processes such as adhesion, angiogenesis, migration, proliferation, thrombosis, lipid metabolism and cell death (8, 10, 11). Perlecan is related to both the early and late stages of embryonic development as well as to the development of cancer and diabetes. In the oral cavity, the overexpression of perlecan has been reported in basal cells, and its expression increases in cancer and pre-cancerous stages (9). The presence of perlecan has been reported in odontogenic tumors, including the AMs, suggesting its participation in

cell proliferation and differentiation (12).

The family of small leucine-rich proteoglycans (SRLPs) participates in ECM organization and in the regulation of cell behavior. The SRLPs facilitate protein-protein interactions between the cellular components of the ECM, and they also participate in hard tissue formation (13). Biglycan belongs to this family; it is an ubiquitous PG that interacts with different types of collagens (I-IV) and elastin. Therefore, it is considered a structural protein that regulates growth factors and cytokines. It acts as a signaling molecule and as a ligand for different receptors (14), participating in cellular differentiation, adhesion and proliferation (15). During the processes of inflammation and tissue injury, soluble forms of biglycan act as a danger signal by uniting the adaptive and the innate immune systems (14). The expression of biglycan has been reported in numerous diseases including cancer (14). Biglycan inhibits the *in vivo* growth of pancreatic cancer cells, and overexpression of the biglycan gene is related to tumor suppression mechanisms in salivary gland carcinoma (15). In hepatocellular carcinoma, the ectopic presence of biglycan suppresses cancer cell growth, and its overexpression is associated with a more favorable prognosis; on the other hand, biglycan overexpression is associated with tumor progression in colorectal cancer (4).

The study of AMs based on the presence of PGs allows us to probe the mechanisms of tumor progression, contributing to the understanding of the role of PGs in AM tumor progression. To date, there are no reports demonstrating the presence of biglycan in AMs. Therefore, the aim of this study is to determine the presence of biglycan and perlecan in UA and CA using immunohistochemistry.

MATERIALS AND METHODS

Tissue samples

Fifty-one tissue samples that had been previously fixed in 10% formaldehyde and embedded in paraffin blocks were obtained from a collection belonging to the Laboratory of Molecular Pathology at the School of Dentistry of the Universidad de la Republica (Uruguay). The blocks contained adequate amounts of tissue and

had no physical alterations. This study was evaluated and approved by the ethics committee of the School of Dentistry at the Universidad de la Republica, folio number 091900/000120/16. Every case was evaluated and classified according to the most recent WHO classification of head and neck tumors (2). The clinical features associated with each sample, such as patient age and gender and tumor size, were grouped as follows: age, ≤ 30 years and > 30 years; gender, male and female; and tumor size, ≥ 50 mm and < 50 mm.

Immunohistochemistry

Blocks were cut into 2- μ m-thick sections and treated with citrate solution (pH 6.2) and Tween 20 for epitope retrieval. Endogenous peroxidase activity was blocked with 0.9% hydrogen peroxide. A monoclonal antibody against perlecan (Santa Cruz/A7L6 Mouse Monoclonal, dilution 1:25) and a polyclonal antibody against biglycan (Santa Cruz/L-15 Goat Polyclonal, dilution 1:25) were used. The sections were incubated with the primary antibodies for 45 min; the sections were next incubated with a biotinylated anti-mouse/anti-rabbit antibody and then with a streptavidin/peroxidase complex reagent for 20 min each. The products of this reaction were visualized with a 3,3'-deaminobenzidine-H₂O₂ substrate. Finally, the samples were counterstained with Mayer's hematoxylin. For the negative controls, we omitted incubation with the primary and secondary antibodies as well as with the streptavidin/peroxidase complex; for the positive control, we did not omit any step.

Evaluation of immunoexpression

Immunoexpression of biglycan and perlecan was considered positive when staining was observed in the plasma membrane, the cytoplasm or the nucleus of the peripheral columnar tumor cells and the stellate reticulum-like tumor cells. Immunoexpression was evaluated semi-quantitatively by calculating the percentage positivity. The percentage of cells staining positive for each protein was determined by evaluating the tumor tissue present in every slide using the 10x and 40x microscope objectives; the result is expressed as the percentage of positive tumor cells for each type of AM. We then stratified the percentages on a four-grade immunopositivity scale: 0% staining was considered negative; 1-10%, low immunopositivity; 11-

50%, intermediate immunopositivity; and $> 50\%$, high immunopositivity (16).

Statistical analysis

The data were analyzed using IBM SPSS Statistics 23.0 (SPSS, Chicago IL, USA) to calculate the means and standard deviations of the clinical features and the percentages of biglycan and perlecan. The Mann-Whitney test (U-test) was used to determine the differences in immunoexpression between the types of AM. The results were considered significant for $p < 0.001$.

RESULTS

Twenty-eight out of 51 samples were classified as UA (54.9%), and 23 were classified as CA. The age range was 10 to 76 years, the mean age was 32.8 ± 17.6 years, and most of the samples were from patients ≤ 30 years old (54.9%). The samples were predominantly from female patients (56.9%). The mean tumor size was 49.4 ± 29.6 mm, and most of the tumors were ≤ 50 mm in size (60.7%).

We identified the presence of perlecan and biglycan in the cytoplasm of tumor cells in UA and CA. Fig. 1 shows perlecan-positive cytoplasmic staining in UA (a) and CA (b), as well as biglycan-positive cytoplasmic staining in UA (c) and CA (d). Interestingly, we found perlecan in the nuclei in one sample of UA (e).

Table I shows statistically significant differences between the mean values of perlecan and biglycan immunoexpression between types of AM; the mean immunoexpression value for both PGs is higher in CA ($p < 0.001$).

On the four-grade immunopositivity scale, most of the samples negative for perlecan and biglycan were UA, and most of the samples rated as having high immunopositivity for perlecan and biglycan were CA. Fig. 2 shows the differences between UA and CA in respect to immunopositivity ratings on the four-grade scale.

Differences were observed when the clinical features of the AMs were related to the immunoexpression of the proteins. These relationships are shown in Table II.

Table I. *Immunoexpression of perlecan and biglycan in UA and CA.*

	UA	CA	<i>P</i> value
	<i>n</i> =28	<i>n</i> =23	
	Mean ± SD (%)	Mean ± SD (%)	
Perlecan	42.32±33.85	47.87±35.13	<0.001
Biglycan	10.89±16.81	20.74±28.33	<0.001

UA: unicystic ameloblastoma; CA: conventional ameloblastoma; SD: standard deviation. The Mann-Whitney test indicates a p value <0.001 for the mean expression of both biglycan and perlecan between the two types of ameloblastoma.

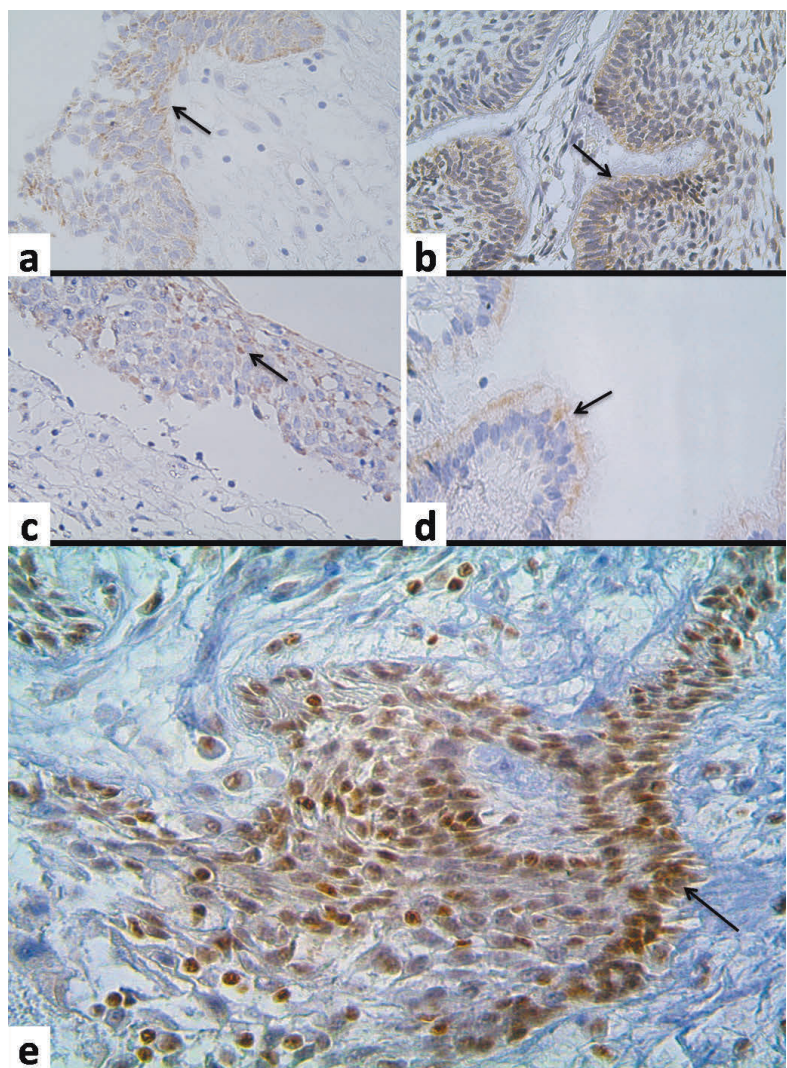


Fig. 1. *Immunohistochemical localization of perlecan and biglycan in ameloblastomas; (a, b) cytoplasmic immunoexpression of perlecan in the ameloblastomatous epithelium of UA (arrow) and in the columnar cells of CA (arrow), 400x; (c, d) biglycan-positive cytoplasmic staining in epithelium of UA (arrow) and epithelial islands in CA (arrow), 400x; (e) immunopositive nuclei for perlecan in epithelial cells (arrow) of an UA sample, 400x.*

DISCUSSION

Ameloblastomas are benign but aggressive tumors that are divided into different histological types. The most common types are UA and CA; CA has been described in multiple studies worldwide as the most frequent among all AMs. However, in our series, we found that UA was more frequent (54.9%) than CA. The authors of other studies in Latin American populations reported similar results, providing evidence that UA is the most common AM type in Latin America (3). Although this observation could be related to the diagnostic criteria used in these studies, the reason that UA is more common than CA in the Latin American population remains unknown. Ameloblastomas show a tendency to be more frequent in women than in men (ratio 1.3:1), they are diagnosed in the third decade of life (mean 32.88 years), and they occur more frequently in the molar area of the maxilla and the mandible. The most frequent radiographic pattern associated with AM is the unilocular pattern (3, 17, 18).

The immunoexpression of perlecan appears to be similar in both types of AM. Most of the perlecan-positive samples were from CA (78.26%), in comparison to UA (75%). In addition, the mean immunopositivity was higher in CA (47.87%) than

in UA (42.32%). In our study, the immunoexpression of perlecan was observed in both peripheral and stellate reticulum-like tumor cells. Ida-Yonemochi et al. (12, 19) reported the presence of perlecan in the intercellular spaces of the central portion of AMs, suggesting that perlecan could be synthesized by the tumor cells and accumulate within the intercellular spaces, acting as a carrier and transporting nutrients within the ameloblastomatous foci. In contrast, the results of the present study indicate that both the reticulum-like neoplastic ameloblasts and the peripheral tumor cells synthesize perlecan, releasing it to carry growth factors that may act as nutrients to stimulate tumor growth.

On the other hand, Tsuneki et al. (20, 21) found that deposits of perlecan were present in odontogenic keratocyst tumors and UA, although they were absent in other cystic but non-neoplastic lesions, suggesting that perlecan participates in tumor development processes. Thus, perlecan might be related to more aggressive behavior in neoplastic tissues.

Perlecan has been demonstrated to promote tumor growth, migration, invasion, angiogenesis and chemoresistance in various malignant tumors through the regulation of different growth factors linked to heparin (22).

Perlecan is a large HSPG expressed in a variety of

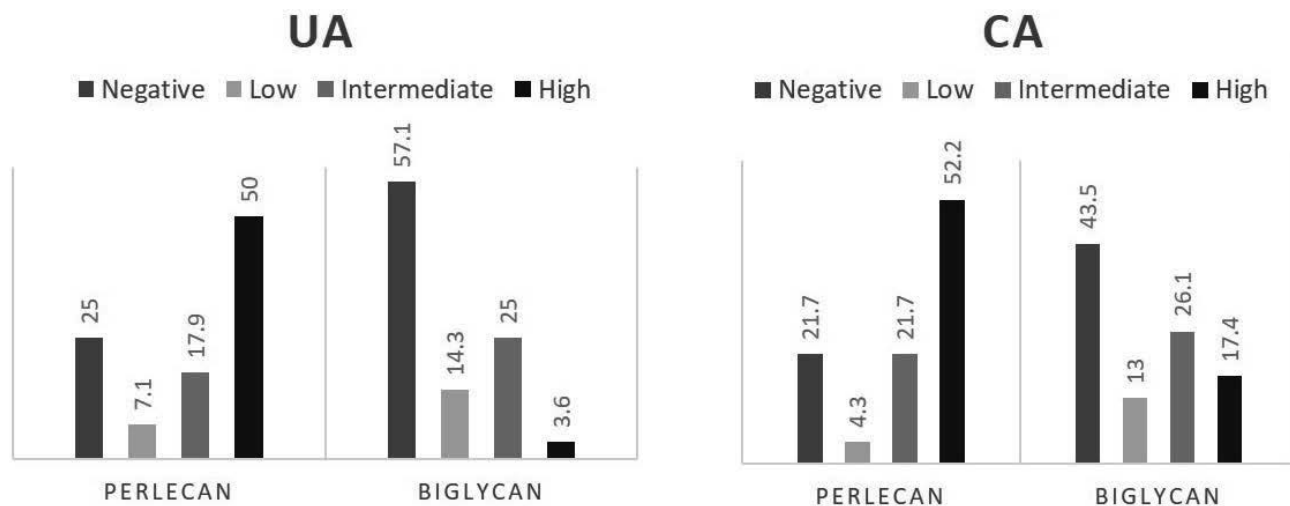


Fig. 2. Percentage of unicystic ameloblastoma (UA) and conventional ameloblastoma (CA) samples classified in each grade of the immunopositivity scale.

tissues, and it regulates both normal and neoplastic processes (23). Perlecan plays an important role in pathological neoangiogenesis due to its ability to link its HS chains to two of the most important angiogenic growth factors: VEGF and bFGF. Perlecan promotes the progression of malignant neoplasms such as breast cancer, prostate cancer, colon cancer and melanoma through this pro-angiogenic role (24). Thus, we suggest that perlecan might participate in the tumor biology of AMs by performing any of the roles previously mentioned. However, elucidating the participation of perlecan in tumorigenic processes and determining its specific role will likely require further studies to identify its coexpression with these growth factors.

One of the most interesting findings of this study was the evidence that perlecan is present in the nucleus of AM tumor cells. To our knowledge, the nuclear localization of perlecan has not previously been reported in AM or any other odontogenic tumor. The presence of HS or HSPG in the nucleus of various types of cells has been controversial since the first report of this localization in the 1970s. Currently, the mechanism by which HS is transported to the nucleus remains unknown. Nuclear HS was originally believed to result from contamination during the lysis procedure; however, the structure of nuclear HS was subsequently demonstrated to be different from the structure of HS present in any other cell compartment (25). In normal fibroblasts, nuclear

heparin sulphate (HS) has been observed to inhibit cell proliferation; however, this anti-proliferative role is not maintained in lung carcinoma, indicating that malignant tumor cells could lose the ability to respond to HS (25). According to multiple authors (26), the presence of HSPGs in the nucleus could be related to the interaction between their HS chains and nuclear HS; therefore, it participates in different cellular processes, such as cell proliferation, inhibition of topoisomerase I, and inhibition of gene transcription. On the other hand, most of the growth factors that translocate to the nucleus bind to HSPGs (26); this observation is notable because it suggests that HSPGs might act as carriers for these growth factors and thus help stimulate tumor growth.

However, despite both our evidence that perlecan is present in the nucleus of AMs and previous reports of its similar localization in other tumor cells, little is known about the role that perlecan plays in the nucleus, in contrast to other HSPGs such as syndecan and glypican (26). Nuclear perlecan deposits have been observed in astrocytes during tissue damage repair processes but have not been detected in normal brain tissue (26).

Biglycan was detected in 49.01% of the AM samples. The mean immunoexpression of biglycan in UA was 10.89%, and its presence was detected in 42.85% of the UA samples, while the mean immunoexpression of biglycan in CA was 20.74%, and its presence was detected in 56.52% of the CA samples.

Table II. Relationships between the clinical features associated with the AM samples and the protein immunoexpression.

Clinical features	n=51	Mean			
		%Perlecan		%Biglycan	
		UA	CA	UA	CA
<i>Age (years)</i>					
10-30	28	48.00	46.44	13.05	17.11
>30	23	30.33	48.79*	6.33	23.07*
<i>Gender</i>					
Male	22	44.08	47.30	12.58	16.90
Female	29	41.00	48.31	9.63	23.69*
<i>Size (mm)</i>					
≤50	31	45.53	52.36	11.12	20.50*
<50	20	37.36	40.89	10.55	21.11*

UA: unicystic ameloblastoma; CA: conventional ameloblastoma

These results cannot be compared or contrasted with other studies because, to the best of our knowledge, this is the first study of this kind performed in AM. However, previous studies have reported the presence of biglycan in other odontogenic tumors. Zhao et al. (5) reported the presence of biglycan in 26.6% of odontogenic myxoma samples in their study. Because biglycan interacts with type IV collagen (one of the primary components of the ECM), they suggested a possible structural role for this protein. Modolo et al. (15) determined that the role of biglycan in odontogenic adenomatoid tumor is related to the host immune response during tumorigenesis. Our results may contrast with their findings; although AMs are also benign tumors, they are more aggressive compared to odontogenic myxoma and adenomatoid odontogenic tumor. Furthermore, AMs are divided into distinct histological types. In the present study, we observed that biglycan expression was higher in CA, which is considered the most invasive and destructive type of AM. Therefore, biglycan could be associated with biological behavior and aggressiveness in this neoplasm.

Other studies report the presence and possible roles of biglycan in diverse diseases, including neoplastic processes (4, 21, 27). Depending on the tissue, biglycan performs different functions: under physiological normal conditions, biglycan is sequestered as an ECM component; however, when tissues undergo stress, biglycan is released by enzymatic activity and acts as a danger signal through activation of the immune response (28). During chronic inflammation, biglycan stimulates tumorigenesis, allowing the accumulation of diverse proinflammatory molecules; however, this activity seems to depend upon tumor type as well as development stage (29).

The overexpression of biglycan has been observed in various malignant neoplasms such as colorectal cancer, endometrial cancer, gastric cancer, and pancreatic cancer, among others. The presence of biglycan has been proposed to be related to tumor progression, whereby it stimulates tumor growth by promoting angiogenesis (22). For example, interactions with VEGFA and VEGFR allow biglycan

to act as a pro-angiogenic molecule (29); therefore, it participates in the processes of invasion and tumor metastasis (30). Biglycan is overexpressed in endothelial tumor cells, but not in normal endothelial cells, demonstrating its participation in angiogenesis (31).

In melanomas, biglycan is believed to enhance tumor stiffness, conferring a greater invasion potential (32). The results of the present study indicate that biglycan immunoreexpression is lower in UA compared to CA. The differential presence of biglycan in the structural composition of each type of tumor may be influencing the invasive behavior of CA, which differs from UA in that it is considered a cystic lesion.

As previously described, both perlecan and biglycan are PGs that participate in tumorigenesis by stimulating angiogenesis through their interaction with VEGF (29). However, this interaction has not been studied in AM, and the mechanism underlying the interaction of these three proteins has not yet been elucidated.

The current study is the first description of the presence of biglycan in AM; thus, determining a specific role of this PG in these tumors is not possible. However, questions regarding the interaction of biglycan with other proteins, such as growth factors, may open the door to more specific lines of investigation. Moreover, the current study is also the first report of the nuclear presence of perlecan in any odontogenic tumor. Only a few studies regarding the presence of perlecan in the nuclei of tumor cells exist; therefore, additional studies using other techniques may help to elucidate the role of perlecan in AM.

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TETRAMETHYLPYRAZINE INHIBITED HYPOXIA-INDUCED EXPRESSION OF CALCIUM-SENSING RECEPTORS IN PULMONARY ARTERY SMOOTH MUSCLE CELLS IN CHICKENS

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Received March 1, 2018 – Accepted March 21, 2018

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Tetramethylpyrazine (TMP) is a biologically active ingredient, which is isolated from a popular Chinese medicinal plant. It has been used effectively to treat ischemic heart problems, cerebrovascular and thrombotic vascular diseases. This study was designed to evaluate the effect of TMP on calcium-sensing receptors in pulmonary artery smooth muscle in chickens. For this purpose forty day-old chicks were distributed into five groups: the control group, the hypoxia group (kept under low Oxygen treatment), and TMP groups (kept under low Oxygen treatment along with treatment of different concentrations of TMP). The pulmonary artery smooth muscle cells were also cultured on 6-well plates in high glucose culture medium and divided into the same five groups. We used *in vivo* and *in vitro* study models by applying immunohistochemistry, RT-qPCR assay and Western blotting analysis. Our results showed that pre-incubation with hypoxia markedly stimulated the activation of calcium-sensing receptor (CaSR) in pulmonary artery smooth muscle cells (PASMCs). The TMP decreased the mRNA and protein levels of CaSR. Treatment with TMP clearly inhibited the activation of all CaSR in a dose-dependent manner. Our data demonstrated that TMP can down-regulate the expression of CaSR. Therefore, these findings provide a new target to treat pulmonary arterial hypertension (PAH) under hypoxic conditions.

Pulmonary hypertension or pulmonary arterial hypertension (PAH) is highly susceptible to metabolic diseases in rapid growing broiler chickens. It is associated with rapid growth, which is not paralleled by algometric growth of the heart and lungs, and

leads to right heart hypertrophy, failure and death (1-3). PAH is a metabolic disorder associated with hypoxia, which mainly exists at high altitude, where the environment is cold and hypoxic (1, 4). Currently, predictable pathogenesis of PAH is considered to be

Key words: pulmonary arterial hypertension CaSR, PASMCs, hypoxia, TMP, chickens

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0393-974X (2018)

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as follows: hypoxia - heart/lung overload - vascular remodeling - PAH - right heart failure - right heart load capacity increase - cirrhosis and accumulation of ascitic fluid into the abdominal cavity (1, 4). Previous studies suggested the calcium steady-state maladjustment plays an important role in pulmonary artery smooth muscle cell in hypoxic pulmonary vasoconstriction (5). Calcium-sensing receptor (CaSR) is important in maintaining and regulating cell proliferation, differentiation, and calcium homeostasis, such as oxygen activation increase vascular CaSR expression, it is the upstream regulatory factor of vascular smooth muscle proliferation and pulmonary artery high pressure formation (6, 7).

Tetramethylpyrazine (TMP) is one of the most important active ingredients of traditional Chinese medicine, and has been identified with several molecular targeting properties (Fig. 1) (8-10). It has been reported that it is widely used to treat pulmonary hypertension secondary to chronic obstructive pulmonary diseases. Additionally, studies have confirmed previously reported characteristics of TMP as a Ca²⁺ antagonist in vascular tissues (8). To date, the effect of tetramethylpyrazine on calcium-sensing receptor in PSMCs in hypoxia has not been reported in chickens. Therefore, this study intended to determine CaSR expression in PSMCs and the effect of tetramethylpyrazine in hypoxia condition to explore its possible signaling mechanisms via CaSR expression. This signaling transduction pathway can be used for treating PAH syndrome in chickens, and our findings provide new experimental evidence to control PAH.

MATERIALS AND METHODS

Animals

Forty day-old chicks were purchased from a commercial hatchery and were raised under standard conditions. Tetramethylpyrazine was purchased from the Deruifeng commercial company (Jinan, China). After 14 days, the birds were distributed into five equal groups (n=8 chickens in each group) the control group (CG), the hypoxia group (HG), in which the chickens were kept under low oxygen treatment (17% O₂); the TMP groups, in which the chickens were kept under low oxygen treatment along with treatment of different concentrations of TMP (50 μM, 100 μM, and 200 μM) according to previous study (11). All the AA broiler chickens were given anesthesia and sacrificed after 24 h. The pulmonary artery was quickly collected and was fixed in 10% formalin for subsequent histopathological experiments.

All experiments were approved and reviewed by the Animal Welfare and Ethics Committee by following the national legislations regarding animal welfare.

Cell culture and treatment

The pulmonary artery smooth muscle cells (PSMCs) were cultured in high glucose culture medium (Servicebio co., Ltd, Wuhan) according to our previous study (12). The PASM cells were seeded onto 6-well plates at 37°C for 24 h. Then, the cells were divided into five groups including control group, the hypoxia group (HG), and TMP groups in which the cells were pretreated with different concentrations (50 μM, 100 μM, 200 μM) of TMP in hypoxia condition according to previous study (11), after which cells were cultured for 24 h.

Table I. Characteristics of primer pairs specific for qPCR.

Primer	Sequence (5'-3')	Reference
CaSR	Forward: TTCGGCATCAGCTTTGTG	Xing et al., 2010
	Reverse: TGAAGATGATTTCGTCTTCC	
GAPDH	Forward: CTCAACTACATGGTCTACATG	
	Reverse: TGGCATGGACTGTGGTCATGAG	

Immunohistochemistry (IHC)

CaSR protein was detected and localized in pulmonary artery, and PASMC level was examined by IHC according to Song et al. (13) and Yang et al. (14).

Quantitative real-time polymerase chain reaction (RT-qPCR) assay

Total RNA (tRNA) were extracted from the cell samples of each group by using Trizol method. All other procedure from tRNA to cDNA and then RT-qPCR was performed in triplicates according to our previous study (14). The primers are shown in Table I.

Western blotting analysis

The total proteins of the collected cells and pulmonary artery were extracted using RIPA lysis solution containing a phosphatase repressor. Whereas, other procedures like Western blotting were performed according to our previous study (15).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software and results are presented as means $\pm$ standard error of means (S.E.M). $P < 0.05$ used for statistically significant.

RESULTS

Immunohistochemical (IHC) evaluation of cells and pulmonary artery

To confirm the expression of CaSR in pulmonary artery and PASMCs, the samples

were examined using immunohistochemical techniques and observed with a light microscope. The immunohistochemical results are presented in Figs. 2 and 3. The results showed that CaSR was highly expressed in the hypoxic group as compared to the control group. In contrast, pulmonary artery and PASMCs treated with different doses of TMP and the expression of CaSR were reduced obviously with the increased treatment of TMP. The immunoreactivity of these samples showed suppressive effects of TMP were increased in a dose-dependent manner. The negative control showed no expression in this study.

Decreased CaSR in TMP induced primary cultured PASMCs

To evaluate the effect of different doses of TMP on CaSR in PASMCs, immunofluorescence staining was performed. As illustrated in Fig. 4, there was a decrease in the expression of CaSR protein with the increase of TMP in a dose-dependent manner by pretreatment up to concentration of 200 μ M.

TMP down regulated the expression of CaSR gene

To further confirm the effect of TMP in hypoxic conditions, the activation of CaSR was assessed by qPCR and Western blotting. As shown in Figs. 5 and 6, pre-incubation with hypoxia markedly stimulated the activation of CaSR in PASMCs. The TMP can decrease the mRNA and protein levels of CaSR. Treatment with TMP clearly inhibited the activation of all CaSR in a dose-dependent manner.

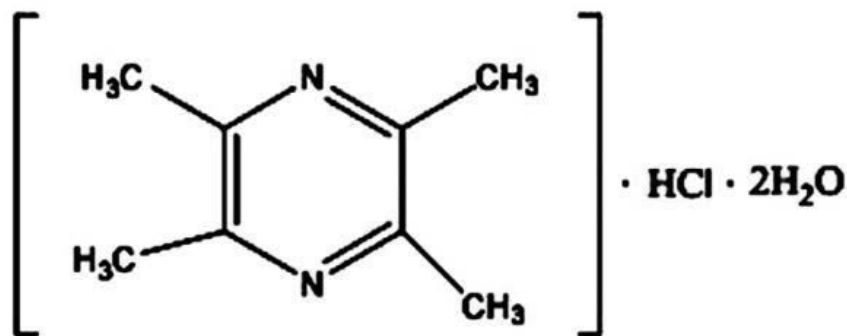


Fig. 1. Chemical structure of ligustrazine.

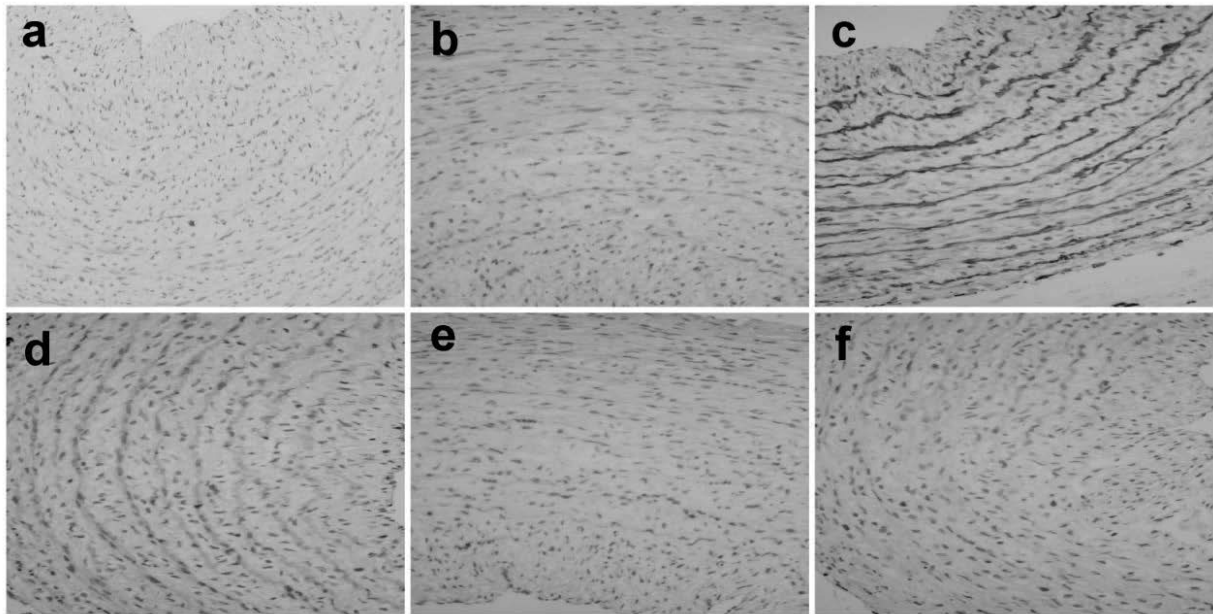


Fig. 2. Effects of CaSR in immunohistochemical sections of pulmonary artery tissues with different treatments. **a)** negative control group; **b)** control group; **c)** hypoxia group; **d)** hypoxia add TMP group (50 μM); **e)** hypoxia add TMP group (100 μM); **f)** hypoxia add TMP group (200 μM).

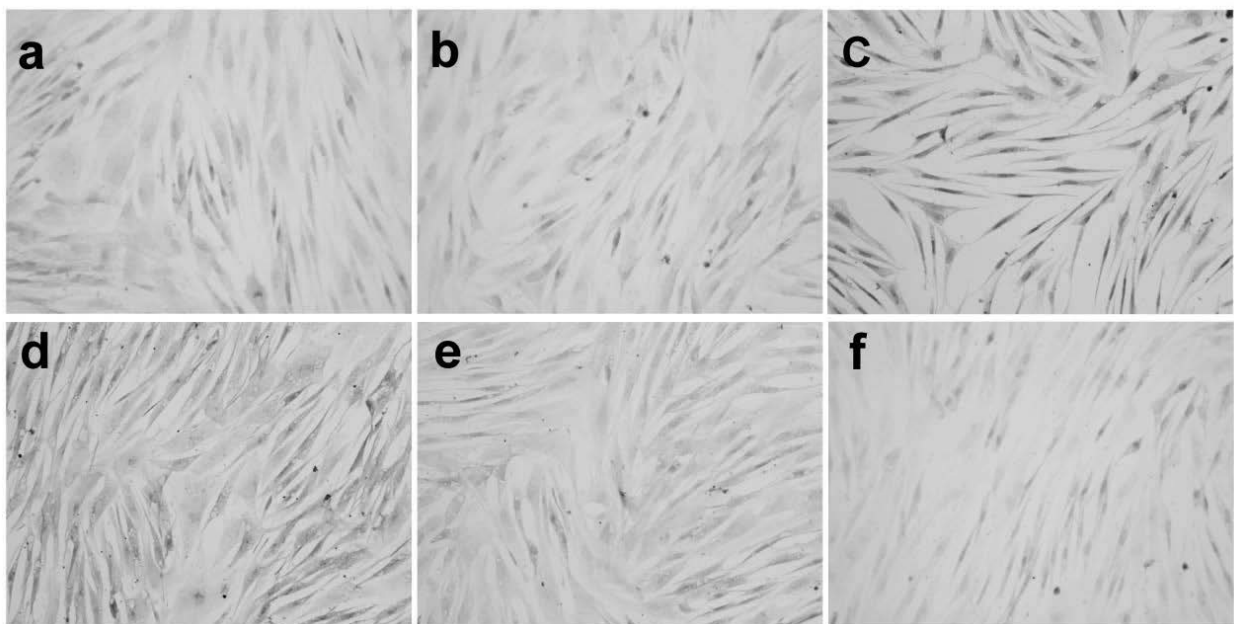


Fig. 3. Effects of CaSR in immunohistochemical sections of PASMCs with different treatments. **a)** negative control group; **b)** control group; **c)** hypoxia group; **d)** hypoxia add TMP group (50 μM); **e)** hypoxia add TMP group (100 μM); **f)** hypoxia add TMP group (200 μM).

DISCUSSION

Pulmonary arterial hypertension is also known as Ascites syndrome (AS), which remains a clinically challenging disease with subsequent

economic losses to the poultry industry, especially in broiler chickens (16-18). Pulmonary arterial remodeling presents a key step in the development of AS (16). In PSMCs, Ca^{2+} signaling pathway is closely associated with PASM contraction

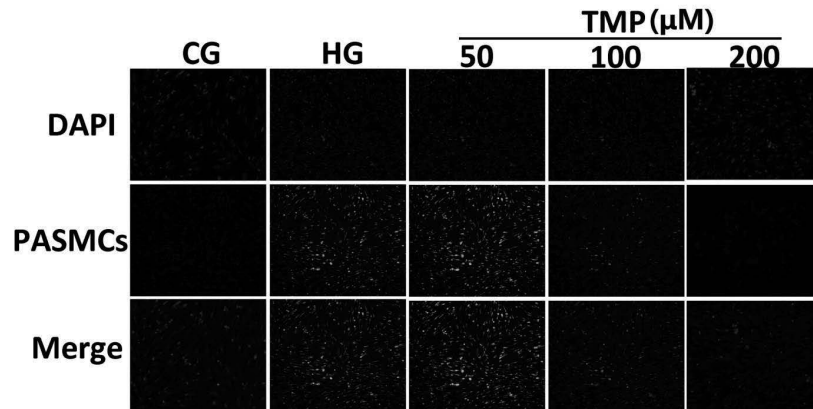


Fig. 4. The effects of TMP on the intracellular calcium concentrations in the PSMCs.

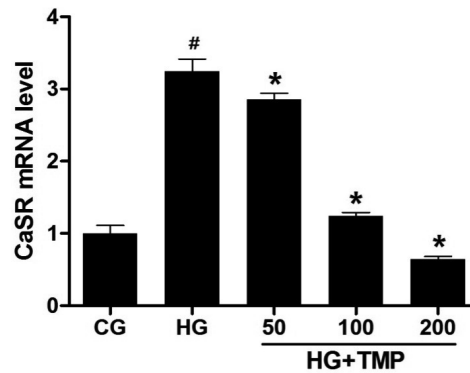


Fig. 5. Hypoxia and TMP regulate CaSR mRNA level in PSMCs. qPCR was performed to assess the mRNA levels of CaSR. CG refers to control group; HG refers to hypoxia group. ^{*} $P < 0.05$ vs CG; [#] $P < 0.05$ vs hypoxia.

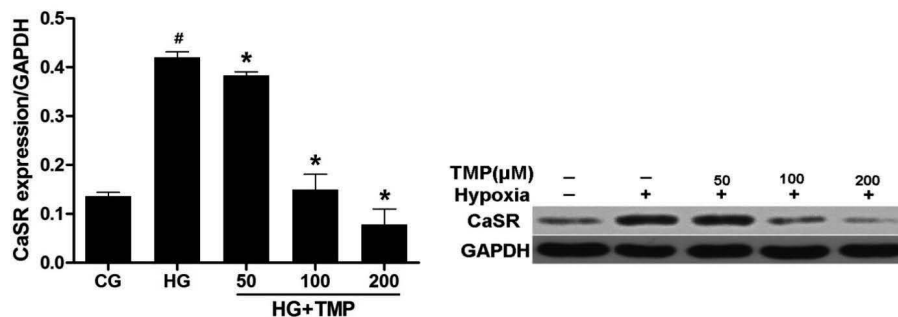


Fig. 6. Hypoxia and TMP regulate CaSR genes expression in PSMCs. Western blotting was performed to detect the protein levels of CaSR. The GAPDH was used as a control. CG refers to control group; HG refers to hypoxia group. ^{*} $P < 0.05$ vs CG; [#] $P < 0.05$ vs hypoxia.

and remodeling that connect with AS via change of Ca^{2+} levels in cells and further elevate the vascular resistance to blood flow and pulmonary hypertension (19, 20). Previous studies have confirmed that the occurrence of hypoxia-induced pulmonary vascular remodeling is linked with PAH (21). It has the same clinical features of hypoxia, pulmonary hypertension, vascular remodeling and pathological features (22, 23).

Tetramethylpyrazine is one of the active ingredients of the traditional Chinese medicine, and it has been reported to protect animals subjected to chronic hypoxia, modulate arterial resistance, platelet function, microcirculation, capillary permeability, inhibit extracellular Ca^{2+} influx in ventricular myocytes and intracellular Ca^{2+} release in vascular smooth muscle (8, 24). However, the effect of TMP on precise molecular mechanism of pulmonary artery smooth muscle cells, especially CaSR remodeling regulating under PAH progression, remains unclear. Therefore, the aim of the present study was to determine the effect of TMP on the expression of CaSR. Based on our results, we found that pre-incubation with hypoxia markedly stimulated the activation of CaSR in PSMCs, and the TMP can decrease the mRNA and protein levels of CaSR. Treatment with TMP clearly inhibited the activation of all CaSR in a dose-dependent manner in chickens. Our data demonstrated a direct positive effect that TMP could down-regulate the expression of CaSR in PSMCs at the mRNA and protein levels in PSMCs in a dose-dependent manner.

Our results confirm that hypoxia plays an important role in CaSR expression in PSMCs. In addition, our findings reveal that TMP has significant effect on the expression of CaSR, which provides new insights for prevention and control of PAH in chickens.

ACKNOWLEDGEMENTS

This study was supported by National natural science youth fund project (No:31502132), Hubei Provincial Natural Science Foundation of China (Grant No: 2014CFB244) and Fundamental Research Funds for the Central Universities (Program No: 2011PYO78).

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REGULATION OF VITAMIN D RECEPTOR AND GENISTEIN ON BONE METABOLISM IN MOUSE OSTEOBLASTS AND THE MOLECULAR MECHANISM OF OSTEOPOROSIS

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Received February 8, 2018 – Accepted April 10, 2018

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The aim of this work was to study the mechanisms of vitamin D receptor (VDR) and Genistein (Gen) on the regulation of bone metabolism of phytoestrogens from cellular and epidemiological perspectives. MC3T3-E1 cells were treated with different concentrations of Gen, and the cell-proliferation rate was detected by an MTT colorimetric assay. The effect of the VDR receptor blocker ZK159222 on the Gen effect was then observed; after adding Gen to MC3T3-E1 cells, we detected the expression of VDR protein via Western blotting. After adding estrogen receptor α -blocker MPP and estrogen receptor β -blocker PHTPP, we observed the effect of Gen on the regulation of the VDR protein. DNA was extracted from the blood samples of 200 postmenopausal women in the early epidemiological survey, and the restriction fragment length polymorphism of VDR gene Apa I and Bsm I in each sample was observed. The results were analyzed using dietary survey and bone mineral density examination. The results show that 10^{-8} mol/L Gen can promote the proliferation of MC3T3-E1 cells ($P < 0.05$). This effect can be canceled by the VDR blocker ZK159222. By analyzing the Apa I and Bsm I genotypes of VDR restriction sites, we discovered no significant difference in bone mineral density (BMD) between different genotypes ($P > 0.05$). In addition, there was no significant correlation between dietary phytoestrogen intake and BMD in different genotypes ($P > 0.05$). In conclusion, VDR can mediate the effect of Gen on the proliferation of MC3T3-E1 cells. The up-regulated expression of VDR protein in Gen is not mediated by the estrogen receptor. Moreover, the VDR gene polymorphism is not related to the BMD in various parts and is not related to the bone metabolism effect of the dietary plant estrogen intake.

Postmenopausal osteoporosis (PMOP) is a bone metabolism imbalance due to the rapid decrease of estrogen level after physiological menopause in women under natural conditions (1, 2). Estrogen replacement therapy can reduce the incidence of fractures and can effectively prevent bone loss (3). In structural terms, phytoestrogens are similar to endogenous estrogens and can produce pseudo-

estrogen-like biological activity (4-6). The role of phytoestrogen in bone metabolism and bone mineral density (BMD) has been repeatedly confirmed, however, conclusions remain inconsistent. Some studies report a relationship between the regulation effect of bone metabolism, estrogen, and VDR (7, 8). Duque et al. (9) showed that the expression of VDR in osteoblasts decreased in female C57BL/6J

Key words: VDR, genistein, osteoporosis, phytoestrogen

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0393-974X (2018)

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mice after ovariectomy, and the expression of VDR was increased after 17β -estradiol supplementation. Estrogen not only up-regulated the VDR, but also enhanced the promoter activity of the VDR gene, which may indicate a relationship (10). The active form of vitamin D is its hydroxylated metabolite $1,25(\text{OH})_2\text{D}_3$ (or calcitriol). The latter is associated with VDR and participates in the metabolism of bone (11-13). A large number of studies have also demonstrated the relationship between VDR expression, dysfunction, and the pathogenesis of osteoporosis. VDR plays an important role in the regulation of bone physiology (14, 15). Therefore, whether phytoestrogen modulates bone metabolism in relation to VDR can be observed by studying the level of cytology. Similarly, whether VDR receptor gene polymorphism is involved in dietary phytoestrogen metabolism can be determined through epidemiological studies.

MATERIALS AND METHODS

Main reagents and materials

Cell lines: mouse osteoblastic cell line MC3T3-E1 was purchased from Celltech (Shanghai) Biological Engineering Co. Ltd.

The main reagents: Gen, Trypsin, NaHCO_3 , MPP, N,N'-Methylene bisacrylate amine, Glycine, PMSF, Agarose, EDTA (Sigma USA), MTT, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, DMSO, Streptomycin, Penicillin sodium, PBS, Tris, PVDF membrane, Protein Extraction Kit (Solarbio); Ethidium bromide, acrylamide, sodium amide, ammonium persulfate, Sodium dodecyl sulfate (SDS), Coomassie brilliant blue G250, Ponceau, Enhanced chemical fluorescent reagent (Ameresco USA); Goat anti-rabbit IgG / HRP enzyme labeling, Goat anti-mouse IgG / HRP enzyme labeling, Restriction enzymes A paI , Restriction enzyme B smI , Blood Genomic DNA Extraction Kit, PCR purification kit, BCA Protein Assay Kit, 2xEasyTaq PCR SuperMix, 6xDNA Loading Buffer, FBS, E2, VDR primary antibody, and β -actin polyclonal antibody (Gibco USA).

Main equipment: Super clean workbench (Hangzhou Haomei Experimental Equipment Co., Ltd.); inverted microscope, micro liquid take-off device (Nanjing Madison Instrument Co., Ltd.); Vortex shaker, PCR

instrument (Gibco USA); high-speed freezing centrifuge, enzyme-linked immunosorbent assay (ELISA) (Nanjing DeTie laboratory equipment Co., Ltd.); PHS-25 pH meter (Chengdu Shangjie Environmental Technology Co., Ltd.); Nucleic Acid Protein Analyzer (Shimadzu, Japan); Mini-PROTEAN3 electrophoresis system, Mini Trans-Blot Transfer System (Bole, USA); and UV-751 UV Spectrophotometer (Shanghai Lingxi Instrument Co., Ltd.).

Experimental modeling and grouping

The culture of MC3T3-E1 cells was carried out first. The cells were evenly inoculated into four 96-well cell culture plates. When about 80% density was reached, new medium was added to each well. The final concentrations of Gen required in each experiment were 0 mol/L, 10^{-10} mol/L, 10^{-9} mol/L, 10^{-8} mol/L, 10^{-7} mol/L, 10^{-6} mol/L. The cell culture plates were incubated at 37 C and 5% CO_2 . After three 96-well cell culture plates were cultured for 24 h, 48 h, and 72 h, the MTT assay was performed. The absorbance of each cell at 490 nm was measured by ELISA. Another 96-well cell plate was cultured for 72 h to extract cell proteins. VDR protein expression was measured by Western blotting. To verify whether the increase of VDR protein expression after joining Gen was related to the estrogen receptor, new experimental groups were designed. The control group did not receive any treatment; E2 10^{-8}M (Estradiol (E2) makes the final concentration of 10^{-8}mol/L); Gen 10^{-8}M (Gen concentration is 10^{-8}mol/L); Gen 10^{-8}M and MPP 10^{-7}M (Gen concentration is 10^{-8}mol/L and MPP concentration is 10^{-7}mol/L), and MPP is an estrogen receptor α blocker; Gen 10^{-8}M and PHTPP 10^{-7}M (Gen concentration is 10^{-8}mol/L and PHTPP concentration is 10^{-7}mol/L), and PHTPP is an estrogen receptor β blocker. When cell density reached 90%, the cells were changed and treated according to the above groupings. The treated cells were incubated at 37 C in a 5% CO_2 incubator. After 72 h, the cell protein was extracted and the expression of VDR protein was measured by Western blotting.

Biological analysis

MC3T3-E1 cells were treated with different concentrations of Gen. Cell proliferation rates were detected by MTT colorimetry. The absorbance of each cell at 490 nm wavelength was detected by ELISA. The

experimental data was recorded, and the experimental results were analyzed. Gen was added to MC3T3-E1 cells. The expression of the VDR protein in MC3T3-E1 cells was detected by Western blotting. The estrogen receptor α blocker MPP was added. The effect of Gen on the regulation of the VDR protein was observed after the estrogen receptor β blocker PHTPP.

In the study, 200 postmenopausal women were examined at the Tonglu County Chinese Medicine Hospital. Unnatural menopause was excluded, such as history of ovarian and hysterectomy surgery, long-term or recent use of bone metabolism-affecting drugs or health products, history of fracture, or rheumatoid arthritis patients with bone disease. The sample survey included two major items: the basic profile (age, weight, etc.) and dietary surveys. Dietary surveys included a history of food frequency (FFQ) and a 24-hour diet review. Next, 10 ml of venous blood samples were taken from each subject, and 5 ml of blood was drawn into the anticoagulant to perform DNA extraction. In addition, 5 ml was used for the detection of biochemical indexes. The BMD of the lumbar L2-L4, the large trochanter, the femoral neck, and other parts of the lumbar spine were also tested. All patients volunteered to participate in the experiments and signed an informed consent. The medical ethics committee of Tonglu County Chinese Medicine Hospital discussed this research and agreed with all the procedures of this study.

By analyzing the FFQ findings for all samples, it was found that the average daily phytoestrogen intake was 51.14 ± 31.65 mg. Based on this average daily intake of phytoestrogens, all samples were divided into low, medium, and high intake groups. The general profile between the sample group lumbar and hip BMD was also compared.

Treatment of blood samples

The genomic DNA was extracted, and the absorbance values of DNA at 280 nm and 260 nm were measured respectively on OD280 nm and OD260 nm in the nucleic acid

protein analyzer. According to the formula: DNA content = $OD_{260nm} \times \text{dilution factor} \times 50 \text{ mg/ml}$; DNA purity was anticipated to be 1.7-1.9. The Apa I and Bsm I restriction sites in the VDR gene were selected. Gene fragments of Apa I and Bsm I enzyme sites in the VDR were amplified. Fragment lengths were 740bp and 822bp. The fragments were then digested with Apa I and Bsm I restriction enzymes, respectively. The primers of the Apa I site were: Forward 5'-CAGAGCATGGACAGGGAGCAAG-3', Reverse 5'-GCAACTCCTCATGGCTGAGGTCTCA-3'; The primers of the Bsm I site were: Forward AACCAAGACTACAAGTACCGCGTCAGTG-3', Reverse ACCAAGACTACAAGTACCGCGTCAGTG-3'.

The candidate gene site was amplified by PCR. The products of PCR were then purified and treated. Lastly, enzyme digestion and identification of PCR products were carried out. There are 3 genotypes that can be obtained by agarose gel electrophoresis: a completely closed homozygous type (AA or BB), the completely cut wild type (aa or bb), and the heterozygous type (Aa or Bb) in which only one homologous chromosome is cut off, as shown in Table I.

Statistical analysis

The data obtained in this study are expressed as mean $\pm$ standard deviation ($\bar{x} \pm SD$). SPSS 19.0 was used to analyze the corresponding experimental data. Each group of experimental data underwent a homogeneity of variance test and one-way analysis of variance. A *t*-test was used to compare between groups. When $P < 0.05$, there was a significant difference between the groups. $P < 0.001$ showed significant difference.

RESULTS

Relationship between phytoestrogen intake and bone mineral density

Table II shows that compared with the low and medium groups high levels of phytoestrogen

Table I. *Fragment length and genotyping of two enzyme cutting sites after endonuclease digestion.*

Site	Fragment (bp)	Genotype		
		Heterozygous	Wild type	Homozygous
Apa I	220/520	Aa	aa	AA
Bsm I	172/650	Bb	bb	BB

Table II. Statistical table of correlation between phytoestrogen intake and bone mineral density.

Intake	Low	Medium	High	P
Cases	66	69	65	-
Total phetoestroGen(mg/d) ^b	16.2±6.4	36.8±7.2	96.6±9.5	<0.001
Weight (Kg)	14.8(7.2,22.4)	37.9(31.5,44.3)	66.9(53.2,80.6)	0.007
Height (cm)	58.2±7.2	59.2±8.1	61.7±7.6 ^{*,#}	0.532
BMI(kg/cm ²)	155.7±6.1	156.7±5.2	157.4±5.1	0.025
Age(y)	23.7±2.7	23.5±3.1	24.5±2.9	0.108
Years since menopause(y)	57.1±5.2	57.6±5.5	55.7±5.4	0.432
Lumbar Spine BMD(mg/cm ²)	8.3±5.9	7.3±6.3	7.2±6.2	0.025
L2-BMD(mg/cm ²)	914±154	920±143	978±175 ^{*,#}	0.042
L3-BMD(mg/cm ²)	821±152	837±133	889±146 [*]	0.036
L4-BMD(mg/cm ²)	882±156	899±139	961±173 [*]	0.187
Total Hip BMD(mg/cm ²)	882±151	912±136	926±176	0.372
Femoral neck BMD(mg/cm ²)	823±122	826±115	852±119	0.835
Trochanter BMD(mg/cm ²)	723±123	710±108	721±117	0.368
Ward's BMD(mg/cm ²)	577±102	603±101	591±98	0.406

BMD, age, and years since menopause were analyzed by ANCOVA; The data statistics of total phetoestroGen are: mean ± SD, median (25th, 75th interquartile); *: Compared with low intake group, $P < 0.05$; #: Compared with mid intake group, $P < 0.05$.

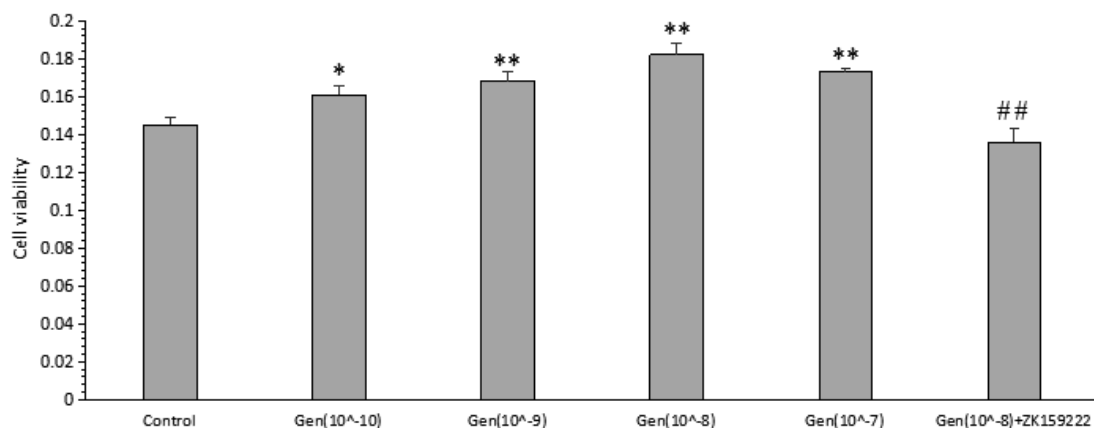


Fig. 1. The effect of Gen on the proliferation of MC3T3-E1 cells. (Compared to the control group, $*P < 0.05$, $**P < 0.01$; ## indicates Gen(10⁻⁸)+zkl159222 compared to Gen(10⁻⁸) $P < 0.01$)

intake were statistically significant ($P = 0.025$). The BMD of L2 and L3 vertebrae with high levels of phytoestrogen intake were higher than those with low and middle levels ($P > 0.05$), whereas there was no significant difference in L4 groups. There was no significant difference in BMD between hip and femoral neck, greater trochanter, or Ward's triangle among all groups.

The effect of Gen on MC3T3-E1 cells

The effects of different Gen concentrations on the proliferation of MC3T3-E1 cells are shown in Fig. 1. Compared with the control group, the proliferation rates of other groups were increased by different degrees ($P < 0.05$). The VDR receptor blocker ZK159222 can cancel the Gen-induced osteoblast proliferation.

As shown in Figs. 2 and 3, different concentrations of Gen acted on MC3T3-E1 cells for 72 hours. Compared with the control group, the expression of VDR proteins in different concentrations of Gen

increased to some extent ($P < 0.05$). The concentration of 10^{-8}M was the most obvious.

As seen in Fig. 4, compared with the control group E2, both Gen groups can make the VDR protein expression increase. The expression in the E2 group was slightly higher than that in the Gen group. There was no significant change in Gen group compared with the Gen+MPP and Gen+PHTPP 22 groups.

Gene polymorphism

Primers for the restriction enzyme sites Apa I and Bsm I were respectively added to the DNA extracted from the blood sample. PCR was performed according to the respective PCR reaction system and conditions. The band size of Apa I was 740 bp, whereas the band size of Bsm I was 822 bp. After the PCR products of VPA gene Apa I and Bsm I were digested with the respective restriction enzymes, the product was subjected to agarose gel electrophoresis. After the site Apa I was digested, the product was

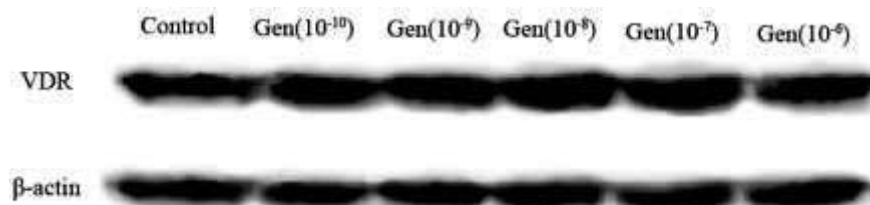


Fig. 2 Western blot analysis of VDR in different Gen concentrations.

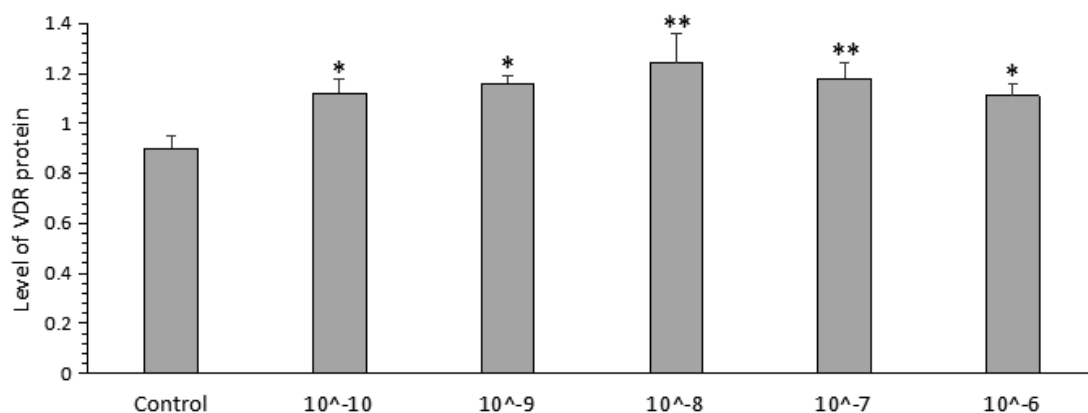


Fig. 3. VDR protein expression in different concentrations Gen. (Compared with the control group, * $P < 0.05$, ** $P < 0.01$)

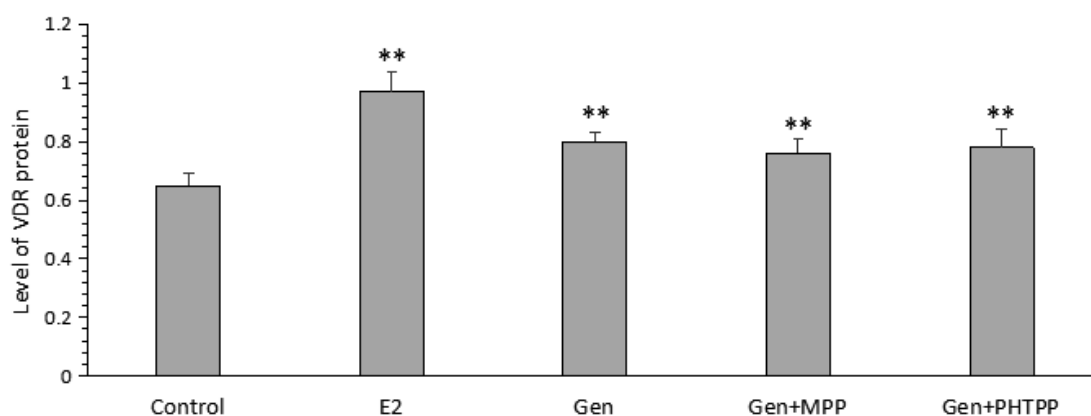


Fig. 4. Expression of VDR protein in different groups. (Compared with the control group, ** $P < 0.01$).

subjected to agarose gel electrophoresis, and the three genotypes were observed: AA with one band (740bp), Aa with three bands (740bp, 520bp, 220bp), and aa with two bands (520bp, 220bp). After the site Bsm I was digested, the product was subjected to agarose gel electrophoresis. Three genotypes were observed: BB with one band (820bp), Bb with three bands (820bp, 650bp, 170bp), and bb with two bands (650bp, 170bp).

Relationship between VDR gene polymorphism and BMD

Samples were then grouped according to the VDR gene polymorphism. RFLP distribution of the Apa I restriction sites was AA (14%), Aa (40%), and aa (46%), respectively. This distribution of genotypes meets the Hardy-Weinberg equilibrium ($\chi^2=3.545$, $0.10 < P < 0.25$, $P > 0.05$). RFLP distribution of the Bsm I restriction sites was BB (3%), Bb (23.67%), and bb (73.33%). This satisfies the Hardy-Weinberg equilibrium ($\chi^2=1.201$, $0.50 < P < 0.75$, $P > 0.05$). The statistical comparison between VDR gene polymorphism and BMD is shown in Table III.

Relationship between genotype and estrogen intake of VDR and BMD

Samples of each genotype were grouped by tertile and analyzed further. After multiple comparisons, we concluded that there was no correlation between femoral BMD, lumbar BMD,

L2-BMD, L3-BMD, and L4-BMD among high, medium, and low groups.

DISCUSSION

In this study, we found that Gen can up-regulate the expression of VDR protein similarly to 17β -estradiol. The effect of Gen on up-regulation of MC3T3-E1 cell proliferation can be reversed by VDR receptor antagonists. These results suggest that phytoestrogen can exert osteocyte proliferative effects through VDR pathways. However, the use of $ER\alpha$, $ER\beta$ antagonists did not abolish Gen up-regulated VDR effects. This suggests that other pathways may be involved. Studies show that estrogen exerts genomic effects in addition to its nuclear receptors α and β . It also activates ERK/MAPK signaling through non-genomic effects. VDR expression in osteoblasts is regulated by this pathway (16-18).

In the VDR gene, two adjacent restriction fragment length polymorphism (RFLP) sites of Apa I and Bsm I were closely studied (19, 20). Bolu et al. demonstrated that individuals with AA genotypes at the Bsm I polymorphism had higher femoral neck BMD than GG genotypes (21). Dunder et al. conducted a study of postmenopausal women and showed that aa genotypes had lower BMD at the lumbar spine than the AA type (22). A case-control study of a post-menopausal woman in Turkey found that the Apa I and Bsm I effects of

Table III. Statistical comparison between VDR gene polymorphism and BMD.

	AA	Aa	aa	P
Cases	28	80	92	-
Total phetoestroGen(mg/d)	27.51±26.13	34.42±33.32	33.88±25.42	0.787
Weight (Kg)	60.67±7.16	58.82±9.83	58.88±7.41	0.746
Height (cm)	157.46±3.87	156.63±5.74	158.12±5.52	0.676
BMI(kg/cm ²)	24.79±2.33	23.90±3.42	23.68±3.12	0.553
Age(y)	57.72±5.12	57.65±5.38	55.44±5.67	0.286
Years since menopause(y)	11.25±7.73	6.51±2.12	6.14±4.38	0.143
Lumbar Spine BMD(mg/cm ²)	908.75±109.4	926.52±171.4	976.91±187.1	0.356
	7	1	8	
L2-BMD(mg/cm ²)	780.97±106.3	759.03±102.6	855.27±185.8	0.247
	6	4	8	
L3-BMD(mg/cm ²)	798.45±104.8	799.36±118.7	887.92±208.3	0.312
	3	1	6	
L4-BMD(mg/cm ²)	778.92±118.3	808.15±116.7	872.03±221.3	0.455
	0	2	1	
Total Hip BMD(mg/cm ²)	912.63±136.1	828.73±129.1	847.03±129.2	0.251
	8	4	6	
Femoral neck BMD(mg/cm ²)	647.45±112.8	689.03±71.45	704.02±124.3	0.534
	3		2	
Trochanter BMD(mg/cm ²)	534.17±89.38	561.04±81.24	566.99±111.32	0.767
Ward's BMD(mg/cm ²)	435.31±117.6	506.17±89.32	517.19±128.7	0.353
	3		3	
	BB	Bb	bb	P
Cases	6	47	147	-
Total phetoestroGen(mg/d)	42.06±31.73	36.25±37.43	30.92±23.13	0.614
Weight (Kg)	55.27±5.23	61.42±10.65	59.84±8.12	0.323
Height (cm)	157.05±7.11	155.25±5.35	158.15±5.63	0.341
BMI(kg/cm ²)	22.28±1.76	25.13±4.43	23.84±2.65	0.131
Age(y)	58.62±8.14	55.27±6.22	57.02±5.18	0.421
Years since menopause(y)	8.66±7.14	8.52±6.46	7.85±6.33	0.872
Lumbar Spine BMD(mg/cm ²)	870.13±141.5	982.64±201.3	936.58±143.6	0.331
	4	7	5	
L2-BMD(mg/cm ²)	802.83±171.2	840.09±213.6	800.93±120.7	0.614
	5	2	3	
L3-BMD(mg/cm ²)	830.25±160.8	869.56±237.0	836.12±135.2	0.725
	3	9	3	
L4-BMD(mg/cm ²)	823.67±183.2	893.81±261.0	830.23±131.9	0.434
		3	8	
Total Hip BMD(mg/cm ²)	763.42±187.5	901.89±153.9	836.01±102.7	0.063
	3	2	6	
Femoral neck BMD(mg/cm ²)	661.62±106.2	705.62±160.7	697.59±79.27	0.558
	9	4		
Trochanter BMD(mg/cm ²)	558.01±98.19	572.16±135.1	566.28±71.02	0.965
		8		
Ward's BMD(mg/cm ²)	476.81±1324	532.73±168.5	503.06±87.13	0.436
		6		

the VDR polymorphic site interact and the AaBb combination increases the risk of osteoporosis by more than 5 times (23). A survey of postmenopausal women in Argentina found that the Bsm I genotype of the VDR gene has lower vertebral and femoral BMD (24). This study found no difference in BMD between different genotypes of Apa I and Bsm I gene polymorphisms in samples. This suggests that VDR gene polymorphism in postmenopausal women has nothing to do with bone metabolism.

Previous studies have found a positive correlation between dietary phytoestrogen intake and lumbar spine BMD in the sample. DNA was re-extracted from blood samples collected from the same batch of samples. The relationship between dietary phytoestrogen intake and BMD at different sites in different genotypes of Apa I and Bsm I RFLP polymorphism sites was analyzed. No significant association was found between the various genotypes at the two polymorphic sites. Brodowska et al. treated 44 postmenopausal osteoporosis patients for one year of HRT (25). The results showed that patients with BB genotype at the Bsm I site of VDR gene had a lower therapeutic effect. Our results suggest that genotypes of Pvu II and Xba I polymorphisms of ER α gene in postmenopausal women have specific effects on the bone metabolism regulation effect of dietary phytoestrogen. This further suggests that analysis of the Pvu II and Xba I polymorphisms of the ER α gene in samples may predict the bone metabolic effects of dietary phytoestrogen uptake.

In summary, similar to 17 β -estradiol, Gen upregulates the expression of VDR protein. The effect of Gen upregulation of MC3T3-E1 cell proliferation rate can be abolished by VDR receptor antagonists, which is similar to previous studies. In addition, the VDR gene polymorphism was not associated with bone mineral density at all sites and bone metabolic effects of dietary phytoestrogen intake. The shortcomings of this study lie in the fact that the number of samples is relatively small, which cannot fully reflect the overall situation. In addition, the regulation of osteoblasts mediated by VDR through phytoestrogens in cell experiments still needs further investigation.

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miR-145 IS CRITICAL FOR MODULATION OF VASCULAR SMOOTH MUSCLE CELL PROLIFERATION IN HUMAN CAROTID ARTERY STENOSIS

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Received March 3, 2018 – Accepted March 21, 2018

miR-145 is highly expressed in vascular cells, where it regulates phenotypic switching and vascular homeostasis, but its role in carotid artery stenosis (CAS) is controversial. In the present study, the expression of miR-145 was assessed by real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in human samples (both plasma and/or endarterectomy samples) from patients with symptomatic CAS and in controls without CAS. The mouse carotid artery ligation (CAL) model was used to determine the role of miR-145 on vascular smooth muscle cells *in vivo* (VSMCs) by using a mimic of or an inhibitor of miR-145. We found that miR-145 expression was significantly reduced in the plasma and plaque from patients with CAS ($p < 0.01$). The expression of miR-145 in the mouse CAL model, as assessed by qRT-PCR, was significantly reduced compared to the carotid arteries of the control group ($p < 0.01$). *In vitro*, enhancement or inhibition of miR-145 in VSMCs demonstrated that miR-145 significantly inhibited proliferation of VSMCs ($p < 0.05$); *in vivo*, enhancement of miR-145 significantly inhibited neointimal formation in the CAL model ($p < 0.01$). These results demonstrate that the expression of miR-145 is reduced in human CAS, miR-145 plays a critical role in CAS by modulation of VSMC proliferation, suggesting that MiR-145 may present a potential therapeutic option for treating CAS.

Stroke burden in China has increased over the past 30 years and has become the leading cause of serious long-term disability. The age-standardized prevalence, incidence and mortality rates of strokes in China have been reported as 1114.8/100,000 people, 246.8 and 114.8/100,000 person/years, respectively (1). Carotid artery stenosis (CAS) is a well-established risk factor

for ischemic stroke, contributing to 10–20% of strokes or transient ischemic attacks (TIAs) (2). Symptomatic carotid stenosis over 70% carries an annual stroke risk of 10-15% (3). The molecular mechanisms underlying stenosis of the carotid artery are therefore good targets for stroke prevention. These molecular interactions are complex and highly regulated, so understanding

Key words: miR-145, carotid artery stenosis, vascular smooth muscle cells, proliferation

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0393-974X (2018)

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the regulatory mechanisms involved in carotid artery stenosis and plaque passivation is crucial for optimal CAS treatment.

MicroRNAs (miRNAs) represent a class of endogenous, noncoding small RNA molecules (~22 nucleotides in length) that comprise a fundamental layer of post-transcriptional regulation of gene expression. miRNAs inhibit translation of mRNAs into proteins by binding to specific sites in the 3' untranslated region (3'UTR) of their target mRNAs, which can serve to negatively regulate gene expression via degradation or translational inhibition of their target mRNAs (4, 5). More importantly, a single miRNA can regulate the expression of multiple genes (5). Accumulating data show that miRNAs regulate a wide range of physiological and pathological processes, including elucidation of a clearly established role in cardiovascular function and disease (6–10).

MicroRNA-145 (miR-145) is the most abundant miRNA in vascular walls (11). It has been reported to control the phenotypic switching of contractile vascular smooth muscle cells (VSMCs), such that miR143/145 KO mice tend to accumulate VSMCs with a proliferation-favoring synthetic phenotype, rather than the WT contractile phenotype (12). miR-145 may also play an anti-proliferative role in the vascular wall (13). The miR-145/miR143 cluster also modulates the response of endothelial cells (ECs) to shear stress and plays a fundamental role for EC/VSMC interaction by modulating EC function and vascular homeostasis (14). In humans, miR-145 has been reported to be downregulated in coronary artery disease and aortic aneurysm (15, 16). While there are published reports of miR-145 expression in CAS, the results are controversial. Some studies reported that miR-145 is overexpressed in symptomatic plaques compared to non-symptomatic plaques (17, 18). Other groups reported significantly lower miR-145 levels in the atherosclerotic plaques in samples taken during carotid endarterectomy, compared to adjacent, “normal,” plaque-free areas (19).

The present study aims to determine miR-145 expression in human CAS and its role in VSMC proliferation and neointima formation using both cultured VSMCs *in vitro* and a carotid artery ligation (CAL) model *in vivo*. We found that the expression of

miR-145 was reduced in human CAS and miR-145 plays a critical role in CAS by modulation of VSMC proliferation, suggesting that miR-145 may present a potential therapeutic option for treating CAS.

MATERIALS AND METHODS

Human sample collection from patients with CAS

The collection and use of human carotid tissues was approved by the ethics committee of Harbin Medical University. Written informed consent forms were obtained from patients or family members of donors. Plasma samples from 37 patients with symptomatic CAS undergoing endarterectomy between August 2015 and December 2016 at the Department of Surgery of the First Affiliated Hospital of Harbin Medical University, were collected. In addition, 50 plasma samples from patients without CAS, matched for age and gender, were also collected as plasma controls. Normal human carotid arteries from 5 otherwise healthy young donors following brain death were collected as control arteries. The clinical characteristics of the 2 groups are shown in Table I.

Mouse carotid artery ligation model

Carotid artery ligation (CAL) was performed in FVB male mice (9–10 weeks of age). Mice were anesthetized with ketamine and xylazine intraperitoneally. The left common carotid artery was dissected and completely ligated near the carotid bifurcation through a midline neck incision, as previously described (20). At 21 days after injury, all animals were anesthetized and perfused at physiological pressure with PBS followed by 10% phosphate-buffered formalin for 5 min. Then, both the left and right carotid arteries were excised, paraffin-embedded and stained for histochemical analysis as described below. All animals and experiments were maintained and performed according to the guidelines of the Animal Research Committee of National Health Research Institutes (USA) and Harbin Medical University.

Histological studies

Five-micrometer sections of paraffin-embedded samples were stained with hematoxylin and eosin staining or elastic van Gieson staining. For morphometric assessment, the outer and inner elastic laminae were traced in 8 carotid arteries 21 days after left CAL. Images were

analyzed with NIH Image J software to quantify lesion size in each animal as described (8 sections per mouse).

Total RNA isolation

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. These RNA samples were used to analyze the expression of miR-145 by qRT-PCR using a SYBR premix Ex Taq kit (TaKaRa, Dalian, China). Expression levels were normalized against endogenous β -actin as controls.

Quantitative real-time PCR (qRT-PCR) for miR and mRNA expressions

For qRT-PCR analysis of miR and mRNA expressions, cDNA from total RNA was obtained by SuperScript® II Reverse Transcriptase (Invitrogen, USA), and then converted into cDNAs using the High Capacity cDNA Reverse Transcription Kit (1708890, Bio-Rad Laboratories, Inc., USA) in accordance with the manufacturer's instructions. MiR expression was validated by quantitative stem-loop PCR technology, as previously described (21). The use of target-specific reverse transcription primers and Universal Probe Library

Probe 21 (Roche Diagnostic, USA) allowed the specific detection of mature miRNAs. A final volume of 20 μ L consisted of 0.5 μ mol/L of each forward and reverse primer, 0.1 μ mol/L Probe 21, 1 LightCycler® TaqMan® Master (Roche Diagnostics, USA), and 2.5 μ L of cDNA. Amplification curves were generated with an initial denaturing step at 95°C for 10 min, followed by 45 cycles of 95°C for 5 sec, 60°C for 10 sec, and 70°C for 1 sec. MiR expression was normalized to the expression of the small nuclear RNA, snU6. qRT-PCR of mRNA was performed with a CFX-96 (Bio-Rad) using the RT2 SYBR Green (1725271, Bio-Rad Laboratories, Inc. USA). All primers used for qRT-PCR are listed in Table II. All values were normalized with GAPDH abundance. Data were presented as the average of triplicates $\pm$ SEM.

Immunofluorescence staining of CAS specimens

Antibodies used for immunofluorescence included primary rabbit polyclonal anti-alpha smooth muscle actin antibody (Abcam, ab5694, dilution 1:100) and primary mouse monoclonal Ki67 antibody (Abcam, ab8191, dilution 1:200). Confocal microscopy images were taken with a Zeiss-LSM 700 microscope and evaluated using ZEN2010 software. For mean fluorescence intensity

Table I. Demographics and risk factors of the patients with CAS and controls without CAS.

Variable	CAS (n=37)	Control (Plasma) n=50	Control (Artery) (n=5)
Age (y)			
<50	67 $\pm$ 5	65 $\pm$ 3	32 $\pm$ 4*
Female/Male	10/27	15/35	2/3
Smoking	16	8*	1
Hypertension	19	11	0
Total cholesterol (mmol/L)	3.85 $\pm$ 1.96	3.23 $\pm$ 1.52	2.89 $\pm$ 1.78
LDL (mmol/L)	2.35 $\pm$ 1.06	1.97 $\pm$ 1.41**	1.92 $\pm$ 1.26
HDL (mmol/L)	1.03 $\pm$ 0.74	1.09 $\pm$ 0.85	1.1 $\pm$ 0.65
Triglycerides (g/L)	1.57 $\pm$ 0.33	0.74 $\pm$ 0.41*	0.81 $\pm$ 0.39
BMI	22.8 $\pm$ 2.8	21.9 $\pm$ 3.2	20.7 $\pm$ 3.5
Diabetes mellitus	5	3	0
Cardiac disease	6	3	0
Peripheral vascular disease	2	0	0
Renal insufficiency	0	0	0

measurements, confocal microscopy images were analyzed with ImageJ.

Human VSMC culture

Healthy human VSMCs (HA-VSMC, C134) were obtained commercially (Mito-Bio Technology, Shanghai, China). VSMCs were grown in smooth muscle cell growth medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% Fetal Bovine Serum (FBS). VSMCs between passages 4 and 7 were used in the experiments.

miR-145 mimic and inhibitor transfection

RNA oligonucleotides, including an miR-145 mimic, an miR-145 inhibitor, and a negative control consisting of a scrambled RNA sequence, were synthesized by GENEWIZ LLC (Suzhou, China). VSMCs grown to approximately 80% confluence were transfected with the miR-145 mimic, the miR-145 inhibitor, or the negative control using Xfect MicroRNA Transfection Reagent (Clontech, Mountain View, CA, USA). Transfection complexes were prepared according to the manufacturer's instructions. The sequences of the miR-145 mimic and the miR-145 inhibitor are listed in Table I.

Cell proliferation assay

The proliferation activity of VSMCs with the miR-145

mimic, the miR-145 inhibitor, or the negative control was ascertained by cell counting assay. To test the proliferation of VSMCs, 5×10^4 VSMCs were seeded on 12-well plates; then, the mimic, inhibitor of miR-145, or negative control were added to the plates. At 24h, 48 h, 72h and 96 h, the number of the VSMCs were counted. For Ki67 staining, 2×10^5 VSMCs were seeded on 12-well plates, FBS starved overnight, then the mimic, inhibitor of miR-145, or negative control with full medium were added to the plates for 24 h. The VSMCs were fixed in 4% paraformaldehyde and stained for Ki67 by immunofluorescence to evaluate cell proliferation as previously described (22).

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis was performed using an independent Student's *t*-test for two groups of data and analysis of variance (ANOVA) followed by Scheffe's post-hoc test for multiple comparisons. A *p* value less than 0.05 was considered significant.

RESULTS

Clinical characteristics of patients and controls

The baseline clinical characteristics of the whole population are shown in Table I. The mean age was 67 years (SEM, 5; range, 45-76) in the

Table II. Primers and RNA sequences used for this study.

Gene	Annealing temperature (°C)	Sequences
miR-145 (Human)	59 °C	F: 5'ACACTCCAGCTGGGGTCCAGTTTCCCAGGA3' R: 5'CTCAACTGGTGTCTGTGGAGTCGGCAATTCAGTTGAGAGGGATTCT3'
hU6	57 °C	F: 5'CTCGCTTCGGCAGCACAA3' R: 5'AACGCTTCACGAATTTGCGT3'
miR-145 (Mouse)	59 °C	F: 5'TCCAGTTTCCCAGGAATCCCT3' R: 5'GTTGCTGTAGCCAAATTCGTTGT3'
mU6	57 °C	F: 5'GCTTCGGCAGCACATATACTAAAAT3' R: 5'CGCTTCACGAATTTGCGTGTTCAT3'
MiR-145 mimic		5'GUCCAGUUUUCCCAGGAAUCCCU3'
MiR-145 inhibitor		5'GGAUCCUGGAAAUACUGUUCU3'

symptomatic CAS group and 65 years (SEM, 3; range, 52-73) in the plasma control group without CAS. Age and gender ratios were not significantly different between the CAS and plasma control groups. The patients with CAS had significantly higher proportions of smoking, hypertension, LDL, triglycerides and peripheral vascular disease. There were no differences in age, diabetes, cardiac disease, high-density lipoprotein (HDL), total cholesterol and body mass index (BMI) between the two groups ($p>0.05$). There was a significant difference in age between the CAS group and the normal carotid artery control group from brain dead donors ($p<0.05$).

miR-145 expression is reduced in human symptomatic CAS

First, miR-145 expression was detected in the plasma samples of patients with symptomatic CAS ($n=37$) and CAS-free controls ($n=50$) by qRT-PCR. MiR-145 expression was significantly reduced in the plasma samples of patients with CAS when compared to CAS-free controls ($p<0.001$, Fig. 1A).

Next, miR-145 expression in the endarterectomy plaques from the CAS patients was measured by qRT-PCR. As expected, miR-145 expression was also significantly reduced when compared to healthy carotid arteries from brain dead donors without CAS ($n=5$) ($p<0.001$, Fig. 1B).

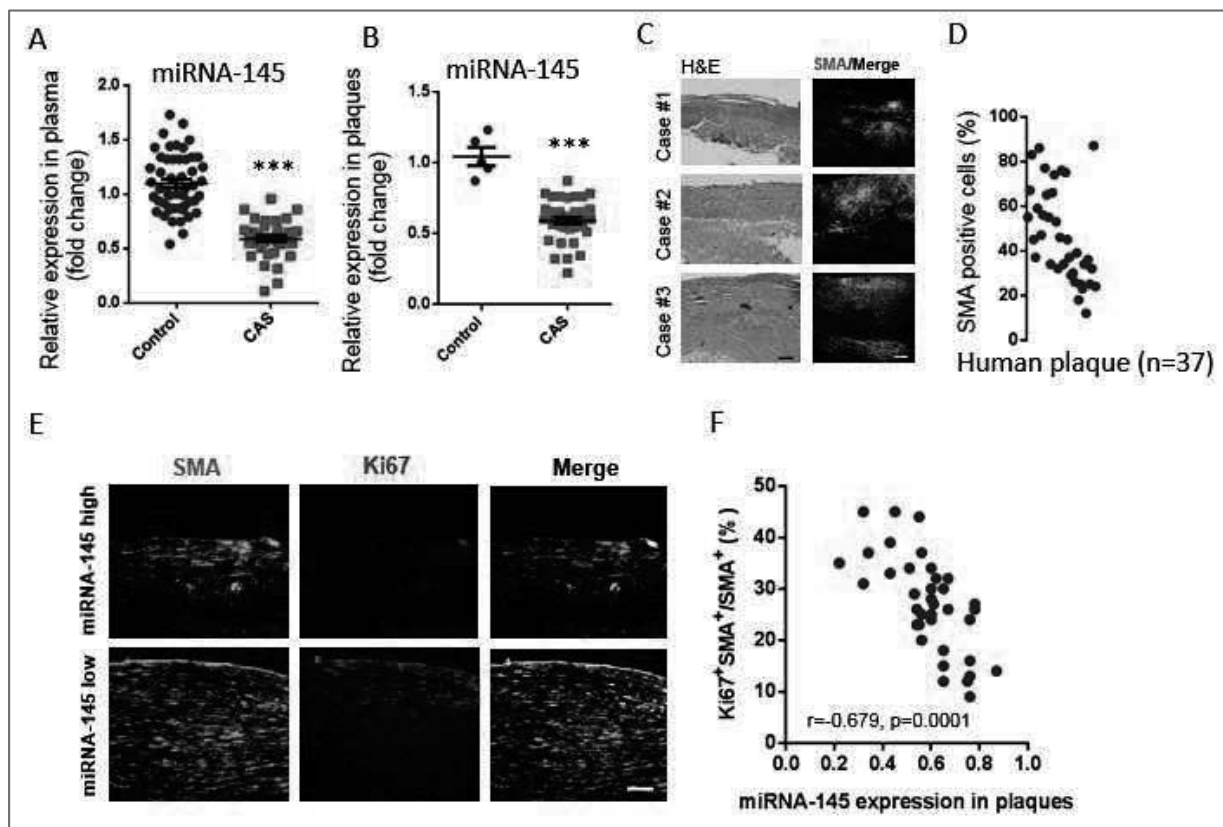


Fig. 1. *miR-145 expression reduces in human symptomatic carotid artery stenosis (CAS).* A. miR-145 expression in the plasma samples of patients with symptomatic CAS ($n=37$) and CAS-free controls ($n=50$) by qRT-PCR; B. miR-145 expression in the endarterectomy plaque samples from patients with CAS ($n=37$) and normal carotid arteries from brain dead donors ($n=5$) by qRT-PCR. C. H&E and immunofluorescence staining of alpha vascular smooth muscle actin (α SMA) in the endarterectomy plaque samples from patients with CAS; nuclei were stained with DAPI. Bar: 100 μ m and 50 μ m. D. Quantification of α SMA positive cell percentage in the plaque samples ($n=37$). E. Immunofluorescence staining of α SMA and Ki67 in the endarterectomy plaque samples from patients with CAS. Nuclei were stained with DAPI. Bar: 50 μ m. F. Scatter plots showing the correlation between miR-145 expression and percentage of Ki67+ α SMA+/ α SMA+ in the endarterectomy plaque samples from patients with CAS. ($R=-0.679$; $p<0.0001$). Data are presented as the mean $\pm$ SEM. $n=37$, ***, $P<0.001$.

To determine the cellular composition and cell proliferation in the plaque samples, immunofluorescence assay was performed for detection of alpha vascular smooth muscle actin (α SMA) and Ki67. As shown in Fig. 1C, there were many α SMA-positive cells in the plaque samples, and quantification of the α SMA-positive cell percentage demonstrated a composition of about 50% α SMA-positive VSMCs. Ki67 was further detected in higher-expression miR-145 plaque samples (normalized miR-145 > 0.59) and in lower-expression $\leq$ miR-145 plaque samples (normalized miR-145 $\leq$ 0.59) by immunofluorescence, and it was found that there were more Ki67-positive cells, co-localized with α SMA, in the lower-expression miR-145 plaques. Correlation analysis showed that there was a significantly negative correlation between miR-145 expression and VSMC proliferation in the endarterectomy plaques from the CAS patients ($p=0.0001$, $r=-0.679$, Fig. 1 D, E)

Taken together, these results demonstrated that miR-145 expression was significantly reduced in both plasma and plaque samples from symptomatic CAS patients when compared to CAS-free controls, suggesting that miR-145 might play important role

on VSMC proliferation in CAS.

miR-145 expression is also reduced in mouse CAL model

Based on the human data — which suggested that there was a significantly negative correlation between miR-145 expression and VSMC proliferation — the complete CAL model in mice was used to better understand the role of miR-145 on VSMC proliferation in CAS *in vivo*, as it can induce rapid proliferation of medial smooth muscle cells, leading to extensive neointima formation in the presence of an endothelial lining (20).

At day 21 post-surgery, the representative HE staining of the arteries showed that there was significant neointima formation and lumen stenosis in the CAL model when compared to the control artery without ligation (Fig. 2A). Quantification of the neointima area showed that there was a significantly larger area in CAL arteries than those in the control arteries with time (Fig. 2B, $p<0.05$ at 1w and 2w, $p<0.01$ at 3w).

We next evaluated miR-145 expression in carotid arteries with CAL or without CAL. qRT-PCR results demonstrated that there was a nearly 2-fold decrease

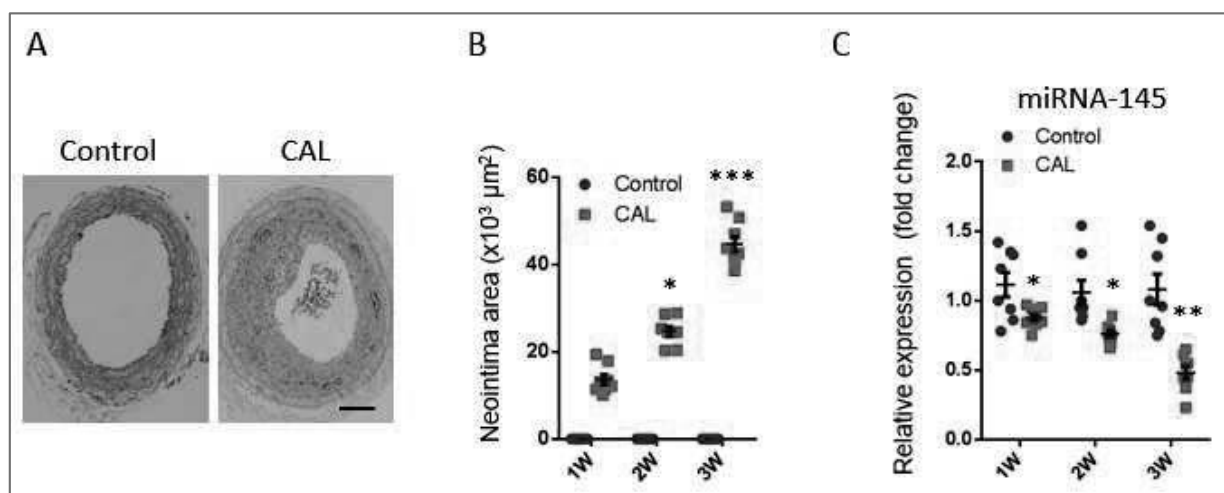


Fig. 2. miR-145 expression also reduces in the mouse carotid artery ligation model. (CAL). A) Representative images of H&E staining of carotid artery sections from control and CAL groups at 21 days after CAL surgery; B) Neointima area of mouse carotid artery in control and CAL groups at 1w, 2w and 3w; C) miR-145 expression in control and CAL groups by qRT-PCR at 1w, 2w and 3w. Data are presented as the mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n=8$ for each group).

of miR-145 expression in the CAL group ($p=0.0075$, Fig. 2C) when compared to the control, non-ligated carotid arteries.

Mir-145 inhibits cell proliferation of human vascular smooth muscle cells (VSMCs) in vitro

To explore the effect of miR-145 on human VSMCs, human VSMCs were cultured *in vitro* for further experiments (Fig. 3A). The VSMCs were treated with an miR-145 mimic, an miR-145 inhibitor, or a negative control, and qRT-PCR results showed that miR-145 expression was significantly reduced after treatment with the miR-145 inhibitor ($p<0.01$, Fig. 3B).

The numbers of cells at various time points were also counted. As expected, VSMCs treated with the miR-145 mimic had significantly fewer cells over time, while the miR-145 inhibitor caused a significant increase in the numbers of VSMCs ($p<0.05$) when compared to the negative control (Fig. 3C). Ki67 staining by immunofluorescence was also used to evaluate the proliferation of VSMCs in response to treatment with the miR-145 mimic, miR-145 inhibitor, or the negative control. A similar effect was observed with the expression of Ki67 significantly reduced after treatment with the miR-145 ($p<0.01$), whereas the expression of Ki67 was significantly increased after treatment with the

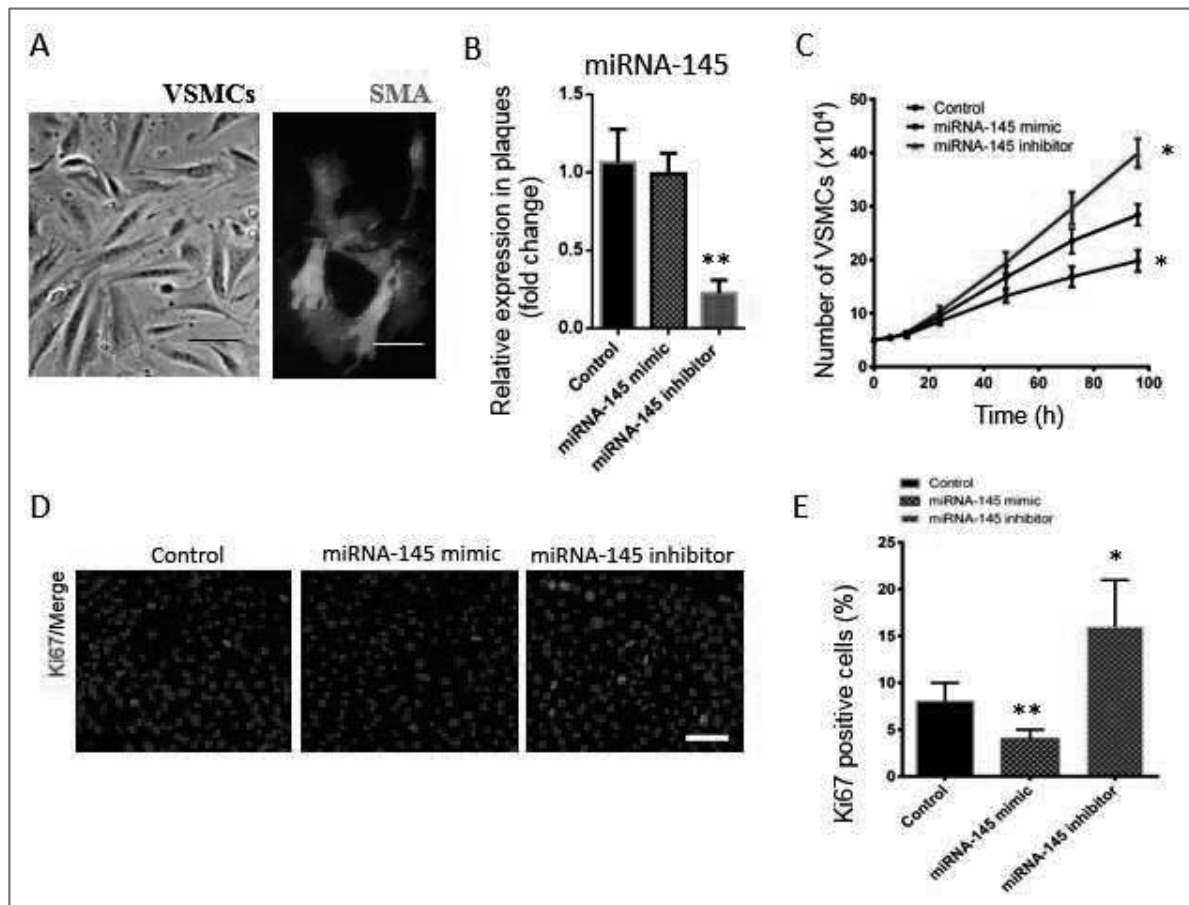


Fig. 3. miR-145 inhibits cell proliferation in human vascular smooth muscle cells (VSMCs) *in vitro*. A) Representative images with α SMA immunofluorescence staining (green) in human VSMCs under 400 magnification, scale bar: 50 and 25 μ m. B) miR-145 expression in the VSMCs treated with control, miR-145 mimic or miR-145 inhibitor by qRT-PCR. C) Cell proliferation of VSMCs (total cell number counting) transfected with control, miR-145 mimic or miR-145 inhibitor was detected at times indicated. D) Cell proliferation of VSMCs transfected with control, miR-145 mimic or miR-145 inhibitor was detected by Ki67 immunofluorescence staining under 400 magnification, scale bar: 150 μ m. E) Ki67 positive VSMCs were quantified in each group. Data are presented as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$ ($n=3$ in each experiment).

miR-145 inhibitor ($p < 0.05$) when compared to the negative control treatment (Fig. 3 D, E).

These results demonstrate that miR-145 plays an important role in manipulating the proliferation of VSMCs *in vitro*.

DISCUSSION

An accumulating number of studies have reported that dysregulation of microRNAs contributes to cardiovascular diseases (6-10). miR-145 is the most abundant microRNA in vascular walls; however, its expression in human CAS is controversial. In line with findings of decreased levels of miR-145 in coronary artery disease and aortic aneurysm (15, 23, 24), the present study demonstrated that miR-145 expression is significantly reduced in human CAS. Through *ex vivo* and *in vivo* approaches, we found that miR-145 plays a critical role in CAS by regulating the proliferation of VSMCs.

miR-145 has an anti-proliferative effect on VSMCs. It is crucial for VSMC function and controls the phenotypic switching of contractile VSMCs towards a synthetic phenotype (11-13). It has been reported that loss of miR-143/145 in VSMCs promotes atherosclerosis (25). VSMC-specific overexpression of miR-145 not only limits the plaque burden in atherosclerosis-prone apolipoprotein E knockout (*ApoE*^{-/-}) mice, but also reduces plaque inflammation and increases features of plaque stability in a fashion consistent with promotion of the contractile VSMC phenotype (26). In human cardiovascular diseases, patients with unstable angina pectoris (AP) had higher levels of miR-21, -133a/b and -199a, and lower levels of miR-145 and -155 when compared to those with stable coronary artery disease (7). A lower level of miR-145 in plasma was significantly associated with the presence and severity of coronary artery disease (21). Cholesterol loading may cause reprogramming of VSMCs towards a macrophage-like phenotype, via down-regulation of the miR-143/145/SRF/Myocardin axis (27). Statin treatment, the most common form of anti-atherosclerotic therapy at present, increases expression of miR-143/145 in ECs (28). In human cardiovascular diseases, decreases in miR-145

may result in loss of anti-proliferative effects, thus promoting disease development. Consistent with these findings, miR-145 expression was found in the present study to be significantly reduced both in plasma samples and in endarterectomy specimens in patients with CAS when compared to controls without CAS. It has been reported that miR-145 may play this crucial role of anti-proliferative effect on VSMCs through its downstream targets, such as myocardin, platelet-derived growth factor D (PDGFD), Kruppel-like factor 5 (KLF5) and serum response factor (SRF) (29).

There is a limited number of studies exploring miR-145 expression in CAS, albeit with controversial results which warrant further investigation. One study reported that miR-145 levels were significantly lower in carotid atherosclerotic plaques than in atherosclerosis-free regions (19). Other groups have reported that miR-145 was overexpressed in symptomatic plaques compared to non-symptomatic plaques (17, 18). miR-145 expression was also shown to be higher in carotid plaques from patients with hypertension, who are more prone to develop stroke if hypertension is not properly treated (17). The results of the present study showed that the expression of miR-145 is reduced in both plasma and carotid artery plaques from patients with symptomatic CAS, and there was a significant negative correlation between miR-145 expression and VSMC proliferation in the endarterectomy plaques from the CAS patients. This suggests that a lack of the miR-145's anti-proliferative effects may play an important role in development of this disease. Variations in results may be due to differences in race, as all of the specimens used in the present study were taken from patients of Asian descent. Other factors potentially affecting the expression of miR-145 include age, BMI, timing of blood collection, and control setting, among other factors.

In summary, this study has demonstrated that miR-145 expression is reduced both in human CAS as well as in a mouse CAL model. miR-145 inhibits the proliferation of VSMCs, and a reduction of miR-145 is therefore critical to the development of CAS due to loss of its anti-proliferative effects. miR-145, as a protective factor in vessel walls, may present

a potential therapeutic option for treating carotid artery disease.

ACKNOWLEDGEMENTS

We thank Hong Zhu, Haijun Wang and Ningning Qiu for their technical help. This study was supported by grants from the National Natural Science Foundation of China, University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2016193) and Harbin Medical University scientific research innovation fund (2017LCZX05). Fund of scientific research innovation of The first affiliated hospital of Harbin medical university (NO.2018B012)

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AMINO ACID AND HYALURONIC ACID MIXTURES DIFFERENTIALLY REGULATE EXTRACELLULAR MATRIX GENES IN CULTURED HUMAN FIBROBLASTS

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Received October 18, 2017 – Accepted March 15, 2018

The aim of this screening study was to evaluate the efficacy of different proprietary mixtures of amino acid and hyaluronic acid (HA) in stimulating the production of extracellular matrix (ECM) components, particularly the neo-synthesis of elastin, and in promoting a more efficient deposition of elastic fibres (elastogenesis), while at the same time maintaining the stimulation of collagen. The study has allowed identification of the optimal ratios between the amino acids (AA) for the production of collagen and elastin. Human primary dermal fibroblasts from a 44-year-old female donor were used as a test system in an experimental design based on the evaluation of the expression of relevant ECM genes using a transcriptomic dynamic approach. The expression of ECM genes was evaluated by RTqPCR from 24 to 120 hours in the presence of the test items. Moreover, the production of ECM proteins was verified by Western blot analysis after a 120 h treatment period. In addition to elastin, collagen IV, a fundamental structural component of the basal lamina responsible for epithelial and connective tissue anchoring, was analysed as potential target for the modulation of ECM protein production by human fibroblast. The first phase of the study demonstrated that alanine and valine are essential to promote production of elastin, of which they are important constituents. The second phase of the study, which was conducted to clarify the interactions between the different clusters of AA, demonstrated that it is necessary to choose a mixture that contains specific amounts of amino acids of both proteins, collagen and elastin, to give a significant response and a significant production of both. This also proves the existence of a ratio between the 2 clusters (AA elastin/AA collagen) that guarantees an adequate and balanced response to gene expression and production by fibroblasts, collagen and elastin. The study has allowed identification of the optimal ratios between the AA for the production of collagen and elastin.

The use of biomaterials, a broad category of materials that also comprises biopolymers for clinical application, is becoming increasingly important and hyaluronic acid (HA), in particular, is one of the most used. Due to its unique physical characteristics (hygroscopicity and visco-elasticity), it is largely employed in several fields of medicine including: ophthalmology, dentistry, rheumatology and regenerative and aesthetic medicine (1-5). In addition to its mechanical and physical properties,

HA has important biological functions, binding specific membrane receptors (6), regulating cellular adhesion, proliferation and migration, cytokine synthesis and deposition of extra cellular matrix (ECM) proteins (7-8).

It has been demonstrated that adding small molecules, such as mixtures of amino acids (AA) (e.g. proline, lysine, glycine and leucine), to HA can enhance its action in many fundamental biological processes, and mainly in wound healing (9-10).

Key words: amino acids, fibroblasts, elastogenesis, elastin, collagen

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0393-974X (2018)

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The biological wound healing process is one the most complicated processes described in relation to the skin and involves different and overlapping phases such as inflammation, proliferation, migration of keratinocytes, new tissue formation and tissue remodelling: all these steps are necessary to achieve tissue regeneration and wound healing (9).

All these phases are characterized by biochemical, molecular and morphological changes and even if not entirely characterized, they can be monitored in the different skin compartments thanks to identification of the relevant bio-markers. During the latter phases (re-modelling), ECM proteins, which are massively synthesized, assembled and reorganized by fibroblasts allowing tissue reconstruction, play a critical role (11).

These proteins include collagens and fibronectin, the biosynthesis of which is strictly connected to the local bioavailability of constituent AA. Collagen triple helix consists of glycine, L-proline and L-lysine in well-determined positions. Following the activation of specific hydroxylases (e.g. LOXL1) by L-lysine, the hydroxylation of glycine and proline residues causes the formation of inter- and intra-molecular bonds, which give collagen

its viscoelastic properties (12). It has been shown that the use of an AA-enriched HA (Aminogam®) promotes the expression of type I and III collagen, the major components of connective and granulation tissues respectively, and of fibronectin genes in human pulmonary fibroblasts (13). Moreover, the expression of ECM deposition regulators [such as TGF β and, connective tissue growth factor (CTGF)] has been shown to be increased by the treatment (13).

Recently, attention has been paid to other components of ECM in order to investigate the further effects of HA mixtures and to discover other potential clinical applications (14). Of the components of ECM, elastic fibres play a fundamental role for the structure and function of elastic tissues such as the bladder, cartilage, ligaments, blood vessels and the skin.

The elastic fibres consist of microfibrils and elastin. Microfibrils mainly consist of fibrillin, and provide a scaffold which guides the alignment and the cross-linking of elastin molecules by one or more members of the lysyl oxidase (LOX) gene family (15).

Elastin is a polymeric protein derived from the

Table I. Amino acid composition of the tested formulations.

		%			
		Mixture A	Mixture B	Mixture C	Mixture D
COLLAGEN	Glycine	50	34.8	33.4	30.2
	L-Proline	37.5	26.1	25.1	22.7
	L-Lysine HCl	5.5	3.8	3.7	3.3
	L-Leucine	7	4.9	8.7	4.2
ELASTIN	L-Valine	-	12.9	12.3	16.8
	L-Alanine	-	17.5	16.8	22.8

Table II. TaqMan probes for genes of interest and endogenous control used in RTqPCR analysis.

Gene	Code	Taqmas assay no.
Elastin	ELN	Hs00355783_m1
Collagen IV	COL4A1	Hs00266237_m1

crosslinking of secreted monomers of tropoelastin precursors.

Elastin has an AA composition similar to that of collagen in terms of glycine and proline residues, but with a specific enrichment of the hydrophobic residues, valine and alanine (16), therefore it can be assumed that increasing the bio-availability of these specific AAs could enhance elastin synthesis.

The aim of this screening study was to evaluate the efficacy of different proprietary mixtures of AAs and HA in stimulating the production of ECM components, particularly the neo-synthesis of elastin, and in promoting a more efficient deposition of elastic fibres (elastogenesis), while at the same time maintaining the stimulation of collagen. The study has allowed identification of the optimal ratios between the AA for the production of collagen and elastin.

For this purpose, human primary dermal fibroblasts from a 44-year-old female donor were used as a test system in an experimental design based on the evaluation of the expression of relevant ECM genes using a transcriptomic dynamic approach. The expression of ECM genes was evaluated by qRT-PC from 24 to 120 h in the presence of the test items. Moreover, the production of ECM proteins was verified by Western blot analysis after a 120-h treatment period.

In addition to elastin, collagen IV, a fundamental structural component of the basal lamina responsible for epithelial and connective tissue anchoring, was analysed as potential target for the modulation of ECM protein production by human fibroblast.

MATERIALS AND METHODS

Test items

Different proprietary formulations of AA-enriched HA (Professional Dietetics, Milan, Italy) were tested in this study. The compositions of the mixtures are reported in Table I.

Cell culture

The human dermal fibroblasts were isolated from a breast surgery resection from a 44-year-old Caucasian woman, who signed informed consent. The epidermis was

separated from the dermis with overnight incubation with Dispase II. The dermis was digested with Collagenase I and fibroblasts were seeded in DMEM F12 medium with 20% fetal bovine serum and antibiotics (1% penicillin/streptomycin) and ascorbic acid P (1 mM), and then used for the assays in steps 1-3.

Selection of treatment dosage by MTT assay

The cytotoxicity of the test items was preliminarily assessed after 120 h. This time was chosen because it was the longest time to be applied in the efficacy test at which the three concentrations used were non cytotoxic. The cytotoxicity was studied at three different concentrations of 10-100-1000 µg/mL in the medium, before proceeding with the efficacy test. The fibroblasts were seeded at 10^4 cells/well in a 96-well plate and cultivated until confluence, then the test item and the negative control were applied in triplicate. After 120 h of product application, the MTT Test was performed to measure cell viability (Table II). Briefly, fibroblasts were incubated in a 0.5 mg/ml solution of MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide) (Sigma) in PBS at 37°C, 5% CO₂. After this period, the tetrazolium ring yielding blue/purple MTT crystals, which were extracted by isopropanol and optical density, was measured at 570 nm using the Tecan Infinite M-200 spectrophotometer. The results are expressed as a percentage of cell viability compared to the negative control.

Experimental design

Human fibroblasts were seeded at a density of 10^4 cells/well in a 96-well plate and cultivated until confluence. The test items were dosed in the culture medium at 1000 µg/mL and maintained throughout the treatment period. The read-out parameters were measured after 24 h, 72 h and 120 h for gene expression and at 120 h for Western blot.

Real Time Quantitative PCR

The RNAqueous method (RNAqueous kit, Ambion, Life Technologies) was used to extract the total RNA from cell samples. Each biological replicate was run in triplicate. The High Capacity cDNA Reverse Transcription kit (Life Technologies) was used to synthesize cDNA from RNA. The instrument used was the Applied Biosystems 7500 Fast Real Time PCR with fluorescent-based PCR chemistry, the TaqMan assay. TaqMan gene expression

assay and cDNA (25 ng) were added to the 2X TaqMan Fast Universal PCR Master Mix for a total volume of 20 μ L. The Thermal condition steps in the ABI 7500 Fast were: 95°C 20 sec; 40 cycles (95°C 3 sec +60°C 30 sec).

GAPDH was used as an endogenous control gene to normalize input amounts. The list of TaqMan probes for genes of interest and endogenous control is reported in Table II.

RT-PCR fluorescence data, generated by the ABI 7500

Fast thermocycler, was collected by the internal SDS 2.0.6 software. Relative gene expression was calculated for each formulation compared to the negative control using the $2^{-\Delta\Delta Ct}$ method. The software uses a 95% confidence level to calculate errors.

A value was accepted as significant when the gene was “one-fold” up- [Relative Quantification (RQ)>2] or down-regulated (RQ<0.5) compared to the calibrator sample (RQ=1). The internal instrument confidence level used was 95%.

Table III. Cell viability by MTT assay after 120 h treatment with products A, B, C, D at 10-100-1000 μ g/mL.

μ g/mL	Viability % at 120H	sd
NC	100	0.6
A 10	97.29	1.1
B 10	96.90	1.5
C 10	103.34	11
D 10	97.42	0.8
A 100	96.25	2.0
B 100	90.62	2.3
C 100	102.81	0.8
D 100	95.22	2.6
A 1000	91.97	1.6
B 1000	92.25	0.9
C 1000	96.09	1.4
D 1000	89.92	0.6

NC: negative control (untreated cells).

Percentages of viable cells are reported.

Both treated cells and NC were tested in triplicate.

Table IV. Relative quantification of elastic protein performed considering Negative Control (NC) at 120 h and calibrator sample.

RELATIVE QUANTIFICATION	ELN	COL4A1
NC	1	1
A	1.478	1.484**
B	1.056	1.330*
C	2.121**	2.463**
D	2.164**	3.197**

** $p < 0.01$ and * $p < 0.05$ (Student t-test)

Western blot

Fibroblast cell samples were placed in Bio-Plex Cell Lysis buffer (BIORAD) with freshly added protease inhibitor mixture (cocktail SRE 0055 Sigma (containing aebfsf, aprotinin, bestatin, e-64, EDTA, leupeptin), homogenized, and stored on ice for 30 min, then centrifuged at 16,000 x g for 10 min at 4°C. Protein determination was performed with RC DC Protein Assay (BIORAD). After adding an equal volume of 2X Laemmli Sample Buffer to the lysate, each sample (10 µg of total protein) was boiled in sample buffer at 100°C for 5 min, separated on Tris-Glycine Extended (TGX) Stain-free precast gels 7.5% (Bio-Rad) and transferred to PVDF membrane (BIORAD).

The membranes were blocked for 1 h at room temperature using 3% blocking solution (BSA) in TBST 0.05%. The membranes were incubated with anti-COL4A (SantaCruz, sc-59814) or anti-ELN (Santa Cruz sc-166352) Mouse monoclonal antibody for 1 h at room temperature and then rinsed three times with TBST for 5 min each time.

Bound antibodies were detected with the recommended dilution of labelled goat anti-mouse IgG secondary

antibody (Invitrogen) in TBST at room temperature for 1 h. After three washes in TBST, the membrane is ready for signal development with Immun Star Westernc Kit (Biorad).

The chemiluminescence bands of COL4A and ELN were visualized by using Chemidoc XRS system (Biorad) and quantified using ImageLab software (250 kDa and 70/55 kDa, respectively). Protein levels were normalized on total protein content.

RESULTS

The study has two evaluation phases:

- Phase one: to clarify whether the proposed mixtures could produce both collagen and elastin
- Phase two: optimization of the ratios between the various AAs

Phase One Results

Cytotoxicity test

All products induced residual cellular viability not lower than 90% for all tested concentrations (Table III).

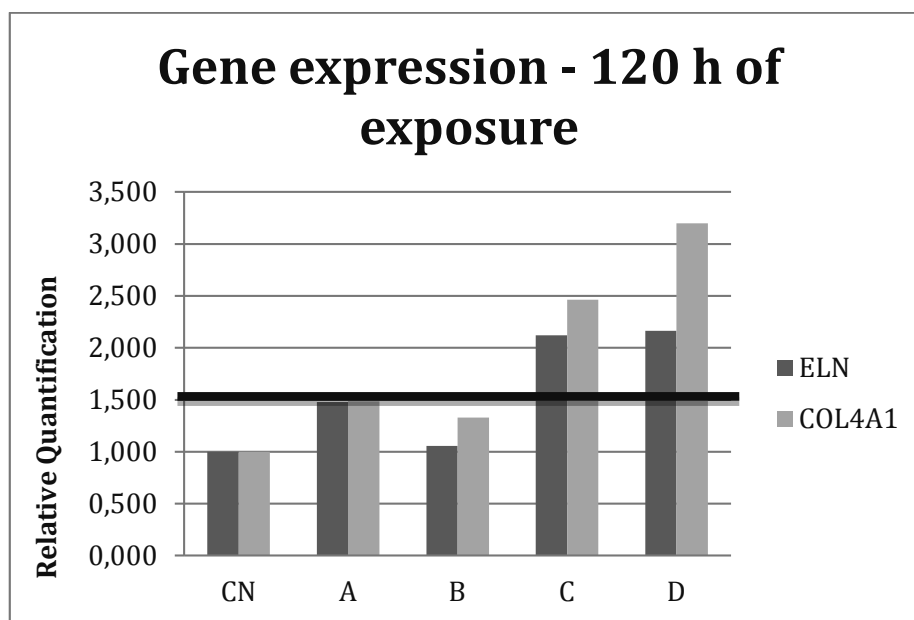


Fig. 1. Expression of ELN, FBN-1, FN-1, LOXL-1 and COL4A1 genes in cultured fibroblasts after 120 h of treatment with different mixture of amino acids and hyaluronic acid. Negative Control (NC) was used as calibrator sample for relative quantification calculation NC=1

Since non-toxic effects were noted, in order to maximize the potential biological response to the treatment, the higher concentration (1000µg/mL) was selected for the subsequent efficacy assays.

Transcriptional study

As expected, since mainly structural proteins were investigated, none of the genes involved in EMC formation were modulated until 72 h (data not shown). On the contrary, as reported in Fig. 1 and Table IV, a significant modulation of ELN and COLIV genes was quantified after 120-h treatment.

After 5 days of growth, compared with the negative control (untreated), mixture A, the effects of which were investigated in a number of studies, seemed not to exert any significant action on the expression of the selected genes. The addition of alanine (17.5%) and valine (12.9%) to mixture B also had no effect on the levels of the transcripts, when leucine was only 4.9%.

Otherwise, mixture C (with the highest L-leucine content 8.7%) and mixture D [with the highest

L-Valine (16.8%) and L-Alanine content (22.8%)] were able to overexpress collagen IV and elastin, thus promoting the assembly of matrix components.

Protein synthesis

Western blot analysis was performed exclusively at 120 h.

Fig. 2 and Table V show the Western blot images and relative quantitative analysis of the bands for collagen IV protein. The protein was found to be significantly modulated in samples treated with mixture C and D and very close to significance in samples treated with mixture B.

Western blot images and relative quantification of elastin are shown in Fig. 3 and Table VI, respectively.

As can be seen in Fig. 3, elastin protein presented two different bands corresponding to the soluble non-cross linked precursor of elastin (tropoelastin, 70KDa) and elastin form of 55 kDa. Quantification of the single bands not only showed a different capacity of the tested mixtures to modulate elastin

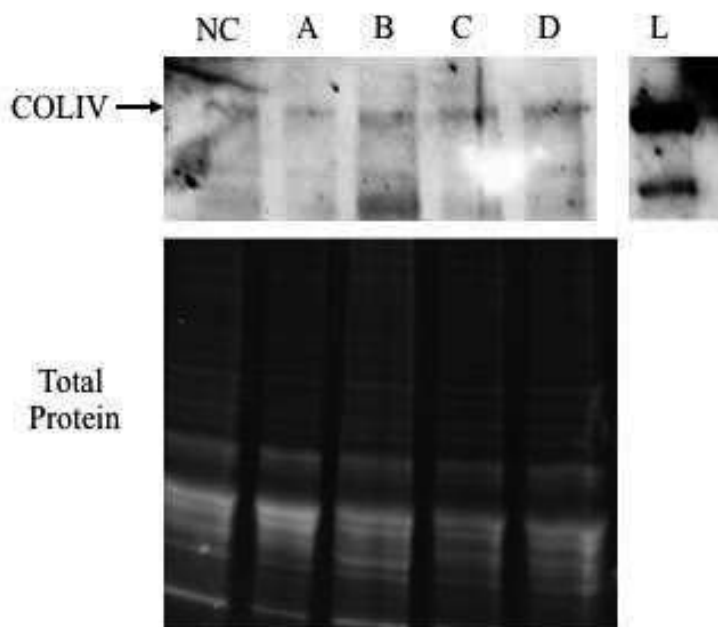


Fig. 2. Western blot analysis of collagen IV. Lanes: NC: negative control; A-D: test items; L: molecular weight ladder

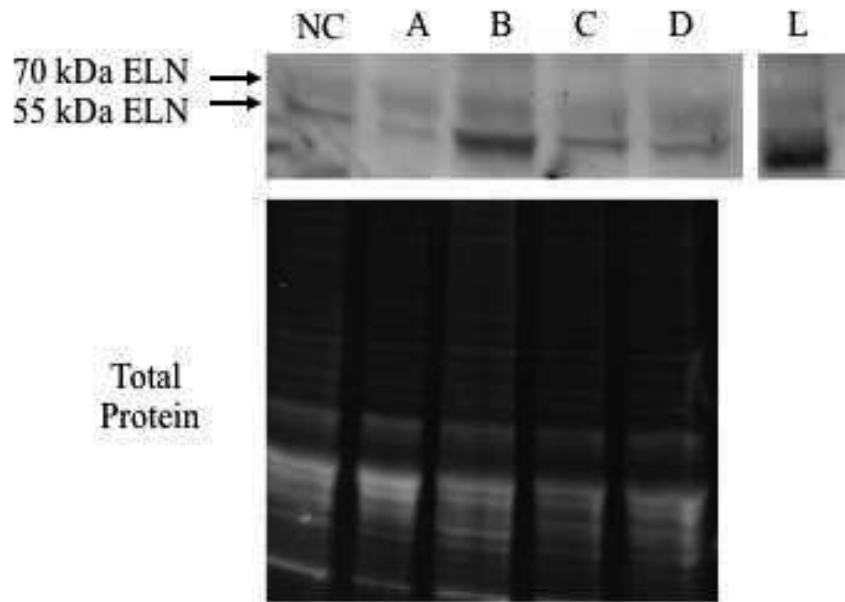


Fig. 3. Western blot analysis of elastin. NC: negative control; A-D: Test items; L: molecular weight ladder.

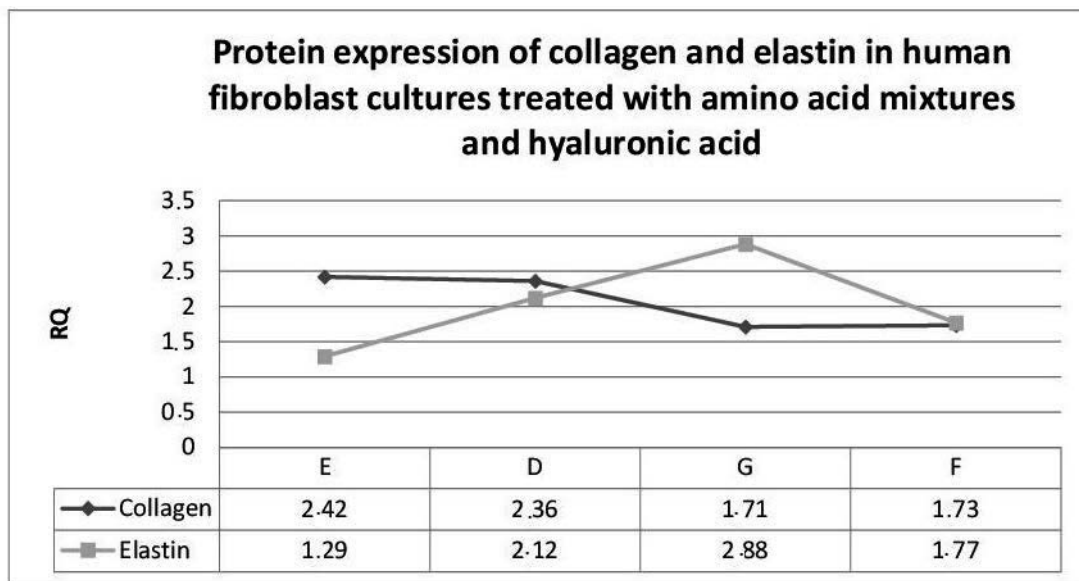


Fig. 4. Relative quantification of collagen IV and elastin proteins performed as negative control (NC = 1) at 120 h as calibrator sample.

production but also a differential action on the two different forms. In particular, mixture B seemed to be the most active formulation, enhancing both the forms with different intensity, mixture C increased only the insoluble α -elastin, while mixture D exerted

a stimulatory action on precursor and final protein in equal manner.

Phase Two Results

In the first study it was shown that the three

different 6 AA (and not 4 AAs as in mixture A)-mixtures are able to significantly produce both collagen and elastin in respect to the control, while the 4 AA mixture is capable of stimulating the production of collagen alone.

In the second part of the study, three different AA clusters were defined to clarify the limits and potentials of the mixture table:-

- More expressed AAs in collagen (glycine, lysine, proline)
- More expressed AAs in elastin (alanine, valine)
- Leucine, essential AA, primer of protein synthesis as demonstrated in several studies (18).

In this second experimental session, all blends contained the same amount of leucine, the lowest of all those tested in the first session of the experiments, in order to more clearly evaluate the effectiveness of the other clusters on the production of collagen and

elastin (Table VII). At the same time, the glycine-proline -lysine ratio and the valine-alanine ratio were kept constant to reduce the number of variables. In this study, the variable becomes the cluster of AAs characterizing the single protein. The compositions of the blends were as follows:

Blend E: Elastin AA Cluster: 29% of the total mixture

Blend D: Elastin AA Cluster: 39%

Blend G: Elastin AA Cluster: 44%

Blend F: Elastin AA Cluster: 49%

The results are summarized in Fig. 4 and show that in mixture E, with the lowest amount of elastin AA, there is no significant production of elastin itself, confirming the need for specific amounts of such AAs in order to produce this protein.

The above results show that the production of elastin is not linearly related to the quantities of the AAs thereof present in the mixtures. It therefore

Table V. Relative quantification of collagen IV protein performed considering Negative Control (NC) at 120 h as calibrator sample.

Sample	COLIV
NC	1
A	1.685
B	1.957
C	2.547*
D	2.278

** $p < 0.01$ and * $p < 0.05$ (Student *t*-test)

Table VI. Relative quantification of elastin protein performed considering Negative Control (NC) at 120 h as calibrator sample.

Sample	ELN 55 kDa	TROPOELN 70 kDa
NC	1	1
A	0.805	1.82
B	4.105**	2.24*
C	2.403	1.65
D	1.999	2.16

** $p < 0.01$ and * $p < 0.05$ (Student *t*-test)

Table VII. Percentage of aminoacid composition of the tested formulations in Second phase.

		Mixture D	Mixture E	Mixture F	Mixture G
COLLAGEN	Glycine	30.2	35.9	25.2	27.9
	L-Proline	22.7	27	18.9	20.9
	L-Lysine HCl	3.3	3.9	2.7	2
	L-Leucine	4.2	4.2	42	4.2
ELASTIN	L-Valine	16.8	16.7	28.2	25.3
	L-Alanine	22.8	12.3	20.8	18.7

** $p < 0.01$ and * $p < 0.05$ (Student *t*-test)

Table VIII. Summary of the effects of tested mixture of amino acids and hyaluronic acid on ELN and COLIV gene and protein expression in human fibroblasts, after 120 h of treatment.

	Gene Expression		Protein	
	ELN	COLIV	ELN	COLIV
A	-	-	-	-
B	-	-	↑	↑
C	↑	↑	↑	↑
D	↑	↑	↑	↑

Up-regulation (↑) or no effect (-), compared to negative control (NC).

follows that increasing the concentration of elastin AAs results in an increased protein response up to a certain point. Indeed, 49% of elastin AAs have a non-optimal elastin production (towards control).

Collagen IV protein production is linearly connected to the amounts of the respective AAs present. Low collagen AA concentrations are directly related to low stimulation of collagen IV. It follows that further reducing the concentration of collagen AAs compared to those tested would certainly not be enough to stimulate collagen synthesis.

DISCUSSION

In addition to the important physical and chemical properties that make it a largely used molecule

in clinical applications, HA has an interesting biological action on cell adhesion, proliferation and migration, cytokine release and the synthesis of extracellular matrix proteins in connective tissues. It has been shown that the biological action of HA can be synergistically enhanced by adding small active molecules such as AAs, thus increasing the possible application of this polymer in different fields of medicine and in the therapy or revitalization of connective tissue in particular.

The aim of this study was to assess the effects of different mixtures of AAs and HA on the production of extracellular matrix proteins, in particular: elastin and collagen IV, a major component of basal lamina.

For this purpose, a transcriptomic dynamic approach was applied to human primary dermal

fibroblast cultured in monolayer (from the breast skin of a 44-year-old donor, responsive to elastin synthesis), which was cultured for 5 days in the presence or absence (negative control) of products, while monitoring the expression kinetics of the selected genes after 24, 72 and 120 hours via qRT-PCR. The production of proteins was verified by Western blot analysis after 120 h of treatment.

Regarding the effects on transcriptional activity, as a general consideration, it can be noted that there was no change in gene expression until 72 h, possibly because the timing was insufficient for the dermal fibroblast monolayer to express and produce functional fibres.

In a recent study (14), it was demonstrated that 6.2% HA containing several active molecules, including AAs, progressively increased ELN expression in dermal fibroblasts, reaching a maximum at 72 hours.

This data supports the hypothesis that, in fibroblast monolayer, the expression of ECM proteins (in particular elastin) is not an immediate process. The presence of stimulation at 24-72 h is attributable to the different experimental design (different mixtures tested and application method)

In our model, an overexpression occurred after 120 h of growth with treatment and, as assumed, the test items exerted a differential action, on ELN and COLIV in particular. As summarized in Table VIII, compared to the negative control (untreated), formulation A, which can be considered an experimental benchmark [since its activity has been investigated in various studies (9-10,13)], it did not induce any up-regulation of the selected genes, although its ability to stimulate the transcription of collagens I and III has been previously reported (13). This formulation contains a combination of AAs, principally glycine and proline (present in large quantities in collagen molecules) and, following the assumption that the ECM protein synthesis can be enhanced by increasing the local bio-availability of specific constituent AAs, it can be argued that the AAs in formulation A are not the most effective for improving non-collagenous ECM proteins.

To verify this idea, other mixtures were formulated based on the AA composition of elastin (15), while

decreasing the general glycine and proline content, introducing the specific hydrophobic residues, valine and alanine (in formulation B), doubling the amount of leucine (in formulation C) and increasing the amount of valine and alanine (in formulation D). Of the three products, C and D induced a significant up-regulation of ELN and COLIV genes. With regard to ELN, it seems that proline, alanine and valine are important for the biosynthesis thereof and this indication seems to be confirmed by the Avantaggiato and colleagues (14) study in which ELN gene expression was stimulated by a product that also contained AAs. Interestingly, collagen IV was also stimulated by mixtures C and D, thus indicating that those formulations are able to act on other components of the EMC matrix.

In order to verify the biological effects of the formulations on ECM component production, the ex-novo bio-synthesis of elastin and collagen IV was verified by Western blot analysis after 5 days of treatment. As reported in Table V, formulation A did not have any effect (as expected from RTqPCR data), while all the other mixtures (B, C and D) induced an increase in protein production. Interestingly, in connection to elastin, the products seemed to act differently on the two protein forms, insoluble α -elastin and soluble tropoelastin, thus indicating that different formulations can enhance the amount of the precursor (which can act as a reservoir for elastin recovery in the event of tissue damage) or promote protein maturation. Surprisingly, mixture B stimulated protein synthesis although no increase in gene expression was detected. This result could relate to the fact that differences between mRNA and protein levels can be observed depending on several factors, including the kinetics of transcription and translation phases, which can be influenced by local culture, cellular response to the stimulus, etc. (17).

The second phase of the study, which was conducted to clarify the interactions between the different clusters of AA, demonstrated that, given that the total amount of AAs in all the mixtures was always the same, a mixture containing AA amounts of both proteins must be selected in order to give a significant response and a significant production of both collagen and elastin. This also proves the

existence of a ratio between the 2 clusters (AA elastin/AA collagen) that guarantees an adequate and balanced response to fibroblast, collagen and elastin gene expression and production.

Moreover, the data of this pilot study must be confirmed and extended in order to allow in-depth investigation of the underlying mechanism of action.

This study, conducted on human dermal fibroblast monolayer using a transcriptomic dynamic approach, has shown the efficacy of different proprietary mixtures of AAs and HA in modulating the biosynthesis of extra cellular matrix proteins and in promoting elastogenesis in particular. It has been demonstrated that varying the quality and quantity of AAs in the mixtures allows elastin expression to be increased at gene and protein level by maintaining collagen stimulation, and of type IV collagen in particular.

This preliminary data can be a starting point for further studies on the biological activity of new mixtures of AAs and HA and on their future application in the medical field.

CONFLICT OF INTEREST: A. Orlandini has received consultancy fees from Professional Dietetics. The other Authors declare no conflicts of interest relevant to this article.

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ROLE OF APC-MEDIATED MDR-1/CLCX-1 SIGNALING PATHWAY IN OVARIAN TUMORS

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We explored the role of APC-mediated MDR-1/CLCX-1 signaling pathway in ovarian tumors. In this study, ovarian tumor cell lines SKOV-3, ES-2 and MCV-152 were used to conduct the research. Fluorescence quantitative PCR and Western-blotting were used to investigate the effects of MDR-1/CLCX-1 signaling pathway in ovarian tumors. The effects of the APC gene silencing and overexpression on proliferation and apoptosis of ovarian tumor cells were detected by flow cytometry. Compared to normal cells, the expression of APC gene mRNA in ovarian tumor cells was significantly decreased ($p < 0.05$). Western-blotting results showed that the level of APC protein in ovarian tumor cells was significantly lower than that in normal tissue, while MDR-1/CLCX-1 related proteins levels were significantly increased ($p < 0.05$). In the APC gene silenced ovarian tumor cell lines, the expression of MDR-1/CLCX-1 was significantly higher than that of the untreated group ($p < 0.05$), and apoptosis of ovarian tumor cells decreased. However, in ovarian tumor cell lines that over-expressed APC gene, the expression of MDR-1/CLCX-1 was significantly lower than that of the untreated group ($p < 0.05$), and apoptosis of ovarian tumor cells was increased. There is a certain correlation between the APC gene and ovarian tumors, and the APC gene mediates the apoptosis of tumor cells through the MDR-1/CLCX-1 signaling pathway.

As one of the three major types of tumors of the female reproductive system, the incidence rate of ovarian cancer ranks third overall (1, 2). Research has shown that currently, the prevalence of ovarian cancer in China is approximately 0.174% and increases by nearly 1.52% per year. Therefore, it has become increasingly important to strengthen the ability to diagnose and treat ovarian cancer. Clinical studies (3, 4) have shown that there were no significant symptoms in the early stage of ovarian tumors, and nearly 78.4% were transferred to different degrees after the diagnosis (5). Yajing et al. (6) found that colonic adenomatous polyposis coli (APC) gene plays an important role in cell proliferation, differentiation and migration in some

tumors and cancer cells.

Further research also showed that the APC gene plays a certain role in inhibiting migration and proliferation of tumor cells, such as colon cancer cells (7) and gastric cancer cells (8). The overexpression of this gene can inhibit the migration of some tumor cells. However, the association between the gene and ovarian tumor is rarely reported. It is suggested that as an important cellular pathway involved in the regulation of tumors and cancers (9), MDR-1/CLCX-1 plays an important role in the occurrence, development, invasion and metastasis of tumor cells (10). Therefore, it has been proven that there is a certain correlation between the MDR-1/CLCX-1 signaling pathway and the differentiation and

Key words: APC gene, MDR-1/CLCX-1 signaling pathway, ovarian tumor, cell apoptosis

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0393-974X (2018)

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proliferation of ovarian cancer cells; the expression of MDR-1/CLCX-1 signaling pathway-related genes in ovarian cancer cells were significantly different from that in normal ovarian cells. In this study, we first explored the effects of APC-mediated MDR-1/CLCX-1 signaling pathway in different ovarian cancer cell lines, and the underlying interaction mechanism was discussed, which lays the foundation for further studies.

MATERIALS AND METHODS

Materials

Ovarian tumor cell lines SKOV-3, ES-2 and MCV-152 were used for this study.

The main reagents were APC goat anti-rabbit primary antibody (Cell Signaling Technology, Danvers, MA, USA), fetal bovine serum (Axygen, Tewksbury, MA, USA), CRISPER kit (ABM, Suzhou, China), and trypsin (Sigma, St. Louis, MO, USA), which were all obtained commercially.

The main instruments were Multiskan spectrum (ABI, MA, USA), protein electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd, Beijing, China), fluorescence quantitative PCR instrument (ABI, MA, USA), incubator (Olympus, Japan), and inverted microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

CRISPER/Cas gene knockout

The APC gene in different ovarian tumor cell lines (SKOV-3, ES-2 and MCV-152) was knocked out by CRISPER/Cas9 technology. The sgRNAs that were used in different ovarian cancer cell lines were designed through the website (<https://chopchop.rc.fas.harvard.edu/index.php>), and were synthesized by the Suzhou Genewiz Biological Engineering Co., Ltd. The sequences are shown in Table I. Experimental procedures referred to the specification and related literature (11).

APC gene overexpression

The APC gene was overexpressed by the pAV32-6 vector. Primers used for the amplification of the APC gene were synthesized by the Sangon Biotech (Shanghai, China) Co., Ltd., and the sequences are shown in Table I. The enzyme restriction sites were BamH1 and Kpn1, respectively.

RNA extraction

Different ovarian cancer cell lines were cultured in 5% CO₂ incubator at 37°C. RNA was extracted according to the RNA extraction kit manual operation.

Fluorescence quantitative PCR

In order to detect the mRNA expression of the APC gene in different samples, the SYBRGREEN1 dye method was used, and specific experimental protocol was followed according to the instructions. The sequences are shown in Table II.

Western-blotting

The total protein was extracted using the cell protein extraction kit in accordance with the Guide of Molecular Cloning (third edition). The primary antibody was diluted according to the ratio of 1:800, and the secondary antibody was diluted according to the ratio of 1:500 (12).

Flow cytometry detection

Different types of ovarian cancer cells were collected (about 1-5x10⁶/ml) at 4°C. After centrifugation at 3800 rpm, the cells were washed 2-3 times with PBS treated by sterilization (pH=7.2). The experimental procedures were carried out according to the reference literature (13).

Statistical analysis

The statistical analysis was performed using the SPSS 20.0 software (Version X; IBM, Armonk, NY, USA). $\alpha=0.05$; $p<0.05$ was considered statistically significant ($\alpha=0.01$, $p<0.01$) was considered statistically significant.

RESULTS

APC gene amplification and construction of overexpression vector

In order to construct the APC overexpression ovarian tumor cell line, we selected the pAV32-6 vector as an overexpression vector and apc-F/R as the primer to amplify the APC gene. BamH1 and Kpn1 were used to carry out the enzyme digestion. The PCR amplification result (Fig. 1) shows that the length of the APC gene was about 4320 bp, and there were two obvious bands after enzyme digestion and connection with overexpression vector, which suggested that the overexpression vector was constructed.

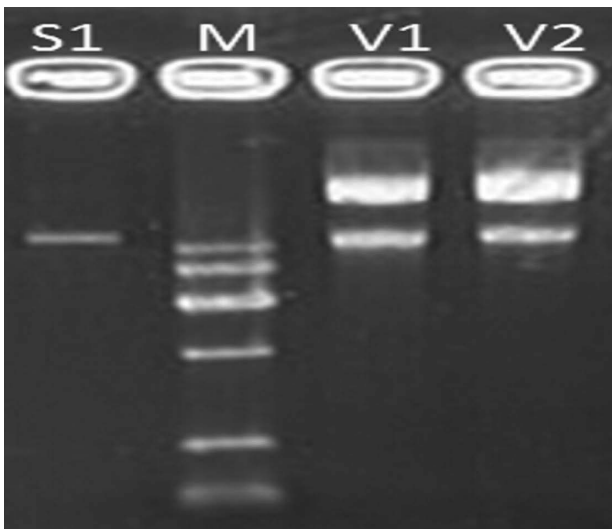


Fig. 1. Results of APC gene amplification and double enzyme digestion of overexpression vector. S1: PCR product of APC gene; M: DL4000; V1: Recombinant overexpression vector 1; V2: Recombinant overexpression vector 2.

Fluorescence quantitative determination of mRNA in different types of ovarian tumor cells

Total RNA was extracted from different types of ovarian cancer cell lines. The expression of APC and MDR-1/CLCX-1 mRNA were determined by a fluorescence quantitative PCR method; the results are shown in Fig. 2. Compared to normal cells, the expression of APC mRNA levels in ovarian cancer was significantly decreased. The levels of APC

mRNA in normal cells is 12.3 times that of SKOV-3 ovarian cancer cells, 17.4 times of that in ES-2 ovarian cancer cells, and 20.6 times of that in MCV-152 ovarian cancer cells ($p < 0.05$). For MDR-1 and CLCX-1 mRNA levels, the expression levels in normal tissues were significantly lower than that in ovarian cancer tissue samples ($p < 0.05$). These results suggest that there were significant differences in the expression of APC and MDR-1/CLCX-1 genes in normal and ovarian cancer cells ($p < 0.05$). Therefore, there is a certain relationship between the APC gene and MDR-1/CLCX-1 gene and ovarian cancer.

Expression of MDR-1/CLCX-1 mRNAs after APC gene overexpression or knockout

In order to explore the correlation between APC and MDR-1/CLCX-1, we detected MDR-1/CLCX-1 mRNA expressions in ovarian cancer cells with either APC overexpression or knockout; results are shown in Fig. 3. In ovarian cancer cell lines with APC overexpression, the expression of MDR-1/CLCX-1 genes were inhibited and the expression levels were all significantly decreased. In contrast, in ovarian cancer cell lines with an APC knockout, the expressions levels of MDR-1/CLCX-1 genes were all significantly increased. These results suggest that the APC gene could regulate the expression of MDR-1 and CLCX-1 genes.

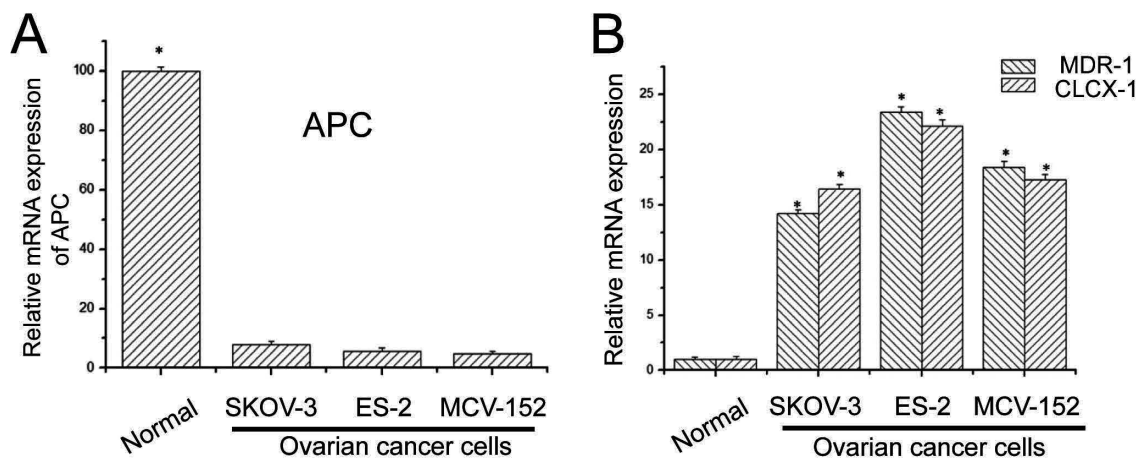


Fig. 2. Determination of mRNA expression of APC and MDR-1/CLCX-1 in different samples. A) Relative expression level of APC mRNA; B) Relative expression level of MDR-1/CLCX-1 mRNA. “*” represents a significant difference between groups.

Table I. *sgRNA sequence.*

Primer	Sequence
SKOV-3-1	AGTGCTAGCTAGTCGATCGATCG
SKOV-3-2	GTCGTAGCTGCTAGCTAGCTGATC
ES-2-1	CGTAGCTAGTCGGCTAGCTAGCAT
ES-2-2	CGTAGCTGATATGCTAGATAGCTAG
MCV-152-1	ATGTCGTAGCTGCGTAGCTAGCTAGC
MCV-152-2	TCGTAGCTAGCTAGCTACGATGAGTGAC
Apc-F	GGATCCATGTGCTAGCTAGCTCGGATCG
Apc-R	GGTACCCGTAGCTAGCTAGCTAGCTAGCTA

Table II. *Primers for fluorescence quantitative PCR.*

Primer	Sequence
apc-F	GTCGATCGTAGCCGGCTAGC
apc-R	CGTAGCTAGGCTAGCTAGCG
MDR-1-F	GTCGATGCTAGCTAGCTACGGAT
MDR-1-R	CGTAGCTGGCTAGCTAGCTAC
CLCX-1-F	CGTAGCTAGGCGTAGCTACG
CLC-1-R	CGTAGCTAGCGATCGTACGTAG
GAPDH-F	CGTAGGGCTAGCTAGCTAGATAC
GAPDH-R	CGTAGCTGAGAGTTAGCTAGCATC

Table III. *Semi-quantitative results of MDR-1/CLCX-1 protein levels after APC gene overexpression or knockout.*

Group	Control group (ug/l)	Ovarian cancer cells with APC overexpression (ug/l)	Ovarian cancer cells with APC knockout (ug/l)
MDR-1	1.05±0.11	0.18±0.07*	9.47±0.12*
CLCX-1	1.26±0.09	0.25±0.09*	10.04±0.15**

*P indicated a significant difference; **P indicated an extremely significant difference.

Table IV. *Apoptosis percentage of ovarian cancer cells with different treatments.*

Group	APC overexpression	No treatment	APC knockout
Apoptosis percentage (%)	12.6	19.6	68.4%

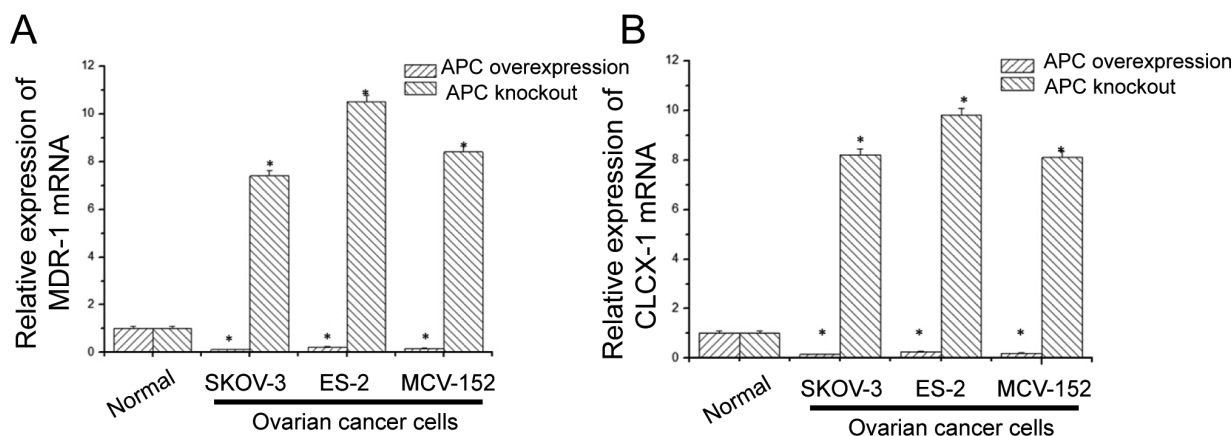


Fig. 3. Expression of *MDR-1/CLCX-1* mRNAs in ovarian cancer cells with *APC* overexpression and knockout. **A)** Relative expression level of *MDR-1* mRNA; **B)** Relative expression level of *CLCX-1* mRNA. “*” represents a significant difference between groups.

Determination of *MDR-1/CLCX-1* proteins after *APC* gene overexpression or knockout with Western-blotting

Compared to untreated ovarian cancer cells, an overexpression of *APC* genes decreased the expression of *MDR-1/CLCX-1* proteins in different ovarian tumor cells. The deletion of the *APC* gene increased the expression of *MDR-1/CLCX-1* proteins. The semi-quantitative results of Western-blotting showed that compared to untreated ovarian cancer cells, overexpression of *APC* gene reduced the levels of *MDR-1/CLCX-1* proteins by 84%-92% in different ovarian cancer cell lines (Fig. 4). While the *APC* gene deletion increased the levels of *MDR-1/CLCX-1* proteins 8-12 times (Table III). The results indicated that the *APC* gene could regulate the expression of *MDR-1/CLCX-1* proteins and participate in the pathogenesis of ovarian cancer.

Detection of apoptosis in different ovarian cancer cells with flow cytometry

In order to study the correlation between ovarian cancer and *MDR-1/CLCX-1* signaling pathway mediated by *APC* gene, we used flow cytometry to detect cell apoptosis in ovarian cancer cells with *APC* overexpression or knockout under the same culture conditions; the results were shown in Fig. 5. Compared to the control group, overexpression of *APC* gene promoted the apoptosis in ovarian cancer cells, while deletion of *APC* gene inhibited cell apoptosis. In ovarian cancer cell lines with *APC* overexpression, the proportion of cell apoptosis was about 12.6%, which was lower than that in untreated ovarian cancer cells (19.6%) and ovarian cancer cells with *APC* knockout (68.4%); this shows a significant difference between the three groups (Table IV)($p < 0.05$).

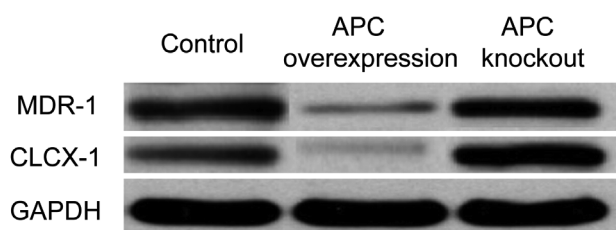


Fig. 4. Determination of *MDR-1/CLCX-1* proteins after *APC* gene overexpression or knockout with Western-blotting.

DISCUSSION

As a common disease of the female reproductive system, the incidence of ovarian tumor in China is showing an increasing trend year by year (14). Clinical studies have indicated that the occurrence of ovarian cancer is affected by genetic factors, environmental conditions and other factors (15). For example, the proto-oncogene mutation and tumor suppressor gene

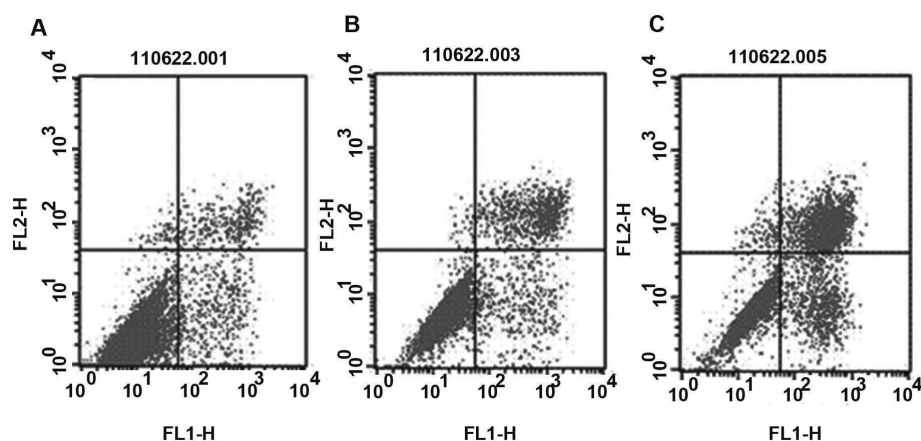


Fig. 5. Detection of apoptosis in different ovarian cancer cells with flow cytometry. *A)* Ovarian cancer cells with APC overexpression; *B)* Untreated ovarian cancer cells; *C)* Ovarian cancer cells with APC knockout.

mutations are likely to induce ovarian tumors and other diseases. In recent years, the environmental condition in China has gradually deteriorated due to water pollution, air pollution and the use of a large number of food additives and hormones. Since the etiology of ovarian cancer is complex, and the early symptoms are not obvious, there is no effective means for the early diagnosis of ovarian tumors at present (16). This directly leads to the fact that most of the patients with ovarian tumors are in the middle or late stage at the time of diagnosis (17).

In recent years, with the development of molecular biology technology, researchers have found that the methylation of some genes could promote the occurrence of ovarian cancer to some certain extent (18, 19). One study suggested that the treatment of SKOV3 ovarian cancer cells with methylation treatment reagents of different concentrations inhibited the growth of ovarian cancer cells (20). It was also shown to inhibit the activation of some genes to a certain extent and then inhibit gene expression, such as promoting ovarian cancer gene expression and activating the proto oncogenes. Further studies have found that the rate of apoptosis was positively correlated with drug concentration (21).

With an increase in drug concentration, the degree of methylation of gene promoters increases gradually, resulting in inactivation of some cancer

genes and ultimately leading to apoptosis. With the deepening of research, a large number of genes associated with methylation in ovarian cancer were found and identified, such as Bcl-2, FANCF, and H-ras. These genes were mainly involved in body cell proliferation, DNA replication, DNA repair and cell transfer. It is known that the APC protein encoded by APC gene is mainly involved in cell differentiation, proliferation and migration (22). Clinical studies have shown that the APC gene showed abnormal expression in colon cancer, gastric cancer, esophageal cancer, and many other cancers, which indicates that APC gene was activated in the above cancers and its expression was closely related to cancers. In addition, the methylation levels of the APC gene promoter were significantly decreased in the above cancers, and the decrease of methylation of gene promoter indicated the increase of gene expression, which was consistent with the increase in APC gene expression in different cancer cells. Therefore, in this study we explored the role of APC-mediated MDR-1/CLCX-1 signaling pathway in ovarian tumors, and results showed that the APC gene could inhibit the expression of MDR-1 and CLCX-1 gene, which further promotes the migration and amplification of ovarian cancer cells. The APC gene, as a tumor suppressor gene, is rarely mutated. However, in this study, the APC gene showed

different degrees of abnormal expression in different ovarian cancer cell lines, indicating that the APC gene might be mutated in ovarian cancer cells and thus, its expression is abnormal. In addition, with molecular technology, it has been found that methylation of the gene promoter region can inhibit gene expression, and demethylation of the promoter region promotes the expression of some genes. Methylation of the promoter region of the APC gene and abnormal expression are found in colon cancer, gastric cancer, breast cancer, endometrial cancer and other cancer cells, which is the focus of the follow-up study.

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DIFFERENCES IN EXPRESSION OF YKL-40 AND TLR4 IN NASAL SINUS MUCOSA OF CHRONIC SINUSITIS PATIENTS WITH AND WITHOUT NASAL POLYPS

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Received April 3, 2018 – Accepted April 17, 2018

This work studies the expression differences of YKL-40 and TLR4 in nasal sinus mucosa of chronic sinusitis patients with and without nasal polyps and its clinical significances. Fifty chronic sinusitis patients with nasal polyps and 50 chronic sinusitis patients without, accepted by our hospital during February 2016-February 2017, were included and taken as group A and group B, respectively. In addition, another 50 patients with nasal deviation were taken as group C (control group). The ostiomeatal complex mucosa of group A and B and the inferior turbinate mucosa of group C were taken and the fluorescence quantitative PCR method was applied to detect the expression of YKL-40, TLR4 and NF- κ B of the mucosa and explore and influence of YKL-40 and TLR4 on NF- κ B. There was a negative correlation between YKL-40 and TLR4 in group A, and the difference was statistically significant ($P < 0.05$) while there was no relationship between YKL-40 and TLR4 expression in group B. The level of YKL-40 protein in group A was higher than that in group B, which was statistically significant ($P < 0.05$). YKL-40 and TLR4 were positively correlated in group A while there was no correlation between YKL-40 and TLR4 expression in group B. The expression of YKL-40, TLR4 and NF- κ B in chronic sinusitis patients with nasal polyps was high. In addition, there was a negative correlation between YKL-40 and TLR4 expression in chronic sinusitis patients with nasal polyps. YKL-40 and TLR4 interacted with each other to activate NF- κ B and promote disease progression.

Chronic rhinosinusitis is one of the common chronic diseases (1, 2), most of which are developed from acute sinusitis attacks which are not well cured (3, 4). Chronic rhinosinusitis seriously affects quality life and work and has been studied by many researchers (5). Suh et al. (6) assisted the treatment of chronic rhinosinusitis with antimicrobial agents, surfactants, cyclic diuretics and macrolide antibiotics, and demonstrated that tissue bacteria attachment and aggregation was an appropriate method to reduce the

sinus inflammation and protect the cilia. Tomassen et al. (7) tried to determine the inflammatory endotypes of chronic nasal-sinusitis through experiments. Patients with chronic nasal-sinusitis and controls underwent surgery, and the expressions of interleukin (IL)-5, interferon (IFN)- γ and IL-17A were analyzed using clustering. Ten clusters were obtained, of which four had low or undetectable IL-5, eosinophilic cationic protein, IgE, and albumin concentrations, and 6 had high concentrations of

Key words: chronic rhinosinusitis, nasal polyps, YKL-40, TLR4 expression

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0393-974X (2018)

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those markers. Moreover, it was found that distinct CRS clusters with diverse inflammatory mechanisms largely correlated with phenotypes and further differentiated them, and provided a more accurate description of the inflammatory mechanisms involved than phenotype information only. Foreman et al. (8) found that biofilm clearly existed in the sinus mucosa of patients with chronic rhinosinusitis and could cause sinusitis, which was associated with severe disease characteristics and surgical difficulty. Therefore, the study of biofilm is the key to treatment of patients with chronic rhinosinusitis. In this paper, the expression of YKL-40 and TLR4 in chronic sinusitis patients was studied to explore the relationship between the two factors and its clinical significance. The results show that the expressions of YKL-40, TLR4 and NF- κ B in patients with chronic rhinosinusitis and those in the control group were significantly different ($P < 0.05$). Furthermore, the expression of YKL-40 and TLR4 in patients with chronic rhinosinusitis with nasal polyps was positively correlated, which was not found in those patients without nasal polyps. Also, YKL-40 and TLR4 could activate NF- κ B and promote disease progression.

MATERIALS AND METHODS

Case selection

Fifty chronic sinusitis patients with nasal polyps (average age: 24.5 ± 65.2 years) and 50 chronic sinusitis patients without nasal polyps (average age: 28.2 ± 65.8 years), admitted to our hospital during February 2016–February 2017, were included and taken as group A and group B, respectively. Another 50 patients with nasal deviation were taken as group C (control group) (average age: 25.6 ± 64.3 years). The selected three groups of patients had no tumor, hyperlipidemia or other chronic diseases. There was no significant difference in the gender and age of the three groups ($P < 0.05$). This study was approved by the medical ethics committee of Qilu Hospital, Shandong, China. Moreover, all the patients signed informed consent to participate in the study.

Materials and instruments

Reagents: universal SP-9000 (Beijing Zhongshan

Biotechnology Co., Ltd.); rabbit source Anti-YKL40 antibody (Jinrui Si Biotechnology Co., Ltd.); rabbit source Anti-TLR4 antibody (Jinrui Si Biotechnology Co., Ltd.); TRIZOL reagent (Shanghai LianShuo Biotechnology Co., Ltd.); reverse transcription kit (Shanghai LianShuo Biotechnology Co., Ltd.); Real Master Mix SYBR Green (Torobo, Japan).

Instruments: PCR instrument (Zhuhai Oumeike Instrument Co., Ltd.); dehydrator [Leica Microsystems (Shanghai) Trading Co., Ltd.]; paraffin slicing machine (Leica Microsystems (Shanghai) Trading Co., Ltd.); biological microscope [Leica Microsystems (Shanghai) Trading Co., Ltd.].

Specimen collection and processing

The blood and tissue fluid on the surface of the collected nasal tissue specimens were washed with physiological saline. The specimens were then divided into two portions, which were stored in sterile test tubes and formaldehyde solution, respectively.

Detection of expression of YKL-40, TLR4 and NF- κ B mRNA in nasal mucosa

Firstly, the stored nasal mucosa tissues were taken out, ground and lysed with cell lysate, then total RNA was extracted with TRIZOL reagent. Next the reverse transcription kit was used to reverse the extracted total RNA into the first template strand cRNA. Finally cRNA was detected using a PCR kit.

YKL-40 and TLR4 protein content detection

The collected tissue was fixed with 10% formaldehyde, dehydrated and paraffin-wrapped for 5 times of slicing. The prepared slices were dried with a dryer and put into a 65°C oven for 40 min. The slices were then dewaxed with xylene and dehydrated with gradient ethanol, and subjected to thermal repair in a citrate solution at 100°C for 20 min. After cooling, the slices were washed with distilled water 4 times, 2 min each time, followed by washing with phosphate buffer (9) for 4 times, 2 min for each time. The washed slices were placed in 3% hydrogen peroxide deionized water, stored at room temperature for 15 min, and then washed 4 times with phosphate buffer. After the addition of anti-YKL40 antibody and rabbit anti-TLR4 antibody, the

slices were placed in a 5°C wet box. After overnight incubation, the slices were cooled and washed using water for four times, 2 min each time, and phosphate buffer solution for four times, 2 min each time. Next, normal goat serum working fluid and biotinylated secondary antibody working fluid were added at room temperature, followed by 15 min of incubation and washing with phosphate buffer solution 4 times, 3 min each time. Color development was performed with diaminobenzidine (10) until the dye was observed to a suitable degree under a microscope. Finally the slices were redyed with hematoxylin and mounted.

Dyeing score criteria

The scoring under 100 times microscope was as follows: no color was recorded as 0 points, light yellow as 1 point, brownish yellow as 2 points and brown as 3 points.

Primer design

Primer sequences (11) were designed and synthesized by Biotech Bioengineering Co., Ltd.

Statistical analysis

SPSS17.0 software (12) was used to perform statistics of the data, and the content was expressed in the form of $\bar{x} \pm s$. $P < 0.05$ was used to indicate statistically significant differences.

RESULTS

mRNA expression of YKL-40, TLR4 and NF- κ B

The detection results of mRNA expression (13) of YKL-40, TLR4 and NF- κ B of the three groups are shown in Table II.

The expression of YKL-40, TLR4 and NF- κ B proteins in group A were significantly higher than

Table I. *Primer sequences.*

	Primer sequence upstream	Primer sequence downstream
YKL-40	5'-TGAG-GCATCGCAATGTAAG-3'	5'-AAGGGGAAG-TAGGATAGGGG-3'
TLR4	5'-TGTC-CTCCCACTCCAGGTAAGT-3'	5'-GATTGCT-CAGACCTGGCAGTT-3'
NF- κ B	5'-TCCAGAAGTATTTCAACCACAG-3'	5'-GCCT-TCACATACATAACGGA-3'

Table II. *mRNA expression of YKL-40, TLR4 and NF- κ B of the three groups.*

	Chronic rhinosinusitis with polyps (group A)	Chronic rhinosinusitis without polyps (Group B)	Nasal septum deviation (Group C)
YKL-40	0.9 $\pm$ 0.15*#	0.74 $\pm$ 0.11*	0.5 $\pm$ 0.09
TLR4	0.89 $\pm$ 0.16*#	0.64 $\pm$ 0.13*	0.48 $\pm$ 0.11
NF- κ B	0.83 $\pm$ 0.12*#	0.67 $\pm$ 0.10*	0.36 $\pm$ 0.12

Statistically significant ($p < 0.05$) compared with group B. * Statistically significant ($p < 0.05$), compared with group C

those in groups B and C ($P < 0.05$); the expression of YKL-40, TLR4 and NF- κ B proteins in group B were obviously higher than those in group C ($P < 0.05$); the relative expression of YKL-40 mRNA in group A was positively correlated with the expression of TLR4 and NF- κ B (Table II, Figs. 1 and 2).

YKL-40 and TLR4 protein expression analysis

YKL-40 and TLR4 protein expression (14) was distributed in the nasal mucosa cells, generally in the same position. YKL-40 positive staining mainly existed in the cytoplasm while TLR4 positive staining was mainly in the cytoplasm and cell membrane (Fig. 3).

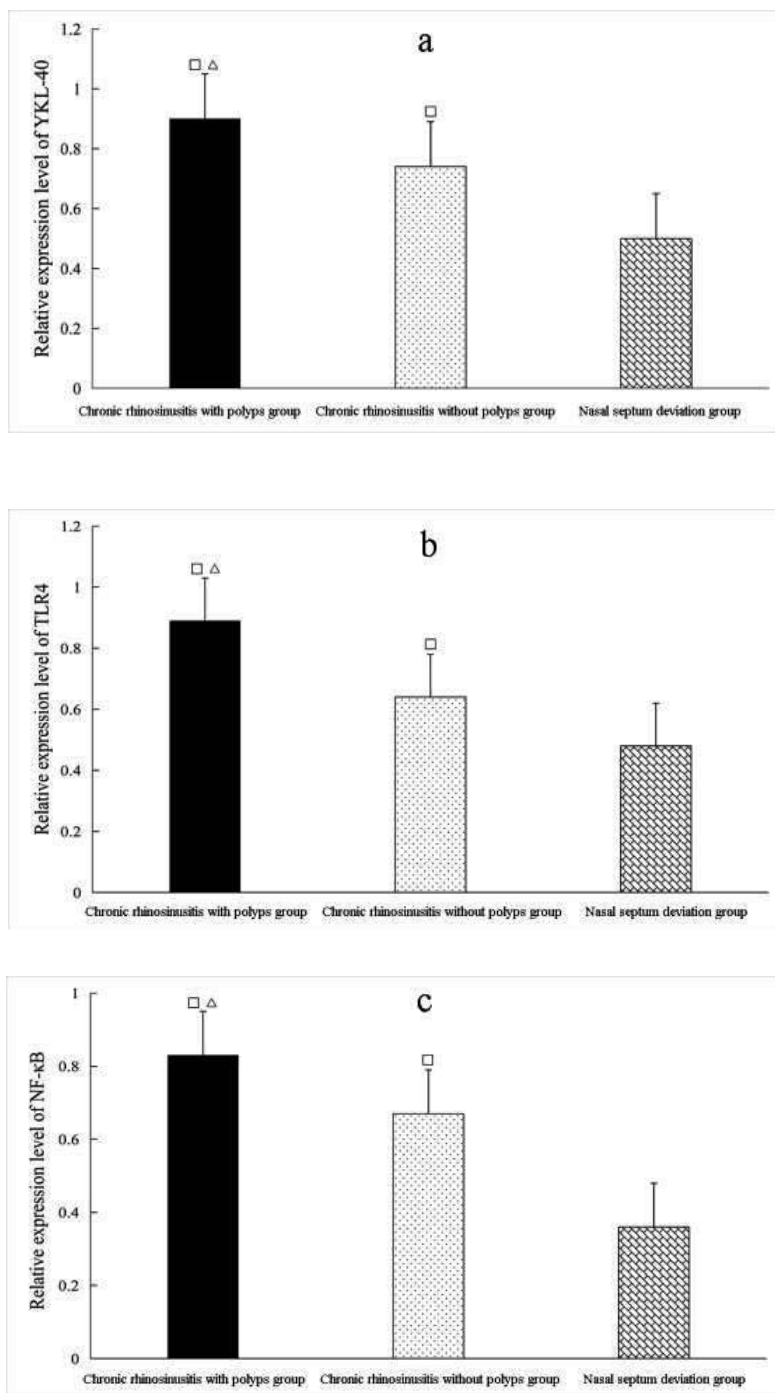


Fig. 1. Histogram of mRNA expression of YKL-40 (a), TLR4 (b) and NF- κ B (c).

□ indicates $p < 0.05$ compared to group B; □ indicates $p < 0.05$ compared to group C;

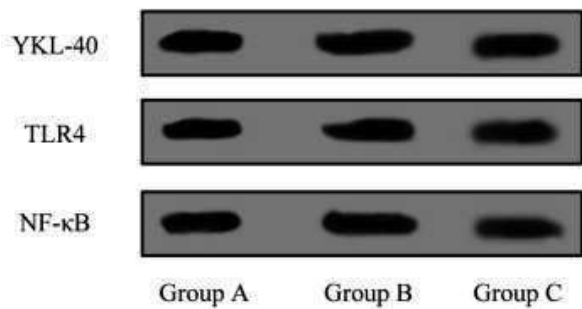


Fig. 2. Western blot of expression of YKL-40, TLR4 and NF-κB proteins in the three groups.

As shown in Table III, the expression of YKL-40 protein in group A was higher than that in group B ($P < 0.05$) while that between group B and C was not obviously different. The expression of TLR4 protein in group A and B was higher than that in group C ($P < 0.05$). There was a negative correlation between YKL-40 and TLR4 protein expression in group A while no such relationship was found between YKL-40 and TLR4 protein expression in group B.

DISCUSSION

To date, the pathogenesis of chronic

rhinosinusitis has not been found (15). Li et al. (16) found that although external risk factors were connected to the development of chronic rhinosinusitis, the mucosal response in the human body was also important. Under inflammatory stress, nasal epithelial injury may occur, causing epithelial hyperplasia, goblet cell metaplasia, stripping, cilia, fibrosis and basement membrane thickening and other rapid remodeling reactions (17). By studying the correlation between TLR2, TLR4 and cytokines and histopathological features, one Author (18) found that TLR2 and TLR4 were associated with TGF-β1 and CRSsNP remodeling, especially with histological features of collagen deposition and neutrophil infiltration, which were closely related to disease development. As a marker of inflammation, YKL-40 and TLR4 are of great use in the diagnosis of chronic rhinosinusitis (19-21). There were some differences in YKL-40 and TLR4 between nasal-sinusitis patients and healthy people as well as between patients with and without nasal polyps (22). It was found that the expression of YKL-40 and TLR4 as well as NF-κB in patients with nasal polyps was much higher than that in patients without nasal polyps ($P < 0.05$). Moreover, the protein expression of the two factors was also higher in patients with nasal polyps, indicating

Table III. YKL-40 and TLR4 protein expression scores of the three groups.

	Chronic rhinosinusitis with polyps (group A)		Chronic rhinosinusitis without polyps (Group B)		Nasal septum deviation (Group C)	
	YKL-40	TLR4	YKL-40	TLR4	YKL-40	TLR4
0 point	3	8	10	9	12	2
1 point	8	22	20	20	20	8
2 point	15	10	10	8	9	20
3 point	24	10	10	13	9	20

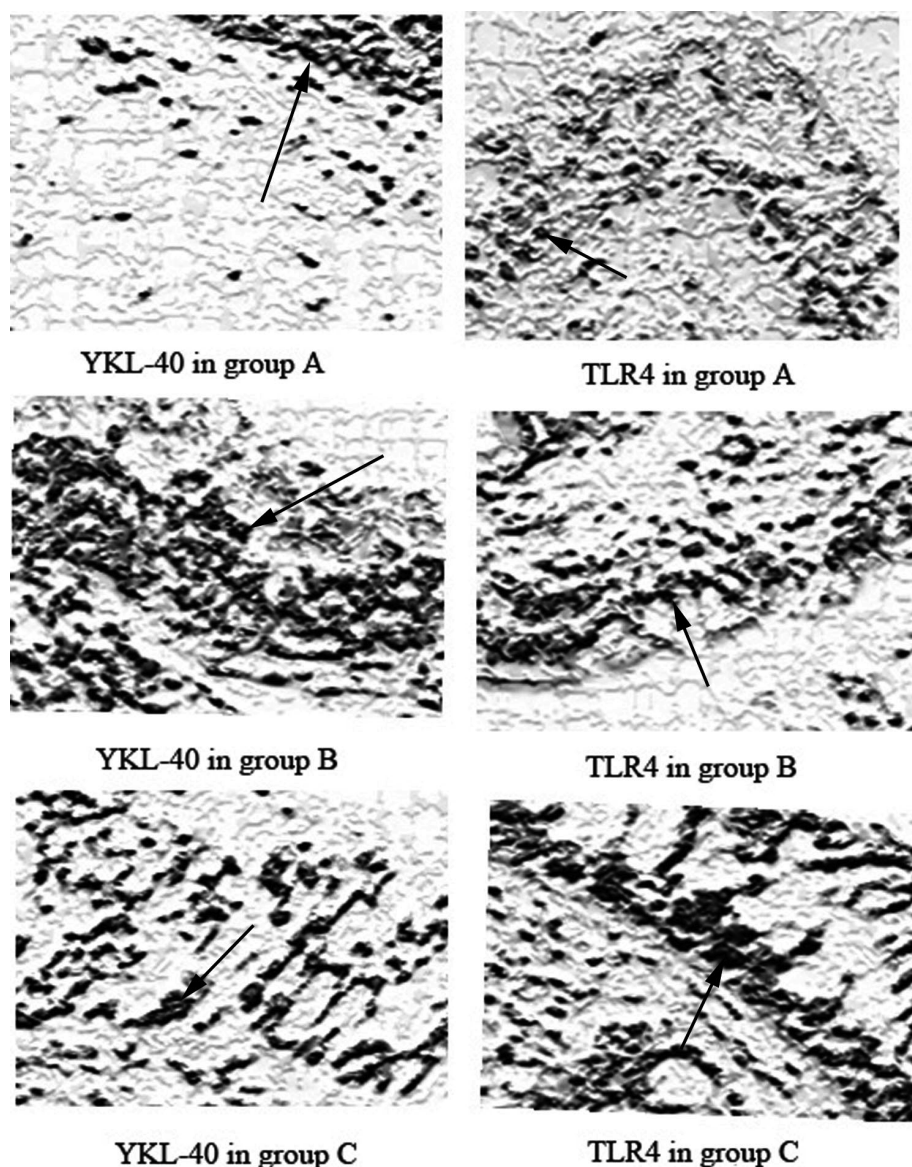


Fig. 3. Expression of YKL-40 and TLR4 in the nasal mucosa in groups A, B and C. Arrows denote the site of the expression of the stained cells with deep colour.

that YKL-40 and TLR4 expression could promote disease progression.

In this study, the expression of YKL-40 and TLR4 in chronic sinusitis patients with and without nasal polyps was studied. The results showed that the expression of YKL-40 and TLR4 mRNA, protein and NF- κ B mRNA were higher in patients with nasal polyps, suggesting that YKL-40 and TLR4 expression could promote disease progression, which provides some reference for the treatment of chronic sinusitis.

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THE INVOLVEMENT AND MECHANISM OF FEBUXOSTAT IN NON-ALCOHOLIC FATTY LIVER DISEASE CELLS

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Received March 9, 2018 – Accepted April 4, 2018

It has been proved that hyperuricemia is associated with non-alcoholic fatty liver disease (NAFLD). The xanthine oxidase (XO) inhibitor, febuxostat, decreases free fatty acids-induced fat accumulation in HFDT-fed mice. Here, it is shown that febuxostat attenuates fat accumulation and reactive oxygen species (ROS) in HepG2 cells. It was further found that the underlying mechanism is related to the reduction in expression of NLRP3/caspase-1/IL-18/IL-1beta and improved insulin resistance (IR). This finding highlights the possible molecular pathways involving NLRP3 activation for management of ROS and insulin IR. In conclusion, febuxostat may be a promising potential treatment for patients with NAFLD.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common health concerns worldwide resulting from lifestyle changes. The disease affects approximately one-third of adults in the United States (1). Recently, non-alcoholic steatohepatitis (NASH) has been ranked as the foremost hepatic disease in China, which shows a high prevalence of 15% in the total population (2). NAFLD comprises a broad spectrum of liver illnesses, extending from simple steatosis, to NASH, fibrosis and cirrhosis, and in some cases even to hepatocellular carcinoma (3-4). These histological liver alterations occur in the absence of alcohol abuse or other causes of chronic hepatic disease (5). Simple steatosis is thought to be benign and asymptomatic with a slow progression over years, whereas it has the potential to progress to cirrhosis and hepatocellular carcinoma (1).

NAFLD is highly associated with obesity, T2DM, insulin resistance (IR) and other metabolic syndromes

(6). Hyperuricemia is a common metabolic abnormality. Several recent lines of epidemiological studies in different populations have demonstrated a significant relationship between serum uric acid and NAFLD. Research from China has shown that the prevalence of NAFLD is positively associated with serum uric acid elevation (1). Elevated serum uric acid significantly increases the risk of NAFLD (7).

Xanthine oxidase (XO), also referred to as xanthine oxidoreductase, is a rate-limiting enzyme that catalyzes the terminal step of purine degradation accompanying generation of reactive oxygen species (ROS) (8). XOR-rich organs include the intestines and the liver. More recently, a role has emerged for the activity of XO in the regulation of lipogenesis and atherosclerosis. The present study also found that the elevated expression and activity of XO are present in cellular and mouse models of NAFLD (1). This phenomenon may molecularly reflect the

Key words: nonalcoholic fatty liver disease, febuxostat, inflammasomes, insulin resistance, therapeutic options

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correlation between NAFLD and hyperuricemia. Elimination of either XO expression or XO activity significantly reduces uric acid generation and exerts a strong protective effect by attenuating hepatocyte fat accumulation in an FFA-induced cellular model of NAFLD (1). It has, moreover, been confirmed that XO has a regulatory effect on hepatic steatosis in HFD-fed and MCD-fed NAFLD mouse models. Prior studies have found that the activity of XO is significantly elevated in NAFLD cases. Reports have revealed that the serum XO activity of patients suffering from familial hyperlipidemia is overtly increasing. The activity of XO is positively related with IR. These results highlight a functional influence of XO on the management of NAFLD. Thus, XO may play a role as a potential new therapeutic target for NAFLD (1).

The NOD-like receptor family, pyrin domain-containing-3 (NLRP3) inflammasome, is a protein complex which is activated through the cellular pathogen- and danger-associated signals through the maturation of inflammatory cytokines such as interleukin (IL)-18. Upon detecting cellular stress, NLRP3 recruits apoptosis-associated speck-like protein (ASC) and procaspase-1 to constitute NLRP3 inflammasomes, which results in maturation and secretion of caspase-1 and pro-inflammatory cytokines, including IL-1 beta and IL-18 (9). Uric acid and ROS are two common activators of NLRP3 inflammasomes. NLRP3 inflammasome activation involves the onset and development of obesity and IR. Recent research has established that NLRP3 inflammasome activation contributes to the development of NAFLD (7).

IR is a critical factor in the development of NAFLD. The activity of XO is also closely related to IR. Previous studies have found that knocking out NLRP3 and caspase-1 expression ameliorates obesity and IR in HFD diet-induced mouse models of NAFLD. Furthermore, related research suggests that NLRP3 recognizes signals released in obesity to contribute to obesity-induced-inflammation and IR (1).

MATERIALS AND METHODS

Cell culture

The human hepatocellular carcinoma cell line (HepG2

cells) was obtained from the Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂. To establish a cellular model of hepatic steatosis, HepG2 cells were exposed to a mixture of free fatty acids (FFA) including oleate and palmitate at a final ratio of 2:1 and final concentration of 1 mM for 48 h. In experiments involving febuxostat, cells were exposed to 1-100 nM of febuxostat.

Cell survival assay

One hundred µl of cell suspension were dispensed (5000 cells/well) in a 96-well plate with media. The plate was pre-incubated for 24 h in a humidified incubator (37°C, 5% CO₂). The cells were then switched to DMEM containing nothing (control) and a mixture of free fatty acids (FFA) including oleate and palmitate at a final ratio of 2:1 and final concentration of 1 mM. The plate was incubated for 48 h and then the cells were then switched to DMEM containing nothing, DMSO, a mixture of free fatty acids (all control), 1 nM febuxostat, 10 nM febuxostat, 30 nM febuxostat, 50 nM febuxostat, 100 nM febuxostat for 3 h. Ten µl of CCK-8 solution were added to each well of the plate and incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader. The absorbance of the solutions was detected at 460 nm using a microplate reader (Bio-Rad, USA).

Oil Red O staining

After washing with PBS twice, cells were fixed with 4% formalin for 15 min in PBS. Cells were washed with sterile double-distilled water and incubated in 0.35% Oil Red O solution in isopropanol for 15 min. Then, cells were washed with sterile double-distilled water and stained with Mayer's Hematoxylin Bio-Opticaready for 5 min and washed again with sterile double distilled water. The images were obtained using Diagnostic Instruments (Nikon, TE2000-U).

JC-1 fluorescence analysis

Cells were rinsed once with PBS and combined with serum-free DMEM and then incubated with JC-1 dye, maintaining room temperature for 20 min. Then the stained cells were washed twice using JC-1 buffer. After adding 2 mL DMEM, the cells were analyzed with fluorescence microscopy, and the images were collected using Diagnostic Instruments (Nikon, TE2000-U).

Western blot analysis

The protein expression levels of NLRP3, caspase-1, IL-18, IL-1 β , p-Akt and p-IRS were determined with Western blotting (China). Proteins were extracted using RIPA buffer (Applygen Technologies Inc., Beijing), protein and phosphatase inhibitor (Sigma), resolved by SDS-PAGE, and transferred to PVDF membranes (Millipore). After blocking with 5% skim milk powder prepared using TBST, the membranes were incubated with primary and secondary antibodies. The primary antibodies used in this study were as follows: anti-NLRP3 (ab16097, 1:500), anti-caspase-1 (SC-514, 1:1000) □ anti-IL-18 (sc-6177-Y, 1:500) □ anti-IL-1 β (A1112; 1:3000) □ anti-P-AKT (Ser473) (AF0908;1:2000) □ P-IRS-1 (Ser307) (AF3272;1:2000) and anti-ACTIN (GB13001-1;1:2000). Horseradish peroxidase-conjugated goat anti-chicken (A21080) and goat anti-mouse (BS 12478) were used as secondary antibodies. Chemiluminescence was visualized using an ECL kit (G2019). The Tanon imaging processing system (Tanon Science and Technology Co., Ltd., Shanghai, China) was used for processing and evaluation of the Western blots by ECL solution.

Statistical analysis

Statistical analysis was performed by SPSS version

19.0 (SPSS Inc., Chicago, IL, USA). Each experiment was performed for at least three times, and results were presented as mean $\pm$ standard deviation (SD). Student-*t* tests were used to evaluate the significance of differences between means. Differences were considered significant when $P < 0.05$.

RESULTS

Febuxostat improves the cell survival rate of HepG2 cells

To elucidate the role of febuxostat in HepG2 cellular proliferation, CCK-8 assays were performed to assess the extent of cell proliferation. Febuxostat improves the cell survival rate of HepG2 cells; as shown in Fig. 1, there was a significant difference between cell growth of the differentiated group (6.4%) and the control group (100%) ($P < 0.05$). As the concentration of Febuxostat was increased (1, 10, 30, 50, 100 nM), the survival rates of the differentiated cells increased. The 30 nM group was significantly different from the differentiated cells group ($P < 0.05$).

Survival comparison of the undifferentiated group, DMSO plus differentiated group,

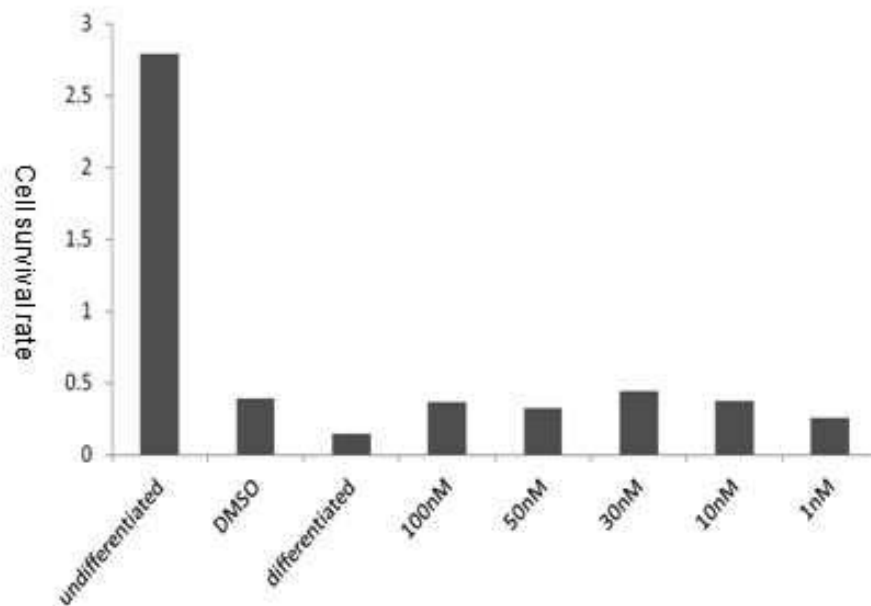


Fig. 1. Effect of febuxostat on survival rate of hepg2 cells.

differentiated group and the treatment group. Each column represents means $\pm$ SD ($n = 6$), $P < 0.05$ compared to undifferentiated group. $\#P < 0.05$ compared to differentiated group.

Febuxostat regulates lipid accumulation in HepG2 cells

The undifferentiated cells (Fig. 2A) are spindle cells which grow rapidly. There are no lipid drops in these undifferentiated cells (Fig. 2B). Febuxostat significantly attenuated FFA-induced intracellular triglyceride accumulation staining red in HepG2 cells (Fig. 2C).

Febuxostat is able to improve the mitochondrial potential of differentiated cells

It is well recognized that ROS is involved in cell death. The reduction of mitochondrial membrane potential is a vital event in the process of cell apoptosis. Dye staining with the cationic, lipid fluorescent dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to detect changes in the mitochondrial membrane potential. There are two types of JC-1 in stained mitochondrial plasma: one is a monomer, which emits green fluorescence when the monomers fail to aggregate in the mitochondrial matrix, leading to less or even no polymer J-aggregates; and the other is a polymer, which is formed by monomers and shows red fluorescence under normal conditions. At the same time, the results of JC-1 reflect ROS in cells. High mitochondrial potential is characterized by red fluorescence. Low mitochondrial potential is characterized by green fluorescence. In the undifferentiated group, red fluorescent signals

tended to be stronger than green fluorescent signals (Fig. 3A). In the CCCP group—as a negative control group—red fluorescent signals had almost disappeared, and green fluorescent signals were greatly enhanced (Fig. 3B). In the differentiated group, the potential was significantly diminished, as reflected by the fluorescent signals shifted from red to green compared with the undifferentiated group (Fig. 3C). The febuxostat treatment significantly attenuated this change. There were stronger red fluorescent signals and weaker green fluorescent signals compared with the differentiated group (Fig. 3D). Overall, the results indicated that cell apoptosis is more obvious in the differentiated group than in the febuxostat treatment group. Above all, febuxostat has the ability to prevent mitochondrial ROS generation in differentiated cells.

Febuxostat suppresses inflammatory response and attenuates IR

ROS could modulate NLRP3 inflammasome activation in cells. NLRP3 recruits apoptosis-associated speck-like protein (ASC) and procaspase-1 to constitute NLRP3 inflammasomes, which results in maturation and secretion of caspase-1 and pro-inflammatory cytokines, including IL-1 beta and IL-18. IL-1 β and IL-18 increase TG and TC accumulation in cells. These data yield certain clues for delineating the mechanism by which febuxostat regulates the accumulation of lipid droplets in cells. Febuxostat downregulates the intracellular expression levels of NLRP3, capase-1, IL-18 and IL-1 β (Fig. 4), which suggests that febuxostat attenuates the expression of ROS in differentiated cells. Compared with the



Fig. 2. Effect of febuxostat on lipid accumulation in HepG2 cells. **A)** undifferentiated cell group; **B)** differentiated cell group; **C)** differentiated plus febuxostat cell group.

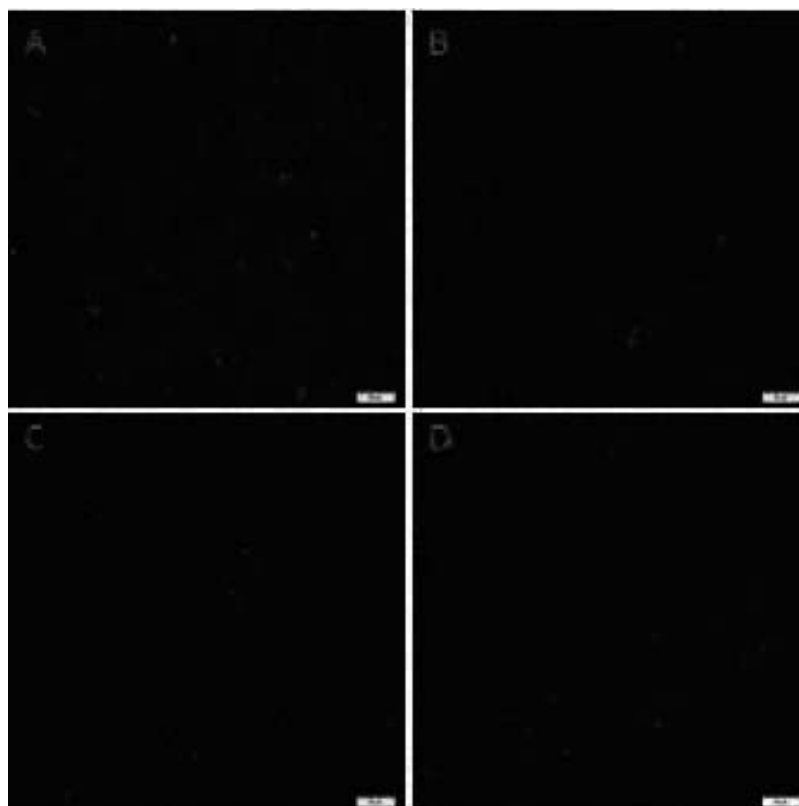


Fig. 3. Effect of febuxostat on mitochondrial potential in HepG2 cells. *A)* represents the undifferentiated cell group; *B)* CCCP group; *C)* differentiated cell group; *D)* differentiated plus febuxostat cell group.

differentiated group, febuxostat treatment suppresses the expression of p-IRS-1 (Ser307) and enhances the expression of p-Akt (Ser437), which demonstrates that febuxostat prevents IR in HepG2 cells (Fig. 4). Therefore, febuxostat may serve as a treatment for both hyperuricemia and NAFLD.

DISCUSSION

Recently, NAFLD is regarded as a global medical problem as a result of lifestyle changes (10). Despite substantial research on NAFLD, its complex pathogenesis is still hard to explain. Different factors involve in the progression include IR oxidative stress, lipid peroxidation and several cytokines. According to the “two hit attack” hypothesis (used to describe the known mechanism of NAFLD development), the first “hit” is IR, which leads to decomposition of

peripheral fat tissue and hyperinsulinemia. As a result of the above changes, increasing fat accumulation occurs in the liver. The vulnerability of liver towards injury is upregulated. The second “hit” is the injury coming from ROS and lipid peroxidation. IR is a vital factor in the total progression of NAFLD, and the interactions between IR and other factors, such as ROS, free fatty acids and cytokines, promote the progression of NAFLD (11).

NAFLD is highly associated with obesity, T2DM, IR and other metabolic syndromes (12). Hyperuricemia is one of the common metabolic abnormalities. Several recent lines of epidemiological studies in different populations have demonstrated a significant relationship between serum uric acid and NAFLD. One study from China showed that the incidence of NAFLD was positively associated with serum uric acid elevation (13).

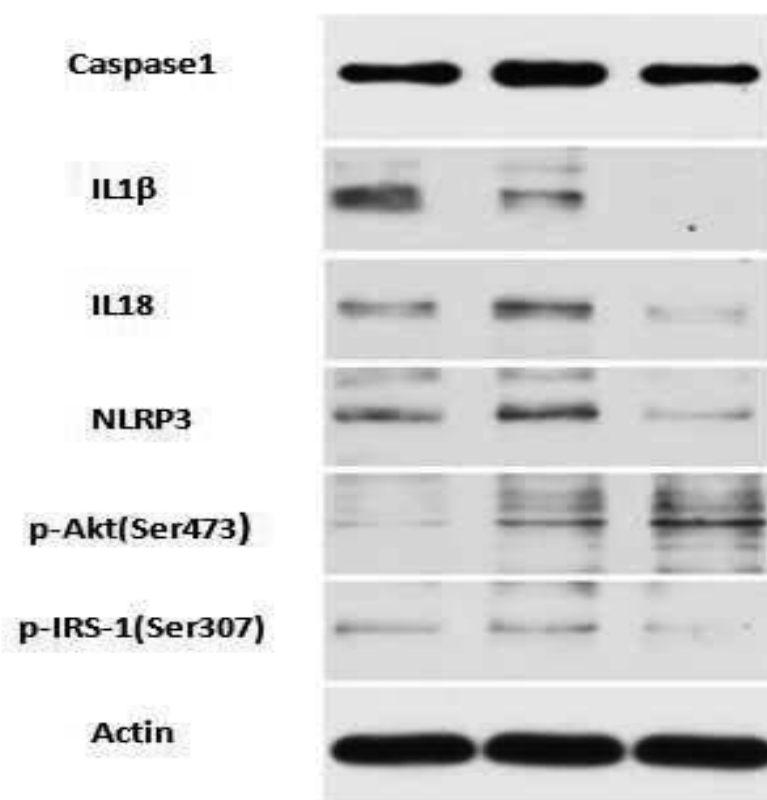


Fig. 4. Mechanism of febuxostat on mitochondrial potential in HepG2 cells.

Xanthine oxidase (XO), also referred to as xanthine oxidoreductase, is a rate-limiting enzyme that catalyzes the terminal step of purine degradation accompanying generation of ROS (14). XOR-rich organs include the intestines and the liver. More recently, a role has emerged for the activity of XO in the regulation of lipogenesis and atherosclerosis (1). Febuxostat not only downregulates lipid accumulation, but also reduces ROS. The present study also found that Febuxostat significantly attenuated FFA-induced intracellular triglyceride accumulation in HepG2 cells. The febuxostat treatment significantly attenuated mitochondrial potential. This reflects that mitochondrial ROS decreased greatly.

ROS could modulate NLRP3 inflammasome activation in cells. NLRP3 plays a vital role in many metabolic diseases, including NAFLD (1). NLRP3 recruits apoptosis-associated speck-like

protein (ASC) and procaspase-1 to constitute NLRP3 inflammasomes, which results in maturation and secretion of caspase-1 and pro-inflammatory cytokines including IL-1 beta and IL-18 (15). IL-1 β and IL-18 increase TG and TC accumulation in cells. These data yield certain clues for delineating the mechanism by which febuxostat regulates the accumulation of lipid droplets in cells (1). Febuxostat downregulates the intracellular expression levels of NLRP3, capase-1, IL-18 and IL-1 β , which suggests that febuxostat attenuates the expression of ROS in differentiated cells. IRS-1 reflects subreceptor activity of the insulin pathway (1). AKT is involved in the modulation of gluconeogenesis in hepatocytes (16). Compared with the differentiated group, febuxostat treatment suppressed the expression of p-IRS-1 (Ser307) and enhanced the expression of p-Akt (Ser437), which demonstrates that febuxostat prevents IR in HepG2 cells. Therefore, febuxostat

may serve as a treatment for both hyperuricemia and NAFLD. Furthermore febuxostat may be a promising potential treatment for patients with NAFLD.

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DIFFERENT MOLECULAR SUBTYPES OF BREAST INVASIVE DUCTAL CARCINOMA

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This study aims to analyze the clinical characteristics of breast invasive ductal carcinoma (BIDC) in patients with different molecular subtypes and identify possible correlation to prognosis. miR-10b expression level was detected using real-time quantitative polymerase chain reaction (RT-PCR). Tissue sections were collected and stained using the immunohistochemical method. The samples were grouped into human epidermal growth factor receptor 2, (HER2) overexpression, Triple negative, Luminal A and Luminal B groups. Age, tumor size, breast cancer molecular subtype, clinical stage, miR-10b positive expression, positive expression of Ki-67 and survival rate of patients diagnosed with BIDC were analyzed. The expression of miR-10b was down-regulated in the breast carcinoma tissues. Age and clinical stage were distinctly different among patients with different molecular subtypes of BIDC ($p < 0.05$). Tumor size was not remarkably different ($p > 0.05$) among different subtypes. The positive expression rate of miR-10b was lowest in patients with Luminal B BIDC; the positive expression of Ki-67 was in different correlation with the expression of different receptors, and there was a remarkable difference ($p < 0.05$); moreover, the survival rate of patients with Luminal A and B BIDC was significantly higher compared to patients with other molecular subtypes ($p < 0.05$). Clinical characteristics and prognosis of BIDC vary among different molecular subtypes. This study provides valuable input on BIDC therapy.

In recent years, female breast carcinoma incidence rates have substantially increased, and it is estimated to be the second leading cause of deaths in women. Breast carcinoma is a heterogeneous disease associated with endocrine disorders including polycystic ovaries, hormonal disorders and severe acne (1) with different subtypes and heterogeneous clinical behavior (2). Histopathological and immunohistochemical examinations are essential for the correct diagnosis of various types of breast cancer (3). Compared to other subtypes, invasive ductal carcinoma has the worst clinical behavior and poorest prognosis; hence there is a growing number

of studies on breast invasive ductal carcinoma (BIDC) (4).

Goldenberg et al. (5) compared BIDC with breast duct carcinoma and intraductal carcinoma using an electron microscope and reported the sparse distribution of invasive carcinoma cells and a clear intracytoplasmic lumen in the invasive carcinoma cells. Sullu et al. (6) identified the role of matrix metalloproteinase (MMP)-2 and MMP-9 in invasion and metastasis of carcinoma through degradation of the basilar membrane and extracellular matrix. Notably, they demonstrated the possible link between the expression of MMP-9 and the clinicopathological

Key words: breast carcinoma, breast invasive ductal carcinoma, clinical characteristics, molecular subtypes

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0393-974X (2018)

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factors of poor prognosis. MMP-9 could be regarded as an auxiliary prognostic marker, though MMP-2 expression manifested a limited role in the prognosis of invasive ductal carcinoma. Dallol et al. (7) studied invasive ductal carcinoma in patients in Saudi Arabia and identified KLOTHO as a tumor-specific methylated gene which was further related to metabolic pathway disruptions that occur in breast cancer. The formation, invasion, and metastasis of malignant carcinoma are in a strong correlation with cell proliferation abnormality. In this study we investigated the age, tumor size, clinical stage, miR-10b positive expression, Ki-67 positive expression and survival rate of patients with different subtypes of BIDC, aiming to shed new light on breast cancer treatment.

MATERIALS AND METHODS

Research subjects

Two hundred patients who were admitted to the Central People's Hospital of Tengzhou between January 2016 and June 2017, and diagnosed with primary BIDC were selected, and their full medical record was obtained. All patients had radical mastectomy, extensive radical treatment and axillary lymph node dissection. No adjuvant therapy was given before surgery whereas patients received chemotherapy or radiotherapy after surgery. The study has been reviewed and approved by the medical ethics committee of the Affiliated Hospital of Jining Medical University, Jining, Shandong, China. Moreover, all the subjects signed informed consent.

Experimental materials

The main materials included citrate buffer solution (Thermo Fisher Scientific Inc., US), peroxidase blocker (Shanghai Yuanmu Biotechnology Co., Ltd., China), normal nonimmune serum (Shanghai Fanke

Biotechnology Co., Ltd., China), polymeric goat anti-rabbit horse radish peroxidase (Shanghai Jianglai Biotechnology Co., Ltd., China), phosphate buffer solution (PBS), diaminobenzidine (DAB), ethyl alcohol, dimethylbenzene, 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride (Solarbio Scientific Co., Ltd., China), TNT buffer, TNB buffer, Vectashield (Shanghai Haoran Biotechnology Co., Ltd., China), anti-DIG-fluorescein (Shanghai Gucheng Biotechnology Co. Ltd., China), paraffin slicing machine (Labsun group, Germany), microscope (Shanghai Dianying Optical Instrument Co., Ltd., China), microwave oven (Henan Geer Microwave Scientific Co., Ltd., China), pressure-cooker (Shanghai Boxun Medical Bio-instruments Inc., China), endogenous peroxide blocker (Beijing Dingguo Changsheng Biotechnology Co., Ltd., China), Ki-67 antibody (ACTIVE MOTIF, US) and enzyme-labelled goat anti-mouse rabbit immunoglobulin G (IgG) polymer (Shanghai Juebo Biotechnology Co., Ltd., China).

The sequences of primers are shown in Table I.

Sample collection

Breast carcinoma specimens were collected during surgery, cut into tissue blocks 1 × 1 × 0.5 cm, transferred to stationary liquid and stored at 4°C. Tissue blocks were washed in tap water for 10 min and sliced. Sections were dehydrated through a series of ethanol baths as described below. Sections were immersed in 70% ethyl alcohol for 3 h, followed by immersion in 80% ethyl alcohol for 3 h, 90% ethyl alcohol overnight, 95% ethyl alcohol for 2 h, 100% ethyl alcohol I for 2 h and 100% ethyl alcohol II for 2 h. Tissues were processed by 30 min impregnation in dimethylbenzene (twice for 30 min each) and immersed in dissolved paraffin at 60°C for 1 h, twice. Tissues were transferred to an embedding apparatus using tweezers. The embedding apparatus was slowly put into water and taken out after the paraffin coagulated. Relevant information such as name was recorded. Paraffin-

Table I. Primer sequences.

miRNA	Primer	Sequence
miR-10b	F-P ^c	GGCTAGCAGCACAGAAATATT
	SL-P ^b	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGCCAAT

embedded tissues were cut into trapezoid with a scalpel and sliced into sections in a thickness of 4 μm , using a microtome. Sections were transferred to a black paper plate in a water bath kettle at 48°C, to discard paraffin ribbon. Tissue sections were picked out of the water using microscope slides and put into an oven at 60°C to dry overnight. Slides were stored at 4°C.

Subtypes and detection of miR-10

Slides were brought to room temperature. After dewaxing and hydration, tissues were washed with PBS for 6 min. Sodium citrate buffer solution (0.01 M) was used for permeabilization and the tissues were repaired in a microwave. After 15 min, tissues were brought to room temperature and washed with PBS for 2 min. One drop of peroxidase blocker was added. After 10 min of incubation at room temperature, tissues were washed with PBS for about 10 min. The excess PBS was removed and samples were incubated with normal nonimmune serum, for 10 min at room temperature. After blocking, samples were incubated with the primary antibody for 30 min at room temperature. Sections were then washed with PBS three times (15 min each wash); PBS was discarded. Samples were incubated with polymerized goat anti-rabbit horse radish peroxidase for 10 min at room temperature and washed with PBS for 10 min. PBS was discarded. DAB solution was used and the tissues were observed under a microscope for 5 min. Sections were washed with distilled water, stained with hematoxylin, washed with

PBS, dehydrated, dried and stored in neutral balsam. Sections were washed with TNT buffer and TNB buffer. Then anti-DIG- fluorescein was added. Thirty minutes later the sections were washed with TNT buffer for 15 min, dehydrated using ethyl alcohol and seasoned by air. Then the sections were stained using DAPI for 10 min and sealed after the addition of Vectashield. Finally, the sections were observed under a fluorescence microscope. The expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) were detected, and the positive expression rate of miR-10b was analyzed.

RNA electrophoresis detection

Firstly, 1 μl of RNA sample was mixed with 5 μl of water and 2 μl of loading buffer. Then 1 μl of ethidium bromide which has been diluted in a ratio of 1:50 was added. Electrophoresis detection and analysis was performed after the gel was cooled and solidified. Marker was added into the well to prevent RNA to fail to run out of the electrophoretic band because of degradation. Then run the gel at 6 V/cm for 20 min, and the corresponding imaging result was recorded, as shown in Figs. 1 and 2.

It could be observed from the image that the three bands, 5S, 18S and 28S, were clear and bright, and moreover the brightness of the 28S band was 1 ~ 2 times that of the 18S band, indicating the total RNA was complete and had no obvious degradation.

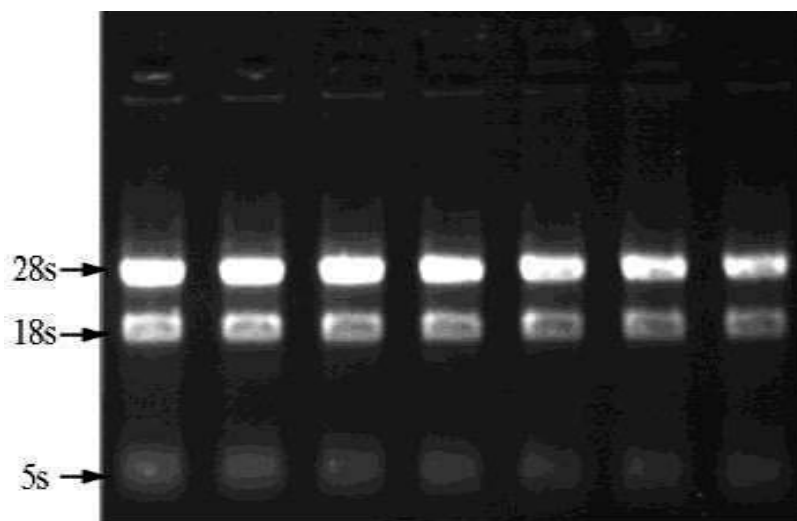


Fig. 1. The electrophoretic result of part of the RNA samples.

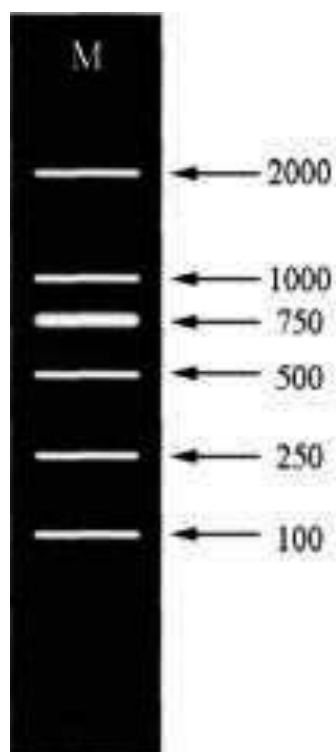


Fig. 2. An example of MarkerDL2000 (unit: bp).

Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted from the breast carcinoma tissues. Reverse transcription product was obtained after reverse transcription. miR-10b was detected according to SYBR primescrptmiRNA RT-PCR kit. U6 was taken as the internal reference gene. Calculation was performed using $2^{-\Delta\Delta Ct}$ relative quantitative method: $\Delta Ct = Ct_{\text{target}} - Ct_{U6}$ and $\Delta\Delta Ct = \Delta Ct (\text{experimental group}) - \Delta Ct (\text{control group})$. If $2^{-\Delta\Delta Ct} > 1$, then it indicated that the target gene was up-regulated; if $2^{-\Delta\Delta Ct} < 1$, then it indicated that the target gene was down-regulated.

Molecular subtypes of breast carcinoma

Breast carcinoma with positive expression of ER and (or) PR and negative expression of HER2 was determined as Luminal A; breast carcinoma with positive expression of ER and (or) PR and negative HER2 was considered as Luminal B; breast carcinoma with negative expression of ER and PR and overexpression of HER2 was determined as HER2 overexpression; breast carcinoma with negative

expression of ER, PR and HER2 was determined as Triple Negative (8, 9).

Detection of Ki-67

The specimens were taken out from the refrigerator and immersed with dimethylbenzene twice, 20 min each time. Then they were immersed in absolute ethyl alcohol twice, 5 min each time. After that the specimens were washed with tap water for 3 min and PBS thrice, 5 min each time.

The citric acid tissue antigen retrieval solution was heated using a pressure cooker. Then the dehydrated sections were transferred to the pressure cooker along with a high-temperature rack for 2-min heating. After 5-min natural cooling and tap water cooling, the sections were washed by distilled water thrice, 3 min each time, and PBS thrice, 3 min each time.

After PBS was abandoned, the detection area was determined. After the addition of endogenous peroxide blocker, it was incubated at room temperature for 10 min, followed by washing with PBS thrice, 3 min each time.

After PBS was removed, 100 μ l of Ki-67 antibody was added and then incubated at room temperature for one hour. Then it was washed with PBS thrice, 3 min each time.

After PBS was removed, enzyme-labeled goat anti-mouse/rabbit IgG was added and then incubated at room temperature for 20 min, followed by washing with PBS thrice, 3 min each time.

After PBS was removed, 150 μ l of DAB was added and incubated at room temperature for 5 min. Then it was observed under the light microscope for 5 min. After redying, it was washed by tap water twice, stained using hematoxylin body cell staining solution, incubated at room temperature for 20 s, and finally washed with tap water.

Finally, it was dehydrated using absolute ethyl alcohol and sealed using neutral resins and cover glass.

Observation of prognosis

The patients were revisited at an interval of twenty months. The outcome of prognosis was determined according to the overall survival time. Overall survival time refers to the period from the start time of surgery to the death of patients or the last time of phone interview (10).

Positive criteria of hormone receptors

According to the immunohistochemical results, when the positive expression in cell nucleus was higher

than 10% of tumor cell expression hormone receptor markers, it was determined as positive expression. When the immunohistochemical staining result of HER2 was $\sim +$, it was determined as negative expression; when the result was $+++$, it was determined as positive expression; when the result was $++$, there was gene copy number amplification and it was determined as positive expression (11). When the percentage of positive Ki-67 cells was less than 14%, it was determined as negative; when the percentage of positive cells was between 14% and 30%, it was determined as weak positive (+); when the percentage of positive cells was equal to or larger than 30%, it was determined as strong positive ($++$) (12).

Criteria of clinical staging

The clinical stages of breast carcinoma include stage I, II, III and IV. Stage I refers to carcinoma tissues in primary sites and no axillary lymph nodes and supraclavicular

lymph node metastasis after excision; stage II refers to local infiltration and lymphatic metastasis in some areas; stage III refers to extensive local infiltration or lymphatic metastasis; stage IV, i.e. advanced stage, refers to metastasis of carcinoma cells (13).

Statistical analysis

Data were statistically analyzed using SPSS ver. 18.0. Measurement data were expressed as mean $\pm$ standard deviation. Enumeration data were processed by Chi-square test. Difference was considered as statistically significant if $p < 0.05$.

RESULTS

In-situ hybridization

The expression of miR-10b in the breast invasive ductal tissue could be seen by observing the in-situ

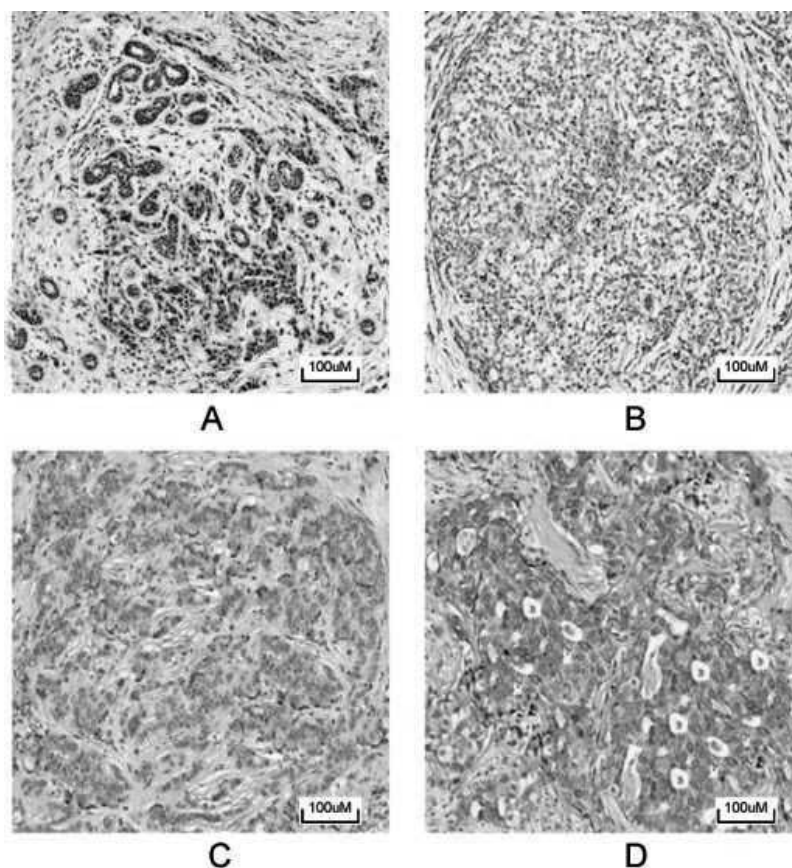


Fig. 3. The expression of miR-214 under the microscope. **A)** Negative expression of miR-10b. **B)** Weak positive expression of miR-10b. **C)** Positive expression of miR-10b. **D)** Strong positive expression of miR-10b.

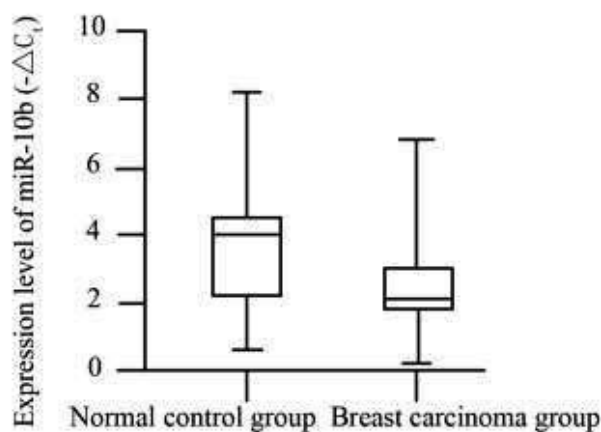


Fig. 4. The expression quantity of miR-10b.

hybridization pathological sections. The expression mainly concentrated on cytoplasm. The brown yellow particles meant positive expression of miR-10b. The expression was divided into negative, weak positive, positive and strong positive, as shown in Fig. 3.

The expression level of miR-10b

The expression level of miR-10b was detected using RT-PCR. The expression quantity was set as 1.00. The relative expression quantity of miR-

10b in B IDC tissues was 0.37 ± 0.41 . Paired t test was performed; the value of p was smaller than 0.5, indicating there was a statistically significant difference. Therefore the expression of miR-10b in the B IDC tissues was down-regulated (Fig. 4).

Table II shows the expression quantity of miR-10b was 6.72 ± 1.38 in the B IDC tissues and 6.97 ± 0.36 in the para-carcinoma tissues. $2^{-\Delta\Delta C_t}$ analysis suggested that the expression of miR-10b in the B IDC tissues was significantly higher than that in the para-carcinoma tissues ($p < 0.05$). The expression quantity of miR-10b in the breast carcinoma tissues with lymphatic metastasis was significantly higher than that in the para-carcinoma tissues ($p < 0.05$). The expression quantity of miR-10b in the breast carcinoma tissues without lymphatic metastasis was close to that of the para-carcinoma tissues which indicated that the expression quantity of miR-10b in the breast carcinoma tissues with lymphatic metastasis was obviously upregulated compared to the breast carcinoma tissues without lymphatic metastasis and para-carcinoma tissues.

Age characteristics

Molecular subtypes were classified in accordance with the expression of ER, PR and HER2 in breast carcinoma tissues (Table III). The ages of patients with Luminal A B IDC were between 32 and 76 years

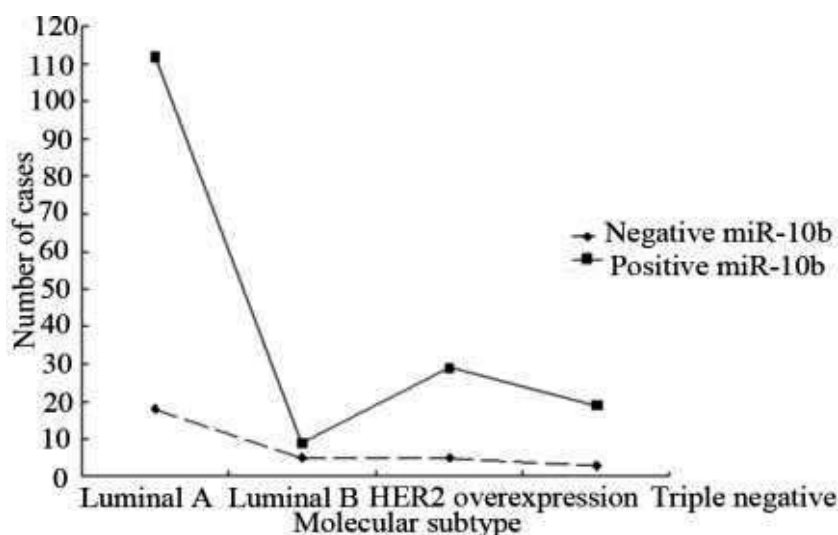


Fig. 5. Expression of miR-10b in B IDC with different molecular subtypes

(average 50.6 years) (Table III). Among the patients who were under 40 years of age, the majority were characterized with Luminal A BDC. The age of the patients with Luminal B BDC was between 34 and 58 years, mainly between 49 and 59 years. Patients with HER2 overexpression were mainly aged from 46 to 62 years. The patients with Triple Negative BDC were aged from 49 to 57 years. Patients in the group of Luminal A BDC were younger, compared to the other three groups. Moreover, there was a statistically significant correlation between age of the patients and the different breast cancer subgroups ($p < 0.05$).

Tumor size and clinical stage

Differences in tumor size were observed in patients with different molecular subtypes (Table IV). The number of patients with Luminal A BDC was larger than that of other subtypes. However, there was no statistically significant difference in the size of tumor among different groups ($p > 0.05$). As for clinical stage, among stage II patients, the number of patients with Luminal A BDC was the largest, followed by Luminal B. The number of patients overexpressing HER2 was the same with the number of patients diagnosed with Triple Negative BDC. The number of patients with stage II was the largest, especially the number of patients with Luminal A; the

number of patients with stage IV was the smallest, and there was one case of Luminal B type and one case of Triple Negative type. These findings demonstrate a remarkable difference in clinical stage among different molecular subtypes ($p < 0.05$).

miR-10b expression

The positive expression rates of miR10b in different molecular subtypes of BDC were 86.2%, 64.3%, 85.3% and 86.4%, respectively (Fig. 5). The analysis results suggest that the positive expression rate of miR-10b was remarkably different among the different molecular subtypes of BDC, especially in Luminal B BDC where the lowest levels of expression compared to the other subtypes were observed ($p < 0.05$).

Expression of Ki-67

The expression of Ki-67 was discussed in aspects of expression of ER, PR and HER2. Table V shows that the positive expression of Ki-67 was in a negative correlation with the expression of ER and PR and HER2 ($r_s = -0.349$ and -0.332) and in a positive correlation with the expression of HER2 ($r_s = 0.201$), and the difference had statistical significance ($p < 0.05$).

Prognosis

Patients with Luminal A and B BDC had better

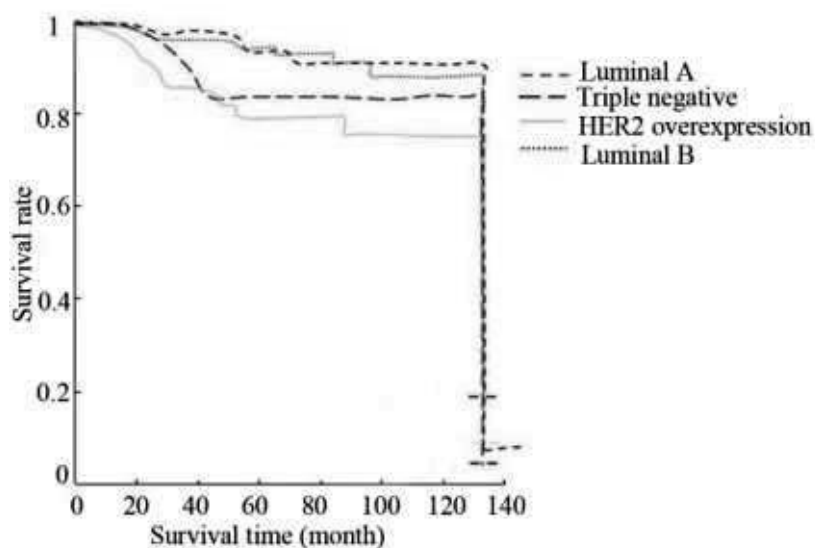


Fig. 6. *The prognosis of patients.*

Table II. The correlation between miR-10b and the clinical pathological characteristics (mean±standard deviation)

Group	Breast carcinoma tissue	Para-carcinoma tissue	With lymphatic metastasis	Without lymphatic metastasis
Number of cases	200	200	103	97
miR-10b ($^{\Delta}Ct$)	6.72±1.38	6.97±0.36	5.29±0.28	7.14±0.51
$2^{-\Delta\Delta Ct}$	3.79±2.86	1.12±0.41*	6.23±1.86	0.98±0.27 [#]

* $p < 0.05$ in the comparison of the breast carcinoma tissues and para-carcinoma tissues, i.e. the difference was statistically significant; [#] $p < 0.05$ in the comparison of carcinoma with or without lymphatic metastasis, i.e. the difference was statistically significant.

Table III. Classification of different molecular subtypes.

Molecular subtypes	Luminal A	Luminal B	HER2 overexpression	Triple Negative
Receptor expression	ER+ and (or) PR+, HER2-	ER+ and (or) PR+, HER2+	ER-, PR-, HER2+	ER-, PR-, HER2-
Number of patients (N)	130	14	34	22
Age (years)	32~76	34~58	46~62	49~57
p value	<0.05			

Table IV. Effects of tumor size and clinical stages.

Clinico-pathological characteristics		Luminal A	Luminal B	HER2 overexpression	Triple Negative	p value
Tumor size (cm)	No larger than 2	40	4	6	10	>0.05
	Between 2 and 5	82	10	14	20	
	Larger than 5	8	0	2	4	
Clinical stage	Stage I	24	8	6	6	<0.05
	Stage II	44	23	13	18	
	Stage III	24	10	12	10	
	Stage IV	0	1	0	1	

Table V. *Expression of Ki-67.*

Expression of receptors		Negative	Weak positive	Strong positive	r_s	p value
ER	+	5	15	41	-0.349	<0.05
	-	17	53	59		
PR	+	10	25	68	-0.332	<0.05
	-	22	43	32		
HER2	+	32	54	64	0.201	<0.05
	-	1	11	38		

prognosis compared to that of other molecular subtypes of BDC (Fig. 6). Moreover, the survival rate of patients with BDC and HER2 overexpression was significantly lower than that of the other three groups ($p < 0.05$). These results indicate that patients with BDC and HER2 overexpression have the poorest prognosis.

DISCUSSION

Constant development in the field of molecular biotechnology has led to the identification of many molecular markers which are sensitive to the evaluation breast carcinoma prognosis. For example, ER, PR and HER2 can provide certain theoretical basis for the biological sensitivity of carcinoma, carcinoma prognosis and sensitivity of carcinoma treatment. miRNA is a kind of endogenous micromolecule single-stranded RNA, which is reported in a close correlation with the occurrence, development and metastasis of carcinoma (14). miR-10b is highly conservative in evolution, and it is important in the recurrence and metastasis of early BDC and prognosis prediction (15). miR-10b is often overexpressed in breast carcinoma cells, which can induce the recurrence and metastasis of breast carcinoma and strengthen its invasiveness. In this study, the expression level of miR-10b was detected using real-time quantitative RT-PCR, and it

was found that its expression was down-regulated in the BDC tissues. Breast carcinoma is a disease with high heterogeneity, and the variability of molecular subtypes is the primary cause of heterogeneity formation (16).

In the early 20th century, American Institute for Cancer Research has proposed that the classification basis of carcinoma is based on morphology and molecular characteristics. Prognosis of breast carcinoma is also different among different molecular subtypes. Generally, the molecular subtypes of breast carcinoma are identified using immunohistochemical method (17). Based on the immunohistochemical results, the cases of invasive breast ductal carcinoma were classified to HER overexpression group, Triple Negative group, Luminal A group and Luminal B group; moreover, the clinical characteristics and prognosis were investigated. It was demonstrated that the age and clinical stage of patients with different invasive breast ductal carcinoma subtypes, were remarkably different ($p < 0.05$), but the size of carcinoma was not ($p > 0.05$).

Unlu et al. (18) considered that the age of females was in a close correlation with breast carcinoma. They made studies by connecting the histopathological characteristics and risk factors of breast carcinoma with the age and menopausal condition of females and found that poor prognosis indicators such as lymphatic metastasis and lymphatic vessel invasion

usually appeared in females aged under 35 years. The positive expression rates of miR-10 b in Luminal A type, Luminal B type, HER-2 overexpression type and Triple Negative type were 86.2%, 64.3%, 85.3% and 86.4% respectively, and the positive expression in Luminal B was significantly lower than that in the other three types, which was because that the positively expressed miR-10b resulted in the increase of ER and HER2 expression. When the expression of ER increased, it was a protective factor. When the expression of HER2 increased, then it was a risk factor; as a result the expression rate of Luminal B type decreased, which may result in recurrence and metastasis. Moreover, the expression of Ki-67 was in a negative correlation with the expression of ER and PR, indicating that the prognosis was favorable when its expression was high; hence Ki-67 was inferred as an unfavorable factor. The expression of Ki-67 was in positive correlation with the expression of HER2, indicating that the prognosis was poor when its expression was low. The survival rate of patients with Luminal A and B invasive breast ductal carcinoma was higher than that of patients with the other two types of invasive breast ductal carcinoma, indicating patients with Luminal A and B invasive breast ductal carcinoma had better prognosis. The survival rate of patients with HER2 overexpression was the lowest, suggesting the poorest prognosis.

In conclusion, clinical characteristics and prognosis of invasive breast ductal carcinoma are different among patients with different molecular subtypes. Molecular subtyping has important significance in the prediction of breast carcinoma prognosis, and can provide valuable information for treatment approach.

ACKNOWLEDGEMENTS

Thank you for the assistance of Qianqian Yuan from Tengzhou Central People's Hospital and Fanhua Kong, Lijun Sun, Shanshan Li, Junye Wang and Shucheng Ye from Affiliated Hospital of Jining Medical University. I would like to express sincere gratitude to the careful guidance and help that they provided for topic selection, experimental design, specific experiments, result analysis, article writing and paper modification.

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LETTER TO THE EDITOR

PERCUTANEOUS TRANSFORAMINAL ENDOSCOPIC DISCECTOMY AND MINI-INCISION SURGERY IN THE TREATMENT OF LUMBAR INTERVERTEBRAL DISC PROTRUSION

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Received August 28, 2017 – Accepted December 18, 2017

Lumbar intervertebral disc protrusion (LIDP) is a frequently occurring disease and 10-20% of patients require surgical treatment. Percutaneous transforaminal endoscopic discectomy (PTED) and mini-incision surgery are currently the most common surgeries for patients. To analyze the efficacy of PTED and mini-incision surgery in the treatment of lumbar intervertebral disc protrusion, this study selected 216 patients with LIDP who were admitted to the hospital between February 2014 and June 2015. The subjects were randomly divided into an observation group and a control group, 108 each. Patients in the observation groups were treated by PTED, while patients in the control group were treated by mini-incision surgery, and treatment efficacy of the two groups was observed. The results demonstrated that the duration of surgery and length of hospital stay of the observation group were significantly shorter than those of the control group, the intraoperative blood loss of the observation group was significantly less than that of the control group and the size of surgical incision of the observation group was much smaller than that of the control group ($P < 0.05$). As to clinical efficacy, in accordance with the Japanese Orthopaedic Association (JOA) score and the Visual Analogue Scale (VAS) score, results of the observation group were superior to those of the control group at 3 months after surgery ($P < 0.05$). In conclusion, treating patients with LIDP through PTED can significantly improve treatment efficacy, shorten surgical and healing time and relieve pain. This therapy is worth clinical promotion.

To the Editor,

With the constant evolving of society and the increase of work intensity, lumbar intervertebral disc protrusion (LIDP) has been the main cause for pain in the waist and lower extremities. According to the statistics of incomplete data, the incidence of LIDP is 38.7% among orthopedic diseases, tending to increase in recent years (1). It manifests pain in the waist, ischialgia and *cauda equina* compression syndrome induced by the stimulated and compressed spinal nerves under the effect of intervertebral disc

degeneration, fiber ring rupture and protrusion or prolapse of *nucleus pulposus* (2). LIDP also impacts the economic system, in terms of days lost to work and reduced productivity, and increases the use of pain medication along with being a physiological burden (3). Many patients with LIDP have no obvious clinical symptoms after conservative treatment, however, 2% of patients require surgery (4). Current surgical treatments for LIDP include traditional open surgery, percutaneous insertion surgery and percutaneous endoscopic discectomy.

Key words: percutaneous transforaminal endoscopic discectomy, lumbar intervertebral disc protrusion, mini-incision surgery

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0393-974X (2018)

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Percutaneous transforaminal endoscopic discectomy (PTED) is currently one of the major therapies for treating LIDP (5-7). In this study, 216 patients were treated with either PTED or mini-incision surgery and various clinical indicators were compared, with the intention of highlighting the pros and cons of PTED and providing a reference for the clinical treatment of lumbar intervertebral disc protrusion.

MATERIALS AND METHODS

Research subjects

Two hundred and sixteen patients diagnosed with LIDP in the Bone Traumatology Department of Binzhou Medical University Hospital between February 2014 and June 2015, were selected. The patients who were diagnosed via the history of the disease, vital signs, X-rays, computed tomography (CT) and magnetic resonance imaging (MRI) were included. Those who had poor compliance, had obvious dissociated or multi-stage protrusion of intervertebral disc, had definite instability of lumbar spine, or excessively narrow foramen intervertebrale of diseased segment because of hyperplasia or congenital factors were excluded. They were randomly divided into an observation group and a control group of 108 each. In the control group, there were 71 males and 37 females, mean age and course of disease was 46.27 ± 3.96 and 5.4 ± 1.5 years, respectively; the segments of disease of 50 patients were at L4/L5 and of 58 patients at L5/S1.

In the observation group, there were 64 males and 44 females, mean age and course of disease 45.3 ± 4.14 and 5.7 ± 1.5 respectively; the segments of disease of 46 patients were at L4/L5 and of 62 patients at L5/S1. The difference of gender, age and course of disease between the two groups had no statistical significance. The hospital ethics committee approved this study and all the patients signed informed consent to participate.

Treatment methods

The patients in the control group were treated by mini-incision surgery. Firstly, the patient was given continuous epidural anesthesia. A C-arm X-ray machine was positioned above the diseased intervertebral space and a 3-5 cm incision was made along the intervertebral disc to highlight the lateral paravertebral muscle. A space of $1.5 \text{ cm} \times 1.5 \text{ cm}$ was opened between the spinal vertebral plates. The ligamentum flavum was removed to fully expose dural sac and spinal nerve root. The protrusion of the intervertebral disc and exfoliated nucleus pulposus were removed successively to expand the shaping lateral recess, and ensure that all tissues compressing nerve roots on the spine had been removed and relax the spinal nerve roots. Finally, the intervertebral space was washed by normal saline. With the drainage tube in place, the incision was sutured.

The patients in the observation group were given PTED. Spinal PTED system (Joimax Company, Germany) and a bipolar radiofrequency machine (Ellman Company, USA) were used. Local anesthesia

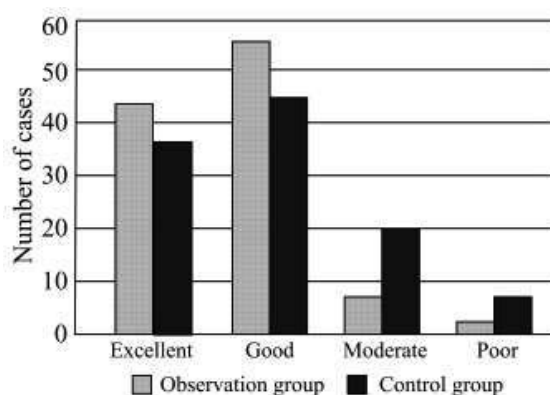


Fig. 1. Comparison of clinical efficacy between the two groups.

was performed on the patient In prone position, using 1% lidocaine. The parameters of the PTED system were preset; external diameter: 7.0 mm, diameter of working catheter: 3.5 mm; distance from the midline of spinous process: 10~15 cm; angle between the inserted guide pin and skin: 10°~25°; skin window: 5~8 mm.

The surgeon slowly placed the catheter into the periphery of the intervertebral foramen and PTED was inserted. The catheter was placed in the diseased spine using the C-arm X-ray machine and the proliferative bone and protruded intervertebral disc on the upper side of the spine were removed under the assistance of Ho: YAG laser. The prolapsed nucleus pulposus was removed using intervertebral disc rongeurs of different sizes according to the imaging results. Finally, Ellman bipolar radiofrequency was used for hemostasis.

Postoperative exercise

Patients in both groups performed early functional exercises, starting on the day of surgery. The patient was assisted in turning around, bending and stretching the lower limbs. Moreover, the lower limbs were massaged. One day after surgery, the patient was required to do active contraction of the quadriceps *femoris*, triceps *surae*, *tibialis* anterior muscle and

dorsal muscles of foot. The lower limbs did straight-leg-raising exercise alternately, starting at 30°~45°, 15~20 times/group 2 or 3 times daily; at 1 week, the angle was 60°~90°; at 2 weeks, functional exercise of waist and back muscles was performed, with the patient taking a supine position and bending the knees and the waist for 10~20 times for each group. Following, swallow skimming over the surface of water was performed; the patient lifted up head and feet with both arms crossing above the waist, 5 s each time, 10~20 times each group, 2 or 3 groups each day. Abdominal muscle exercises started after the sutures were removed; they were performed by clasp the arms around the knees and breathing deeply. When exercising, the patient was in a supine position and bent the knees and hips, 15~20 times each group, 2 or 3 groups each day. When doing this exercise, the patient extended the lower extremities and shrank the abdomen during inspiration as much as possible, 15 to 20 times each group, 2 or 3 groups each day.

Observation indicators

Surgical indicators included intraoperative blood loss, length of incision, duration of surgery and length of hospital stay. The improvement of lumbar vertebral function was evaluated through the Japanese Orthopaedic Association (JOA) score (8);

Table I. Comparison of relevant clinical indicators between the two groups.

Group	Observation group	Control group	t	P
Duration of surgery (min)	62.2±5.6	75.4±10.5	3.437	<0.01
Intraoperative blood loss (mL)	20.4±6.5	67.51±12.57	2.794	<0.01
Length of surgical incision (cm)	1.4±0.3	6.2±2.1	2.919	<0.01
Length of hospital stay (d)	5.1±3.6	8.5±2.6	2.011	<0.01

Table II. Comparison of JOA and VAS scores between the two groups.

Group	Observation group	Control group
JOA Before surgery	11.82±2.06	11.87±1.75
(point) 3 months after surgery	20.3±1.4 ^{*#}	23.2±1.8 [*]
VAS Before surgery	4.8±2.3	4.9±2.3
(point) 3 months after surgery	1.1±0.5 ^{*#}	1.6±0.8 [*]

^{*} $P < 0.05$ compared to before surgery; [#] $P < 0.05$ compared to the control group

a lower score indicated severe dysfunction. Pain in waist and lower extremities was evaluated through the Visual Analogue Scale (VAS) score (9) (1~10 points); a higher score indicated severe pain. The curative effect was determined by Macnab function evaluation criteria in the 3rd month after surgery. Treatment efficacy was determined as: a) excellent, if the symptoms totally disappeared and the patient could work and live as usual; b) good, if there were mild symptoms which slightly limited activities but did not affect work and life quality; c) moderate, if there were symptoms which limited activities and affected work and life quality; d) poor, if symptoms were no different or aggravated after treatment.

The calculation for excellent or good results was: number of excellent cases+number of good cases/total number of cases $\times$ 100%.

Statistical analysis

Data were statistically processed using SPSS ver. 21.0. Measurement data were expressed as mean $\pm$ standard deviation (SD) and processed by *t*-test. Enumeration data were expressed by percentage and processed by *Chi*-squared test. Difference was considered having statistical significance if $P<0.05$ and as remarkable if $P<0.01$.

RESULTS

Relevant clinical indicators

The duration of surgery and length of hospital stay of the observation group were significantly shorter than those of the control group, the intraoperative blood loss of the observation group was apparently less than that of the control group and the length of surgical incision of the observation group was obviously smaller than that of the control group. Difference was statistically significant ($P<0.01$; Table I).

JOA and VAS scores

The differences of the VAS and JOA scores between the observation and control groups before surgery had no statistical significance ($t=0.531$, $P>0.05$; $t=0.764$, $P>0.05$). The VAS and JOA scores of the observation group before surgery were significantly different from those in the third month

after surgery ($t=3.178$, $P<0.05$; $t=4.113$, $P<0.05$) and were remarkably superior to those of the control group at the same time points ($t=2.982$, $P<0.05$; $t=4.344$, $P<0.05$). The details are shown in Table II.

Rating

The excellent and good ratings of the observation group and control group were 91.7% and 75%, respectively. The improvement of the observation group was superior to that of the control group ($\chi^2=5.167$, $P<0.01$) (Fig. 1).

DISCUSSION

Currently, conservative treatment and surgical treatment are the main therapies for LIDP (10). Many patients refuse to undergo conservative treatment due to the long duration, poor efficacy and high risk of recurrence. In the treatment of lumbar vertebra, PTED has more advantages, such as safety, accuracy, less trauma, shorter recovery time and low risk of complications compared to traditional open surgery and percutaneous puncture technology (11).

This study compared the efficacy of mini-incision surgery and PTED in the treatment of lumbar intervertebral disc protrusion. The results demonstrated that the indicators including duration of surgery, intraoperative blood loss, length of incision and length of hospital stay of the observation group were evidently better than those of the control group, which was similar to the research results of Postacchini et al. (12). The reasons may be that the manifestations of intervertebral disc tissues and clinical reactions are clearly observed in PTED and therefore various protruding tissues in the intervertebral disc can be more effectively removed. Moreover, compared to mini-incision surgery, PTED can effectively avoid operations such as artificial probe and removal and help doctors observe the specific characteristics of the intervertebral disc, reducing the surgical incision. Therefore, PTED is better than mini-incision surgery in the overall aspects of surgical effect and prognosis.

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LETTER TO THE EDITOR

PRE- AND POST-TREATMENT COMPUTED TOMOGRAPHIC FINDINGS OF A PRIMARY INTRANASAL TRANSMISSIBLE VENEREAL TUMOR IN A CANINE PATIENT

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Received December 7, 2017 - Accepted March 2, 2018

A two-year-old, female intact, cross-breed dog presented with a two-month history of nasal discharge. Computed tomography (CT) demonstrated obliteration of both nasal cavities by soft tissue density, destruction of the nasal and ethmoidal turbinates, and lysis of the frontal and palatine bones and maxilla. Frontal sinuses and maxillary recesses were obscured by soft tissue/fluid density. Histopathological examination of the mass was diagnostic of transmissible venereal tumor. The dog was clinically normal 3 months after treatment initiation with vincristine sulphate and amoxicillin/clavulanate. Six months after the completion of treatment no mass-like lesion was demonstrated in CT sections. Nasal cavities, maxillary recesses and frontal sinuses were filled with air. The reticular turbinate nasal plexus appeared atrophic with focal loss of the nasal turbinates on both sides. The ethmoidal turbinates were well-defined; however, focal loss of turbinates was also seen. Lysis of the frontal and palatine bones were still evident.

To the Editor,

Canine transmissible venereal tumor (TVT) is a contagious venereal tumor that is commonly observed in stray and wild dogs that exhibit unrestrained sexual activity or are in close contact with one another. Tumor cells are transmitted by allotransplantation from affected dogs during copulation or through licking, biting, or scratching, especially in the presence of wounds or loss of surface integrity (1-3). Naturally occurring TVT is usually located at the external genitalia of both sexes (1, 2, 4). Extragenital primary sites can also be noticed in the nasal and oral cavities, rectum,

skin and ocular membranes (conjunctiva) when an animal has been sniffing, licking or scratching the preputial or vaginal discharges of an infected dog (3, 5-8). The present study emphasizes the pre- and post-treatment computed tomographic (CT) findings of primary intranasal TVT in a dog as, to the authors' knowledge, CT findings of the nasal TVT have not been previously reported.

Case history

A two-year-old, intact, male cross-breed dog was referred to the Companion Animal Clinic, Faculty of Veterinary Medicine, Aristotle University of

Key words: nasal transmissible venereal tumor; computed tomography

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0393-974X (2018)

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Thessaloniki, Greece, with a two-month history of nasal discharge. On clinical examination bilateral nasal serosanguineous discharge was detected. Results of hematological and biochemical analyses



Fig. 1. Transverse CT image at the level of the rostral part of the nasal cavities using a soft tissue window. The right and left nasal passages are completely opacified with soft tissue attenuating material. There is destruction of the nasal turbinates. Nasal septum is not evident and vomer is deviated toward the left (black arrow). Focal lysis of the right nasal bone is seen (white arrow) (R=right, L=left).

were within normal limits, except for mild neutrophilic leukocytosis (19.0×10^3 cells/ μL range: $6.0\text{--}17.0 \times 10^3$ cells/ μL , neut: 82.5% range: 42.5–77.3%). Serological tests for *Leishmania infantum* and *Ehrlichia canis* were negative.

A helical volumetric set of data was acquired with a 3-mm slice thickness and a pitch of 1, on a single-detector-row helical CT scanner (GE CT/e, Paris France, 2008). The field of view extended from the nares to the occipital condyle. Transverse non-overlapping 1-mm slices were reconstructed using bone, lung and soft tissue algorithms. CT scans demonstrated obliteration of both nasal cavities by soft tissue/fluid density, more severe on the right side. The Housfield units (HU) of this density was 52.2 compatible with soft tissue mass. In the rostral part of the nasal cavities there was complete destruction of the turbinates which were totally replaced by the mass. The nasal septum was not evident and vomer bone was deviated toward the left (Fig. 1). In the mid and caudal part of the nasal cavities there was focal loss of the reticular pattern of the ethmoidal turbinates (more severe in the mid-part) and replacement of the air by soft tissue mass. The nasal septum appeared intact, but in some areas was thinned. There was focal lysis of the

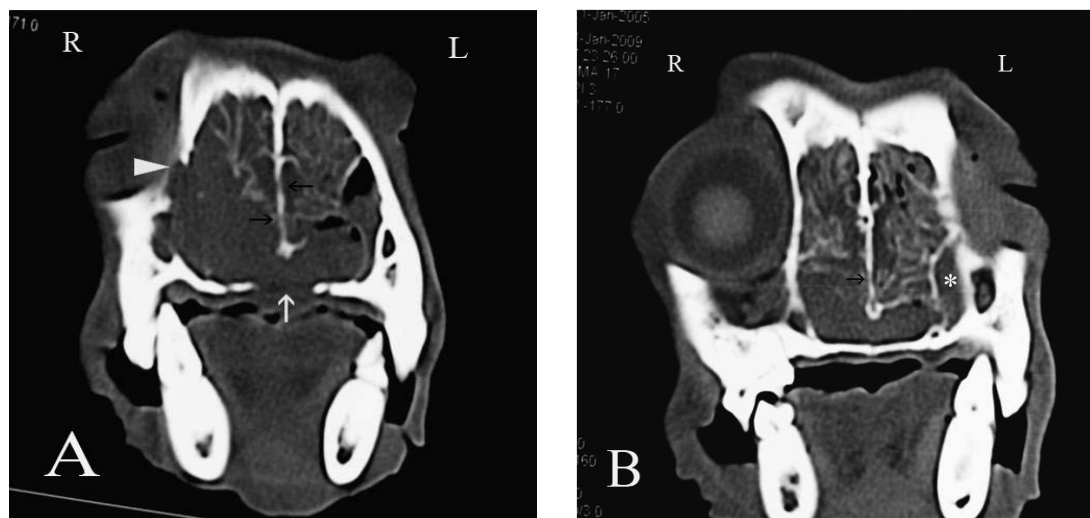


Fig. 2. Transverse CT images (slightly oblique sections) at the level of the mid (A) and caudal (B) part of the nasal cavities using a soft tissue window. There is diffuse increased density of both nasal passages (more severe at the ventral part) and maxillary recesses (asterisk), predominantly right sided turbinate destruction and lysis of the palatine bone (white arrow) and right frontal bone (white arrowhead). The nasal septum is located in the middle line of the nasal cavities and appeared intact but in some areas is thinned (black arrows) (R=right, L=left).

frontal and palatine bones and maxilla. Both frontal sinuses and maxillary recesses were obscured by soft tissue mass (Fig. 2 A and B). The cribriform plate was intact. The mandibular lymph nodes were found enlarged. Contrast medium was not administered. On ultrasonographic examination of the abdomen and CT scan the thoracic and abdominal cavity were found normal.

Endoscopy detected a bilateral, friable and easily bleeding mass in the nasal cavities. Aspiration cytology examination of the mass revealed round to oval shaped cells with lightly basophilic or pale blue cytoplasm, where numerous well-demarcated vacuoles were scattered randomly. Nuclear chromatin appeared clumped with one prominent nucleolus present. Mitoses were numerous and the nuclear to cytoplasmic ratio was relatively high. Inflammatory cells were also noticed among the neoplastic cells. Those findings were considered compatible with TVT. In histopathological examination of the mass the nasal mucosa was diffusely infiltrated by neoplastic cells. The round, oval, or polyhedral shaped cells were arranged in solid sheets interlaced by a fibrovascular network. Mitotic figures were frequent and atypical mitoses were also observed (Fig. 3). The histopathological appearance of the

examined samples was compatible with TVT.

Treatment consisted of vincristine sulphate (Vincristine, Chemipharm, Athens, Greece) 0.5 mg/m² body surface area, i.v., at weekly intervals for six times, and amoxicillin/clavulanate (Augmentin, GlaxoSmithKline, Athens, Greece) 22 mg/kg body weight p.o., q12 h for 25 days. The dog was without symptomatology 3 months after treatment initiation.

Six months after completion of treatment no mass lesion was demonstrated in CT sections of the nasal cavities. Frontal sinuses, maxillary recesses and nasal cavities were filled with air. In the rostral part of the nasal cavities there was near complete absence of the reticular turbinate plexus on the right side and atrophic on the left side.

The nasal septum appeared intact but was deviated toward the left (Fig. 4). In the mid and caudal part of the nasal cavities the ethmoidal turbinates were well-defined; however, focal absence of turbinates was also seen. Lysis of the frontal and palatine bones were still evident (Fig. 5 A and B).

DISCUSSION

Primary extragenital involvement of TVT is quite rare and can be noticed in the nasal and oral cavities,

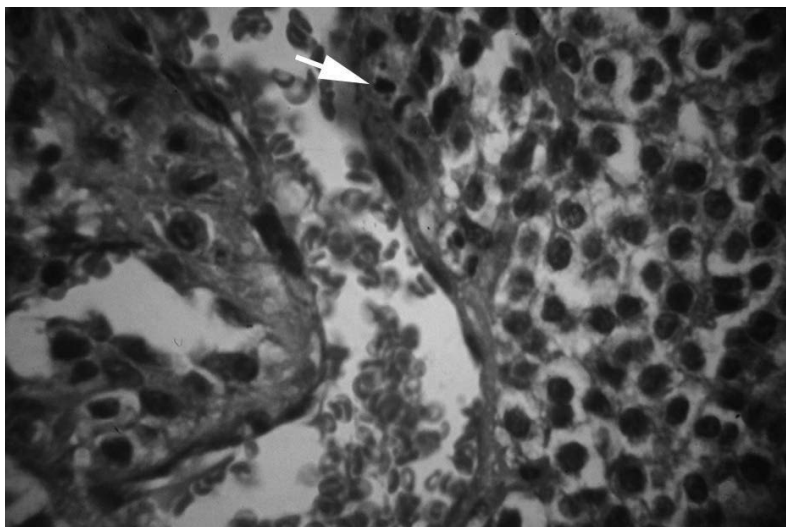


Fig. 3. Histological appearance of the intranasal transmissible venereal tumor: A cluster of round to oval neoplastic cells, with fine granular and more often highly vacuolated cytoplasm and central round nucleus are obvious on the right side of the vessel. Similar cells are sparsely seen on the left side, intermixed with a few inflammatory cells (lymphocytes and macrophages). The arrow points to a mitotic figure. HE, original magnification x40.



Fig. 4. Transverse CT image of the nasal cavities at about the same level as in Fig. 1 using a soft tissue window, six months after completion of treatment. No mass lesion is seen. There is near complete absence of the reticular turbinate plexus on the right side and atrophic on the left side. Deviation of the nasal septum toward the left is seen. Focal lysis of the right nasal bone is still evident (arrow) (R=right, L=left).

rectum, skin and ocular membranes (conjunctiva) (1-3, 5, 7, 8). In cases with extragenital localization, clinical diagnosis is usually more difficult because TVTs cause a variety of signs depending on the anatomical localization of the tumor (1, 2). In the dog presented herein bilateral nasal serosanguineous discharge was detected on clinical examination. In previously published studies, affected dogs with intranasal form of TVT presented with epistaxis, sneezing or nasal bleeding, which are more often seen in the chronic severe form of monocytic ehrlichiosis and leishmaniasis which can be easily excluded with serological and haematological examinations (1, 8, 10). However, primary extragenital cutaneous TVT associated with *Leishmania infantum* infection has also been described (2, 4, 9).

The bilaterally asymmetrical obliteration of the nasal cavities, maxillary recesses and frontal sinuses, destruction of the nasal and ethmoidal turbinates, bone lysis and obliteration of the nasal septum, that were demonstrated on the CT scan of the dog presented in this study were consistent (but not diagnostic of) with nasal neoplasia, since in rare cases of eosinophilic granulomatous rhinitis, aggressive imaging findings including mass lesions and bone destruction may be seen (10). In mycotic rhinitis, marked turbinate destruction resulting cavitation in the affected nasal cavity, a soft tissue mass component in the nasal cavity or frontal sinus and in advanced cases bone erosion and lysis are common CT findings, making the differentiation from neoplasia difficult (11, 12)

CT findings of the primary TVT, as demonstrated in this case report, resemble those of aggressive nasal neoplasia (12). Similar findings appear to be more pronounced with nasal carcinomas, soft tissue sarcomas and lymphomas, however, the differentiation is not possible without histopathological examination. All patients with nasal neoplasia have a soft tissue mass, but as was the case in this dog, the soft tissue mass is not clearly delineated in CT sections because of the summation with adjacent nasal mucosa and underlying disrupted turbinates (10). Contrast medium to demonstrate the mass was not administered in our study because nasal tumor contrast enhancement is variable and often does not assist in delineating intranasal tumor margins because of the concurrent contrast enhancement of adjacent nasal mucosa (10). However, contrast medium is extremely useful as a guide for biopsy, especially for CT-guided biopsy, as it shows areas of viable tissue or areas of necrosis. Moreover, contrast medium is very important for metastases check, mediastinal lymphnodes, etc.

In the dog presented herein, nasal turbinates were more severely affected than ethmoidal turbinates, possibly due to the location of the allogenic cells in the rostral part of the nasal cavities at the transplantation time. During the disease, TVT expanded to the caudal portion of the nasal cavity, and in severe chronic cases invasion of the cribriform bone may occur (13).

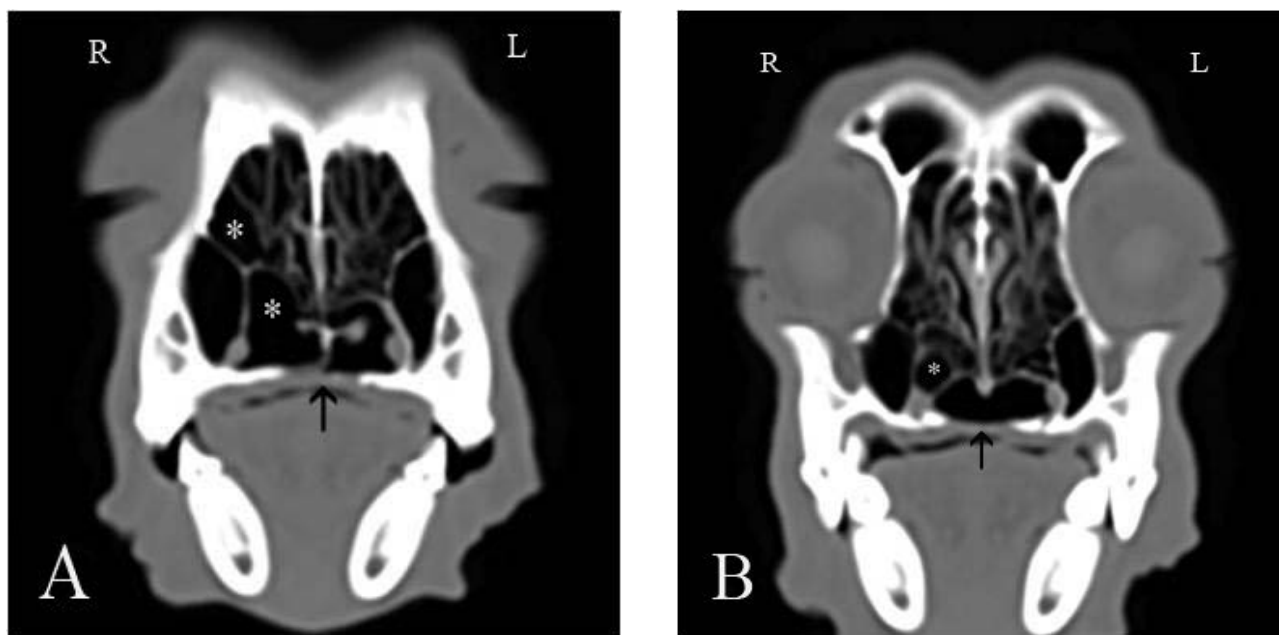


Fig. 5. Transverse CT images (A) and (B) of the nasal cavities at about the same level with the images A and B of Fig. 2 using a soft tissue window, six months after completion of treatment. No soft tissue or fluid attenuating material is seen in the nasal passages. Frontal sinuses, maxillary recesses and choana are filled with air. The reticular turbinate plexus appeared atrophic (more evident on the right nasal cavity in A). Focal absence of turbinates (asterisks) and lysis of the palatine bone (arrow) are seen (R=right, L=left).

It is interesting that the post-treatment CT findings of the nasal cavities in the dog of this case were not as dramatic as was expected after the presence of a neoplastic process. This could be explained by the fact that TVT as benign neoplasm is growing by expansion and not by invasion of the adjacent structures. It is not clear whether the nasal and ethmoidal turbinates seen on post-treatment CT images were the result of a reorganization process or just not-affected turbinates. Since the turbinates are composed of hyaline cartilage and bone, they could be able to start reorganizing their structures after the end of the disease. In that case, a serial of post-treatment CT examinations of the nasal and ethmoidal turbinate cavities should be useful. Unfortunately, in the dog in our study the owners refused any further imaging investigation.

CT findings of this case report were in agreement with radiographic findings reported in other cases of nasal TVT (8, 13). However, CT is more sensitive to provide detailed information concerning the bony

structures, the nasal and ethmoidal turbinates and paranasal sinuses and to delineate the expansion of the disease. CT and MRI are equally accurate in diagnosing nasal neoplasia, although CT has a slightly increased sensitivity for detecting bone lysis (14).

In conclusion, CT findings of primary intranasal TVT in the dog presented in our study resemble those of an aggressive nasal disease. Post-treatment CT findings showed no mass lesion, air-filled nasal cavities, maxillary recesses and frontal sinuses and focal destruction or atrophic turbinates. CT may provide detailed information on the severity and extension of the intranasal TVT lesions as well as on the efficacy of the treatment used.

ACKNOWLEDGEMENTS

The authors would like to thank the radiology technician M. Marinou for assistance with handling this case.

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LETTER TO THE EDITOR

EFFECT OF SILDENAFIL ON PULMONARY HYPERTENSION ASSOCIATED WITH LEFT HEART FAILURE

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This study aims to evaluate the effect of sildenafil on pulmonary hypertension (PH) associated with chronic left heart failure. Twenty patients with PH and left heart failure were divided into treatment group (10 cases, with an oral dose of sildenafil 75 mg daily for 8 weeks) and control group (10 cases, with treatment of cardiac glycosides, diuretics, angiotensin-converting enzyme inhibitor, angiotensin II receptor blocker and beta-blockers). Left ventricular systolic function (LVEF), the pulmonary artery systolic pressure (PH), the left ventricular fraction shortening (LVFS), the left atrium diameter (LAD) and the left ventricular end-diastolic diameter (LVD) were measured by echocardiography, the left ventricular mass index (LVMI) was also calculated. The level of N-terminal pro-brain natriuretic peptide (NT-proBNP) was detected by electrochemiluminescence and high sensitivity C-reactive protein (hsCRP) by immune transmission. The walking distance in 6-minute walk test (6-MWT) was calculated. Before treatment, there were no significant differences in LVEF, LVFS, NT-proBNP, hsCRP, 6-MWT, LVD, LAD and LVMI between the treatment group and control group. After four weeks intervention in the treatment group, LVEF, FS and 6-MWT were significantly increased, while NT-proBNP, hsCRP, 6-MWT, LVD and LVMI were significantly decreased, when compared with the control group. In conclusion, sildenafil can improve cardiac function and reduce pulmonary artery pressure. In addition, it can attenuate myocardial remodeling through its anti-inflammatory effect.

To the Editor,

Congestive heart failure (CHF) complicated with pulmonary hypertension (PH) increases the right ventricular afterload, leading to right heart dysfunction and higher mortality (1). However, there is no efficacious therapy for PH associated with CHF. Pharmacotherapy (diuretics, angiotensin-converting enzyme inhibitor and beta-blockers) or specific intervention measures (left ventricular assist device, valve surgery, cardiac re-synchronization

therapy and heart transplantation), may reduce PH and restore the pulmonary capillary wedge pressure (PCWP) by lowering the left ventricular filling pressure. However, the mean pulmonary artery pressure (mPAP) is still higher in some patients with PH associated with heart failure, though the PCWP was decreased after the treatment. Therefore, efficacious therapy is still needed to reduce PH.

The traditional therapies for PH are calcium channel blockers (CCBS), oxygen therapy,

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0393-974X (2018)

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anticoagulation and treating right heart dysfunction, but their effects are limited. In recent years, according to the pathogenesis of PH (pulmonary vascular smooth muscle cell proliferation and endothelial dysfunction), the targeted therapy drugs, including prostacyclin, endothelin receptor antagonist and phosphodiesterase type 5 inhibitor, are effective in PH. However, the first two drugs expensive, which limit their clinical application. NO (nitric oxide) is useful for the treatment of PH only in emergencies.

Sildenafil is a selective PDE-5 inhibitor, and previous studies reported that sildenafil might exert beneficial effects in PH associated with CHF through decreasing the cGMP, increasing the intrinsic NO production and dilating pulmonary artery. Therefore, sildenafil may have better curative effect in the

treatment of PH associated with CHF. In fact, several studies have reported that sildenafil is an effective therapy for secondary PH (2-8). However, at present, convincing evidence in the Chinese population is still lacking. In this study, 20 cases were recruited to evaluate the effect of sildenafil on the PH associated with chronic left heart failure.

MATERIALS AND METHODS

Written informed consent was obtained from all the participants before the study. Individuals with diabetes, valvular heart disease and atrial fibrillation were excluded. Twenty PH patients associated with CHF, who were admitted to the First Hospital of Zhengzhou University in 2010-2016, were recruited. The basic characteristics of

Table I. The basic characteristics of the 20 PH patients associated with left heart failure.

Patient ID	Sex	Age (Years)	PAP (mmHg)	EF	Pro-BNP	Weight (Kilos)
1	female	52	90	48	3998	41
2	female	44	75	47	10900	35
3	female	46	90	49	7960	65
4	male	51	71	45	2597	55
5	male	54	35	46	2327	53
6	female	35	65	46	1487	56
7	female	41	75	43	2659	71
8	male	43	76	45	335	81
9	male	65	85	49	724	69
10	female	64	92	48	9115	58
11	female	23	108	46	2805	43
12	female	76	87	47	1880	63
13	female	61	52	48	465	57
14	female	32	91	42	2455	60
15	female	53	68	44	592	65
16	female	38	95	43	2350	48
17	female	44	95	49	1435	54
18	female	41	93	43	612	61
19	female	53	85	42	3250	52
20	female	41	90	48	2280	50

the 20 patients are shown in Table I. They were divided into a treatment group (10 cases, with an oral dose of sildenafil 75 mg daily for 4 weeks) and a control group (10 cases, with treatment of cardiac glycosides, diuretics, angiotensin- converting enzyme inhibitor, angiotensin II receptor blocker and beta-blockers). Left ventricular systolic function (LVEF), pulmonary artery systolic pressure (PASP), left ventricular fraction shortening (LVFS), left atrium diameter (LAD) and left ventricular end-diastolic diameter (LVD) were measured by echocardiography; the left ventricular mass index (LVMI) was calculated by Devereux's formula (9) as recommended by the American Society of Echocardiography, using left ventricular internal diameter (LVID), interventricular septal thickness (IVST) and left ventricular posterior wall thickness (PWT):

$$\text{LVMI (g/m}^2\text{)} = (0.8(1.04((\text{IVST}+\text{LVID}+\text{PWT})^3 - \text{LVID}^3)) + 0.6\text{g})/\text{BSA}$$

LVH was defined as LVMI greater than 116 g/m² in males and LVMI greater than 104 g/m² in females.

The level of N-terminal probrain natriuretic peptide (NT-proBNP) was detected by electrochemiluminescence,

and high sensitivity C-reactive protein (hsCRP) by immune transmission. The walking distance in 6-minute walk test (6-MWT) was also calculated.

Nitric oxide production assay

The plasma level of NO concentration in each group was measured. Spectrophotometric analysis was taken to measure the concentration of NO production as previously described (10) with an Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China). In brief, samples were incubated with nitrate reductase enzyme for 1 h at 37°C and incubated at room temperature for 10 min after Griess reagent was added. Absorbance was measured at 550 nm followed by the calculation of NO concentration according to the formula provided in protocol.

Statistical analysis

Data were described as mean $\pm$ SD. The mean of two groups were compared using the unpaired Student's *t*-test. A two-sided *P* < 0.05 was considered significant. Statistical analysis was performed using the statistical software SPSS Version 18.0.

Table II. Results of LVEF, PH, NT-proBNP, NO, Hs-CRP, LVEDD and 6MWT in control and treatment groups.

Observed index	Prior to treatment	Control group after	Post-treatment
LVEF (%)	39.68 $\pm$ 7.06	41.12 $\pm$ 8.31*	50.79 $\pm$ 7.68*
PH (mmHg)	47.80 $\pm$ 11.93	42.87 $\pm$ 12.08*	39.40 $\pm$ 11.34*
NT-proBNP (pg/ml)	5516.60 $\pm$ 1102.48	4396.40 $\pm$ 1022.37 ^a	3671.87 $\pm$ 873.08*
NO (umol/L)	19.8 $\pm$ 8.2	59.7 $\pm$ 2.3	48.2 $\pm$ 3.5
Hs-CRP (mg/L)	10.75 $\pm$ 3.77	8.40 $\pm$ 4.37	3.93 $\pm$ 1.80
LVEDD (mm)	62.73 $\pm$ 8.26	51.17 $\pm$ 0.94	54.43 $\pm$ 0.81
6MWT (m)	312.18 $\pm$ 10.87	340.12 $\pm$ 22.31	369.93 $\pm$ 20.75

*: Compared with control group *P* < 0.05

RESULTS

There were no significant differences in LVEF, LVFS, NT-proBNP and hsCRP levels between the treatment group and control group before treatment. However, there were significant differences in 6-MWT, LVD, LAD and LVMI between the two groups. Therefore, the paired design analysis was used to remove the statistical difference.

Compared with the control group, the LVEF and 6-MWT were significantly increased, while NT-proBNP, hsCRP, 6-MWT, LVD and LVMI were significantly decreased after 8 weeks treatment.

The plasma levels of NO were increased after the treatment with sildenafil in patients with PH associated chronic left heart failure. All the results were summarized in Table II.

DISCUSSION

In this study, we found that sildenafil could improve LVEF and 6-MWT, decrease NT-proBNP, hsCRP, 6-MWT, LVD and LVMI. In addition, sildenafil significantly elevated the plasma levels of NO. These results suggest that sildenafil combined with the traditional measures could improve heart function, decrease pulmonary hypertension and exert an anti-inflammatory effect. However, these effects may have been related to the traditional therapy, such as ACEI/ARB, β -blockers and diuretics. Therefore, further studies are still needed to elucidate the exact role of sildenafil in PH associated with chronic left heart failure.

Sildenafil is a strong PDE-5 inhibitor that was approved for the treatment of PH (to improve exercise capacity) in 2005 in the US and the European Union, and then in 2009, to delay clinical worsening (11-13). Sildenafil can increase downstream cGMP signaling and potentiate the beneficial effects of NO. PDE5 inhibition leads to a significant increase of intracellular cGMP and then vasodilatation, and probably smooth muscle cell mitosis inhibition. PDE5 inhibitors acutely reduce pulmonary vascular resistance (PVR), increase cardiac output and reduce pulmonary artery occlusion pressure (PAOP). These agents can improve clinical end-points in PH. PDE5

inhibitor may also exert vasodilatory effects through PDEIII inhibition and augmenting RV function. A single dose of 50 mg nasogastric sildenafil was studied in a small cohort of consecutive acute respiratory distress syndrome patients, sildenafil reduced mean arterial pressure (MAP), but it also increased the risk of worsening arterial oxygenation due to increased V/Q mismatch.

In summary, sildenafil can improve cardiac function and reduce pulmonary artery pressure. In addition, it can attenuate myocardial remodeling. Detecting the plasma levels of NO might be useful for evaluating the severity of heart failure and the effect of sildenafil on PH associated with CHF.

ACKNOWLEDGEMENTS

This paper was supported by the 2013 Key Laboratory of Science and Technology Funding Projects of Zhengzhou Municipal Science and Technology Bureau. Project number: N2013SC0586.

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LETTER TO THE EDITOR

VEGF-A EXPRESSION IN SOFT TISSUES REPAIRED BY SHOCKWAVE THERAPY: DIFFERENCES BETWEEN MODALITIES

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Received December 7, 2017 – Accepted March 1, 2018

Shockwave therapy has found its place in the medical treatment of various diseases of the locomotor system such as acute fracture, nonunion, chronic tendinitis and pseudarthrosis. Focused shock waves enable maximum energy in the therapeutic zone, and depth of penetration can be adjusted. Radial shockwave therapy primarily affects superficial tissues, so its application in medicine is doubtful. Our study aimed to assess long bone fracture healing in regard to soft tissues. For this investigation, 84 female Wistar rats were divided into a focused shockwave group (n=36), a radial shockwave group (n=36) and a control group (n=12). Conclusively, long bone fracture repair was enhanced in the shockwave groups. Comparison between focused shock waves and radial shock waves suggested that this latter strongly stimulated the processes of the healing, as 75% of vascular spaces were VEGF-A positive on the 5th day of bone healing, and 85% on the 22nd day of healing.

To the Editor,

Shock waves are basically sound waves that travel through the tissue fluid at the sound speed (1). These sound waves of high energy, produced by specific generators that are transmitted through the special transmission system to the body and are precisely focused on the treated area. Shockwave therapy has been used in the therapy of soft tissue disorders for several years. Most of the investigations were conducted with a focused wave, by choosing a variety of physical wave parameters. The results of radial shockwave activity were occasionally investigated.

It is well-known that mechanical stimulation exerts some biological reactions at the tissue level. The most commonly mentioned biological effects are reactive hyperemia, angiogenesis, stimulation of

fibroblasts, tenocytes, osteoblasts and chondrocytes, and mechanisms of destruction (2).

One of the main biological responses to mechanical stimulation of shockwave therapy is the formation of nitric oxide (NO), which stimulates angiogenesis (creating new blood vessels from existing ones). Angiogenesis is by itself an important stage in the whole tissue and is caused by various growth factors and angiogenic factors.

Some studies suggest that the shockwave therapy-induced mechanical stimulation may have a direct effect on the extracellular matrix, which subsequently induces a cytoplasmic and nuclear response, depending on the experimental model, energy density, number of impulses and cell type. Endothelial cells are particularly sensitive

Key words: focused shockwave therapy, radial shock wave therapy, VEGF-A

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0393-974X (2018)

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to mechanical stimuli. Mechanic stimuli induce the hemodynamic response of endothelial cells at different time periods:

- i. early (immediate) response: within a few seconds/minutes, characterized by ion channel activation and secondary messengers, such as NO;
- ii. intermediate response: within minutes, characterized by endocytosis, cell division, up-regulated by genes;
- iii. late response: within a few hours/day, when the structural and functional adaptation of endothelial cells mediated by NO is monitored (3).

The main therapeutic goal in physiatry and orthopedics is not the destruction of tissues but the stimulation of tissue regeneration (4). This might be due to the ability to convert mechanical stimulation into a variety of biological reactions at the tissue level. This mechanism is manifested in all cells responding to physical stimulation. Shock waves produce microtraumas that stimulate the biological processes of tissue rebuild: increasing the ability of self-contained tissue, improving metabolism, increasing vascularization, and the injured tissue regenerates.

To date, the exact mechanism of action of shockwave therapy on the soft tissue of the locomotor system is not completely clear (as opposed to the clear mechanism of urolith destruction), so we aimed this research at elucidating the role of VEGF-A.

MATERIALS AND METHODS

Animals

We performed assessments on laboratory animals – 84 female Wistar rats, aged three months, with an average body weight of 250 g. As a target organ, soft tissue surrounding fibula - patellar ligament and peroneal muscles (*peroneus longus* and *peroneus brevis*).

The research was carried out conforming to the applicable legal regulations and bioethical standards on the experimentation of experimental animals (avoiding unnecessary experiments and experimental animals) and on the basics of so-called experimental animals.

After the animals were anesthetized with 3.6% chloral hydrate, the skin and subcutaneous tissue were cut with a no. 10 surgical knife. Sharp curved-tip scissors (Metzenbaum-

Fino, curved 5³/4") were used to separate the tissue and fibulas from the lateral side and to make a fibular fracture (diameter 1 mm) at the site of the bone body.

The animals were divided into two groups: experimental group 1 consisting of 36 animals, divided into three subgroups of 12 animals each, treated with a focused striking wave of different energy; and experimental Group 2 consisting of 36 animals, divided into three subgroups of 12 animals each, treated with a different radial curve of different pressure.

In both groups, three treatments were administered at intervals of 5 days on 3rd, 8th and 13th postoperative days. The treated area was the bones and surrounding tissues in the radius of 3 cm. From each subgroup of half of the animals were sacrificed on the 15th day and the remaining animals were sacrificed on the 22nd postoperative day.

The control group consisted of 12 animals that also had a fracture of the fibula and injured surrounding soft tissues but were not treated with a striking wave. This group was also divided into two subgroups of 6 animals each. Six animals of the first subgroup were sacrificed on the 15th day, the remaining six animals of the second subgroup on day 22.

Shock waves

Swiss Duoclast® (EMS - Electro Medical Systems) was used to treat all animals, and its applicators were used accordingly: Dolorclast applicator for radial shock waves and Piezoclast for focused shock waves.

In focused shockwave treatment we used an applicator with a transmitter to reach a depth of 15 mm wavelength, with the action of the surrounding tissue in a 30-mm radius, and pressures applied were: 1.6; 2.2; 3.0 bar, 500 strikes per treatment, frequency 5 Hz. We used a 15-mm diameter piezoelectric applicator that generated impulses (shock waves) of: low energy density (0.1mJ/mm²); medium density (0.2mJ/mm²) and high density (0.3mJ/mm²).

Preparation of the sample

The animals were sacrificed after application of an excessive dose of anesthetic. Afterwards the proximal part of the fibula, the surrounding muscle, and part of the patellar ligament were extirpated. The muscular and soft tissue samples were immediately fixated in 4% paraformaldehyde and then embedded in paraffin and cut into 5-µm thick slices.

After decalcination in Osteodek (Bio Optica), bone samples were successively rinsed in physiological solution and fixated in 4% paraformaldehyde and dehydrated in a series of ethanol. Finally, they were impregnated with xylene and incorporated in paraffin. After insertion into paraffin, tissue blocks were cut using a Leica RM550 rotating microtome. Serial longitudinal cuts, 5- μ m thick, were made at intervals of 150 μ m in the middle part of the samples used later for immunohistochemistry and histology.

The obtained cuts were mounted on silanized glass and dried in a thermostat at 37°C until processing microscopy.

Histomorphometric analysis

Histomorphometry was carried out using a Sony digital camera (Sony, Yokohama, Kanagawa) mounted on an Olympus BHA (Olympus, Tokyo, Japan) light microscope. Micrographs were processed using the Issa computer program (VAMS, Zagreb, Croatia). The cross-sectional area (PPP, in mm²) was measured: cartilage, mineralized cartilage, bone grafts in cartilage, bone grafts and cartilage.

100% cells of interest were first labeled in five vision fields and then the percentage of immunopositive cells among them was determined.

Histological staining

Tissue cuts were stained with standard histological staining of toluidine blue and hematoxylin-eosin by the protocols hereunder.

Toluidine blue

The tissue slices were deparaffinized, rehydrated in a series of alcohol concentrations (100%, 96%, 75%, respectively) and rinsed with water. Then the tissue cuts were stained with 1% toluidine blue solution for 5 min and washed with water. Dehydration of tissue sections was carried out in acetone, not in alcohol because the dye dissolves in alcohol. After cleansing in xylene, the colored sections were embodied in the balm. The dyeing result was violet blue staining of interstitial material rich in proteoglycans; the cell cytoplasm was colored light blue, the nucleus was dark blue.

Hematoxylin-eosin

The tissue slices were deparaffinized, rehydrated in a series of alcohol concentrations (100%, 96%, 75%, respectively) and finally immersed in water. Afterwards,

tissue was stained with 1% hematoxylin solution for 2 min and washed with water. Dehydration of tissue was performed in acetone. After cleansing in xylene, the stained slices were fit into the balm. The result was a red colored cell cytoplasm and dark blue nucleus.

Immunohistochemistry

For immunohistochemistry analysis, tissue sections were placed on 3 aminopropyltetroxylan (APES, Sigma, St. Louis, MO, USA) coated glasses and then dried in a thermostat at 40°C overnight. The glass was stored at room temperature until the staining procedure by the indirect immune peroxidase method. The staining process involved deparaffinizing tissue sections in xylene and gradual rehydration in alcohol.

Afterwards, the samples were processed with 0.5 M TrisEDTA of pH 9 for 10 min at 60°C. Non-specific binding of the antibody was blocked in 10% non-immunogenic pork serum for 15 min. Tissue samples were subcutaneously incubated on primary antibody overnight at 4°C. Anti-VEGF-A antibodies (Abcam PLC, Cambridge, UK) in 1:200 dilution was used, followed by incubation with secondary biotinylated antibody and blockade of endogenous peroxidase activity.

Subsequently, DAB (DakoCytomation, Denmark) was used for visualization. The nuclei were stained with hematoxylin.

Statistical analysis

Statistica 8.1 (StatSoft) computer software (StatSoft Inc., Tulsa, USA) was used for statistical analyses. For each of the above-given parameters, differences between the controls and ESWT group, as well as differences between focused shockwave and radial shockwave methods were explored on the 15th and 22nd days, respectively.

The Fisher Highly Significant Difference test was used post hoc to identify specific differences between the groups.

RESULTS

Histology of the muscle and tendon healing

The results of histological analysis of muscle recovery indicate the good organization of muscle fibers on the 15th day of healing with radial shock waves and the same degree of fiber organization was still reached on the 22nd day of healing with focused

shock-wave (Fig. 1). This was visualised using a microscope and a camera system. The obtained micrographs of histological cuts were transferred to a computer system monitor for quantitative microscopic image analysis.

VEGF-A immunohistochemistry

A similar finding was found during the healing of the tendon, where on the 15th day of healing with radial shock waves a significant multiplication of collagen fibers was observed. The same change was

still seen after 22 days of healing with focal shock waves (Fig. 2).

The multiplication of collagen fibers was significantly higher in the group of animals treated with the striking waves than in the control group.

Accordingly, 75% of vascular spaces were anti-VEGF-A positive on the 15th day of bone healing stimulated by radial shock waves, and 85% on the 22nd day of healing. At sites of intense multiplication of collagen fibers, VEGF-A positive blood vessels were found. This was more obvious in specimens

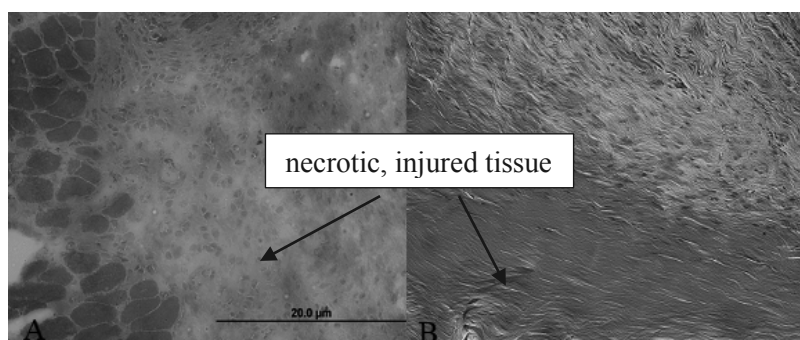


Fig. 1. Histological staining (H&E – henatoxylin and eosin) on the 15th day of focused wave healing, 20X magnification. **A)** Muscle; **B)** tendon.

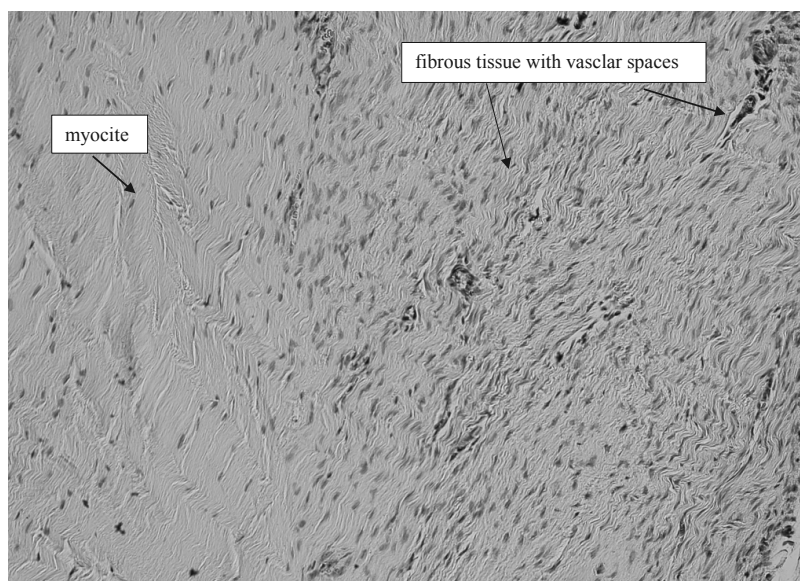


Fig. 2. Immunohistology of the area of the healing tendon as visible on the 15th day after treatment with radial shock-waves. Contrast dyeing with DAB. Magnification 20X. There are numerous VEGF-A positive blood vessels (arrows) in the sites of collagen fibers.

yielded from mice treated with radial shock waves. Fig. 3 shows an abundance of collagen fibers accompanied by the scarce VEGF-A positive blood vessels (arrows).

DISCUSSION

By analyzing slices of mouse tendons of long and short peroneal muscles stained immunohistochemically by anti-VEGF-A antibodies, the highest intensity of positivity was found in bones treated by radial shock waves. Namely, 75% of vascular spaces were VEGF-A positive on 5th day of bone healing, and 85% on 22nd day of healing.

A member of the PDGF/VEGF family (platelet-derived growth factor/vascular endothelial growth factor), vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) and as VEGF-A, stimulates the proliferation of new blood vessels of fetuses and adult individuals. Its common function is the formation of new blood vessels during embryogenic development and collateral circulation after injury. It increases vascular permeability, stimulates angiogenesis and growth of endothelial

cells, promotes cell migration, inhibits apoptosis and affects tumor growth. It is also a vasodilator that increases microvascular permeability (5, 6).

Circulating VEGF-A binds to VEGF receptors on endothelial cells, stimulating tyrosine kinase, leading to angiogenesis. It is part of a system that allows the delivery of oxygen to the tissues by inadequate blood circulation (7, 8). In the absence of oxygen, cells are created by HIF (hypoxia-inducible factor).

Increased VEGF-A levels can lead to disease (6). Solid tumors cannot grow above certain limits without adequate blood circulation; VEGF-A-susceptible tumors have the ability to grow and create metastasis. VEGF-A is also liberated in response to TNF in inflammatory rheumatoid diseases, in increased endothelial permeability and swelling, as well as whenever stimulating angiogenesis is in demand (formation of capillaries). Antiviral agents such as bevacizumab inhibit the formation of VEGF-A.

VEGF-A SDF-1 (stromal cell-derived factor 1) is essential in the activation of circulating endothelial progenitor cells (EPCs), during acute ischemia. However, in chronic ischemia, their expression is frailer, resulting in insufficient mobilization of the

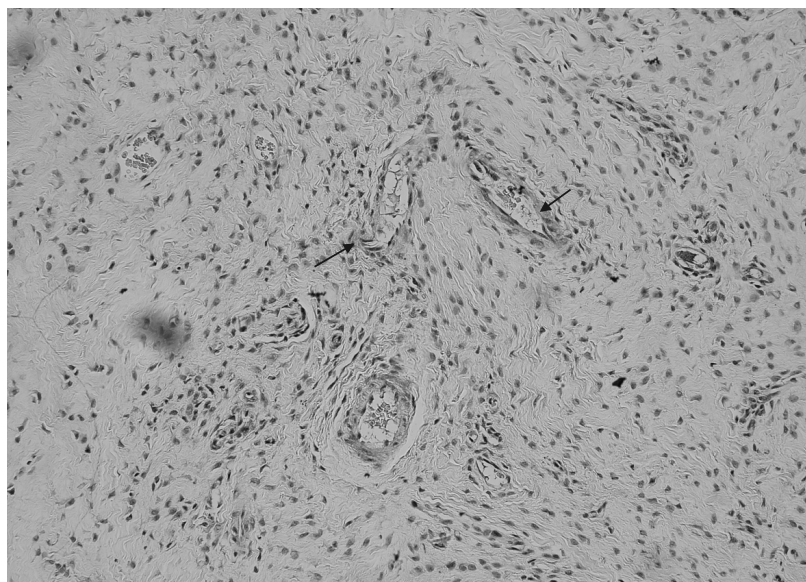


Fig. 3. Immunohistological staining of the area of the healing tendon as it appears on the 22nd day, after treatment with radial shock waves. Contrast staining was carried out with DAB. Magnification 20X. The reaction intensity is weaker than the 15th day of radial shockwave treatment. Collagen fibers are more numerous and VEGF-A positive blood vessels are weaker (arrows).

EPC in the affected tissue. Aicher et al. investigated the effect of focused shock waves on better mobilization of EPC in healthy and chronic ischemic tissue of rats (9). Expression of mRNA SDF-1 factor and histological number of VEGF- in myocytes were significantly increased after treatment. In healthy tissue as well as chronic ischemic tissue treated by shock waves, there is activation of EPCs, which can be used in the treatment of chronic ischemia.

Waguespack et al. evaluated the effect of the shock waves on the accessory ligament of the deep flexor muscle of horses' legs. Lesions were performed by injecting type I collagenase (10). After 15 weeks of shock waves, microstructure, mRNA collagen type I and III levels, and collagen and glucosamine glycol content were established. In the group receiving the shockwaves treatment, a significantly higher level of collagen type I mRNA was demonstrated, while the other parameters tested did not show significant differences.

Silveira et al. considered the effect of shock waves on the healing of superficial wounds on horses' forelimbs in the projection of the III metacarpal bone (11). On specimen yield by soft tissue biopsy taken weekly for 6 weeks, the treatment had not accelerated the healing course, but the treated animals had less granular tissue with less pronounced chronic inflammatory reaction, leading to a conclusion necessitating further studies.

Among other investigators, Wang et al. evaluated the levels of NO, factors involved in angiogenesis (VEGF, vWF, FGF and TGF-beta1), osteogenesis (BMP-2, osteocalcin, AF, DKK-1, IGF) and inflammation (sICAM, sCAM) in humans with fractures treated with shockwave therapy (12). They noticed a significant elevation of all assessed parameters one month after the application of the shock waves.

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LETTER TO THE EDITOR

PURE RED CELL APLASIA WITH T-CELL LARGE GRANULAR LYMPHOCYTIC LEUKEMIA

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Received February 3, 2018 – Accepted March 16, 2018

Pure red cell aplasia (PRCA) develops as a result of erythroid precursors failing to reach maturity in the bone marrow, which eventually leads to anemia. Here we present a case of a 64-year-old Asian male with a medical history of colorectal adenocarcinoma who had been treated with 6 cycles of oxaliplatin and capecitabine four years ago. The patient was diagnosed with PRCA and T-cell large granular lymphocyte leukemia.

To the Editor,

Pure red cell aplasia (PRCA) is a relatively rare disorder. The condition may develop as primary hematological disorder, but often presents as a secondary disorder linked to a number of other diseases (1, 2). Acquired secondary PRCA has been associated with thymoma, hematological malignancies, non-hematological solid tumors, infections, drug and chemical exposure, hemolytic anemia, collagen vascular disease, pregnancy, severe renal failure and severe nutritional deficiency (1-4). Most patients with chronic PRCA develop autoimmune diseases caused either by autoantibodies to erythroid progenitor cells or by T-cell autoimmunity.

Multiple factors are involved in the pathogenesis and development of this condition. The possibility of PRCA should be considered in patients with a slow progressive normocytic-normochromic anemia and reticulocytopenia without leukopenia and thrombocytopenia, who do not show abnormal findings on physical examination, except pallor (5).

Here, we describe a rare case of PRCA that was likely linked to the previous repeated exposure to chemotherapeutic treatment.

Case report

A 64-year-old Asian male with a medical history of colorectal adenocarcinoma, who had been treated with 6 cycles of oxaliplatin and capecitabine four years previously, began experiencing chronic fatigue and shortness of breath. Approximately 5 months after his initial presentation in 2017, the patient became progressively anemic, with a hemoglobin nadir of 4.5 g/dL (normal baseline level - 12.0g/dL) and a mean corpuscular volume nadir of 50 fL (normal baseline values - 80-90 fL/red cell). The patient complained of xerostomia and xerophthalmia. He was markedly overloaded with iron, with ferritin level of 1000 ng/mL and ferritin saturation at 50%. All other blood cell parameters were within the normal range. The patient was referred to the hematology clinic and was treated for profound anemia. The blood test revealed that he had

Key words: pure red cell aplasia, T-cell large granular lymphocytic leukemia, chemotherapy, colorectal adenocarcinoma

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0393-974X (2018)

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severe anemia and reticulocytopenia (hemoglobin: 4.6 g/dl, reticulocytes: 1000/ μ l). The bone marrow analysis revealed severe erythrocytopenia in the absence of abnormal findings of myeloid cells and megakaryocytes. The bone marrow showed diffuse lymphocytosis and rare erythroid precursors, some of

them with cytoplasmic vacuoles (Fig. 1). Myeloid and megakaryocytic series were normal. The phenotype was as follows: CD34+ cells (the proportion of CD34+ among nucleated cells) - 0.4%, lymphocytes - 21.3%, monocytes - 11.3%, granulocytes - 61.2%, eosinophils - 2.0%, blast cells - 0.6%, erythroblasts

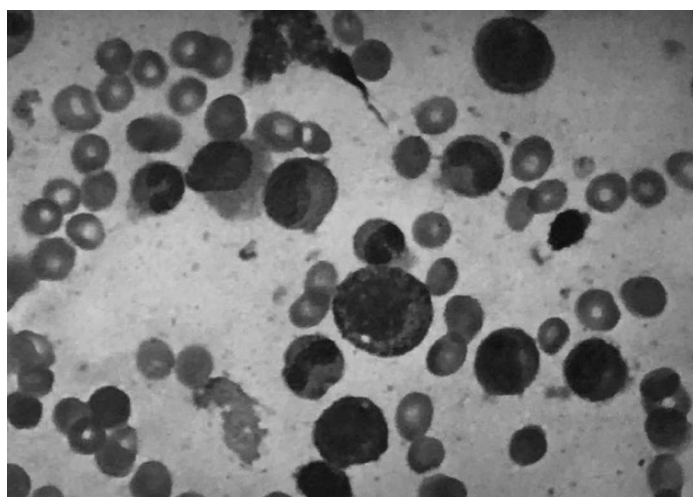


Fig. 1. Bone marrow showing diffuse lymphocytosis and rare erythroid precursors, some with cytoplasmic vacuoles.

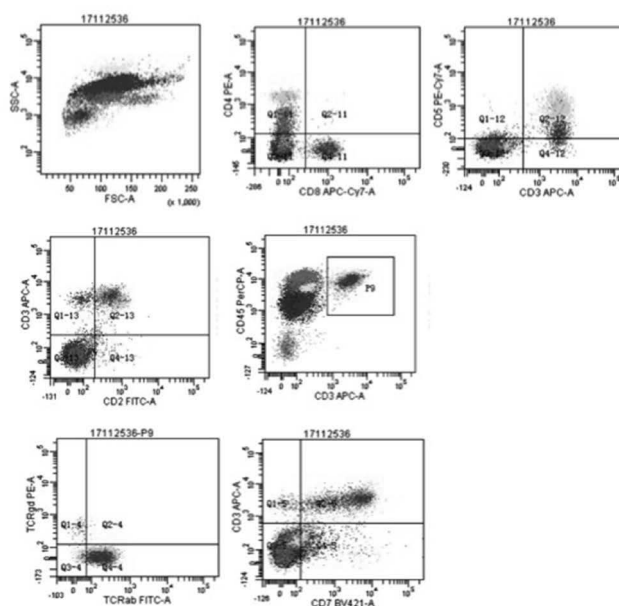


Fig. 2. Antigens examined in this test: HLA-DR, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD33, CD34, CD38, CD42b, CD56, CD64, CD71, CD117, cCD3, cIgM, TCR $\gamma\delta$, MPO, TdT, CD45. The following percentage of cells was observed: lymphocytes (green) 21.3%, monocytes (purple) - 11.3%, granulocytes (blue) - 61.2%, eosinophils (sky blue) - 2.0%, blast cells (red) - 0.6%, erythroblasts (gray) - 3.6%. CD3+ lymphocytes accounted for 92.9% of all lymphocytes, CD3+ CD5+ lymphocytes - for 36.7%. The latter expressed CD7+, CD8+, and TCR.

- 3.6%. CD3+ lymphocytes represented 92.9% of all lymphocytes. CD3+ CD5+ lymphocytes accounted for 36.7% of all lymphocytes, these cells expressed CD7+, CD8+ and TCR. The test for TCR gene rearrangements showed TCRBC(+) and TCRG A(+) (Fig. 2). Repeated serum tests to parvovirus B19 antibodies (enzyme-linked immunosorbent assay, ELISA) showed negative IgM and negative IgG antibodies. Based on these findings, the patient was diagnosed with PRCA and T-cell large granular lymphocyte (LGL) leukemia. Chromosomal analysis revealed no abnormalities. More than 30% of lymphocytes were of the LGL type. A monoclonal rearrangement of T-cell receptor (TCR) beta chain gene was detected by Southern blot analysis.

This study was approved by the Bioethics Committee of the Department of Anesthesia, China-Japan Union Hospital of Jilin University, China. The mode of consent was approved by the ethics committee, and all patient records were provided with informed, signed consent for publishing the case details under strict institutional guidelines.

DISCUSSION

The major diagnostic criterion for LGL leukemia is a granular lymphocytosis greater than 2,000/ml lasting for more than 6 months (6). In the case presented here, the diagnosis of LGL leukemia and PRCA was synchronal, with absolute granular lymphocyte count lower than 2,000/ml. During the patient monitoring period, his hemoglobin level decreased from 10.7 to 4.5 g/dl, which resulted in the need for red blood cell transfusions. LGL leukemia was diagnosed based on granular lymphocyte morphology, surface marker analysis and T-cell receptor gene assay.

PRCA is an acquired anemia that may be idiopathic (7) or secondary to a variety of neoplastic, autoimmune or infectious diseases or exposure to drugs. The majority of PRCA cases are considered to be autoimmune-mediated (1, 8). The pathophysiology of PRCA is heterogeneous, but most cases of acquired PRCA are mediated by diverse autoimmune mechanisms, including antibody-mediated, NK cell- or T-cell-mediated

mechanisms (5). LGL leukemia is a clonal disorder of T-cells or natural killer cells and is often associated with pure red cell aplasia (9).

LGL leukemia-associated pure red cell aplasia accounts for a significant portion of secondary cases of pure red cell aplasia. Usually, patients with primary or secondary PRCA not responding to treatment of the underlying disease should be treated with immunosuppressive drugs, such as corticosteroids (CS), cyclophosphamide (CY), Cyclosporin A (CsA), and anti-thymocyte globulin (1), among which CsA exhibits the most favorable effect for PRCA. Mamiya et al. (10) analyzed the clinical features of 150 patients with acquired PRCA in Japan and demonstrated that CsA was the most effective form of treatment, with the response rate of 82%. The patients receiving CS or CY showed 49% and 29% response rates, respectively. Thus, the authors recommended CsA as the first-line therapy for acquired chronic PRCA. Immunosuppressive therapy with Cyclosporin A and the presence of Epstein-Barr virus infection increase the risk of post-transplant lymphomas. CyA is often used in these patients (6, 11). As an immunosuppressive agent, CyA is believed to inhibit the production of interleukin (IL)-2 and interferon gamma (IFN) by T-lymphocytes, as well as the activation of cytotoxic T-cells by IL-2 (10). In the study on 918 European renal transplant patients, the cumulative risk of neoplasia over 20 years rose to 40% compared with 6% in the age-matched control population.

Since PRCA is rare, has heterogenous pathophysiology and may be associated with broad spectrum of other disorders, the correct diagnostics and treatment of this disease remain difficult. The case described in this report demonstrates the challenges presented by this disease and may be helpful for practitioners dealing with this condition in clinical settings.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Department of Anesthesia, China-Japan Union Hospital of Jilin University, China for providing financial support for this study.

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LETTER TO THE EDITOR

AUTOLOGOUS PLATELET-RICH PLASMA FOR TREATMENT OF VENOUS LEG ULCERS: A PROSPECTIVE CONTROLLED STUDYD. ETUGOV¹, V. MATEEVA² and G. MATEEV¹¹*Department of Dermatology and Venereology, Medical University Sofia, Bulgaria;* ²*Department of Dermatology and Venereology, Military Medical Academy Sofia, Bulgaria**Received December 5, 2017 – Accepted March 12, 2018*

Venous leg ulcers (VLUs) are chronic difficult-to-treat wounds which affect around 1-2% of the world population. Conventional methods for treatment such as mechanical debridement, occlusive dressings and local antibiotics in case of infection, often lack effectiveness. Autologous platelet-rich plasma (PRP) is an alternative method in the treatment of chronic wounds. PRP contains inflammatory mediators, growth factors, and cytokines that modulate the wound microenvironment to create a better chance for healing. The aim of this prospective clinical study was to evaluate the efficacy of intralesional injection of PRP in the management of VLUs. This study included 23 patients with VLUs. For each patient, two ulcers located in the same anatomical zone and at the same clinical stage were selected. One was treated with a single application of autologous PRP. The other ulcer was used as a control and was treated by conventional methods. The size of the ulcers was assessed at baseline (visit 0), 15 days (visit 1) and 30 days after the procedure (visit 2). Results showed a significant reduction of the size of the ulcer both in the group treated with PRP (mean surface 1368.2 mm² at visit 0 and 596.3 mm² at visit 2) and in the control group (mean surface 880.3 mm² at visit 0 and 582.8 mm² at visit 2). Statistical analysis showed a significant change in the size of the ulcer between visit 0 and visit 2 in both groups ($p < 0.0001$). The application of PRP in difficult-to-treat venous leg ulcers may be a promising new method for therapy of this condition. The results of this study correlate with the data from the majority of previous studies and confirm the effectiveness of PRP. Nevertheless further research in the area is needed to evaluate the therapeutic significance of the method and eventually show its superiority to conventional treatments in larger cohorts.

To the Editor,

Venous leg ulcers (VLUs) are a common medical problem in everyday practice. The condition is encountered in around 5% of the elderly over 65 years of age (1) and in 1.5% of the general population (2). The management of VLUs is interdisciplinary and presents a significant economic burden. Furthermore, the importance of VLU is increasing due to the worldwide demographic tendency for aging.

Conventional methods for treatment of VLUs

include: compressive therapy, local wound care such as mechanical debridement and local antibiotics in case of infection; systemic medication; and surgery. Often these methods require prolonged treatment and lack in efficacy.

The fundamental pathophysiological process in VLUs is inflammation of the venous wall due to increased hydrostatic pressure in the vascular system of the lower extremities (3).

The induced inflammatory response consists

*Key words: venous leg ulcers, autologous platelet-rich plasma**Mailing address:*

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0393-974X (2018)

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of an interplay between inflammatory cells such as leukocytes, macrophages and monocytes, T-lymphocytes, and inflammatory molecules such as mediators, chemokines, growth factors. (4, 5).

Growth factors derived from the thrombocytes modify the microenvironment of the wound and influence the early stages of inflammation and cicatrization through stimulation of the capillary angiogenesis, fibroblastic migration and proliferation, collagen synthesis and reepithelisation. The most important thrombocytic growth factors are: platelet-derived growth factor (PDGF), platelet-derived angiogenesis factor (PDAF), platelet-derived epidermal growth factor (PDEGF) and platelet factor 4 (6).

The role of the platelet-rich-plasma (PRP) in stimulating the healing process in difficult-to-treat ulcers has been investigated in the past 25 years. It is suggested that PRP is capable of transforming the difficult-to-heal skin ulcer with low mitogenic activity into a healing ulcer with increased capacity for tissue regeneration (7). A number of clinical studies (8-16) suggest efficacy of PRP in the treatment of VLUs, but this observation is not confirmed by others (17).

The aim of this prospective clinical study was to evaluate the efficacy of the intralesional injection of

PRP in the management of VLUs.

MATERIALS AND METHODS

This study included 46 VLUs from 23 patients (9 male, 14 female), aged 55-78 years. All patients signed an informed consent prior to their entry in the study. For each patient, two VLUs with similar localization and clinical stage were selected for treatment. One of the ulcers was treated with PRP, the other with conventional therapy and served as a control.

Treatment with PRP

After applying topical anesthesia on the treated area (lidocaine, prilocaine, Emla® cream-5%) under occlusion for 50 min., 10 ml blood was extracted from the patient and centrifuged at 3100/min for 4 min (RegenLab 80-2C). A standardized kit was used for the procedure - RegenCT [Ref: R-CT-3, Regenlab Lab SA (CH- 1052, Le Mont-sur-Lausanne, Switzerland)] The separated platelet-rich-plasma was aspirated with a 20G needle in a 20 cc syringe and was injected in the bottom and edges of the ulcer and 3-5 cm in surrounding skin. The surface of the ulcer was divided into 1 cm² squares and 0.2 cc PRP was injected into the middle of each of them. Occlusive dressing with sterile gauze was applied for 5 days. After removal of the

Table I. Size of the ulcer (sq. mm.) in the PRP group and in the CG, V0-V2.

	N	mean	st. deviation	max.	median	min.
PRP group V0	23	1368.2	1185.0	4686.0	988.0	428.0
PRP group V1	23	1029.8	955.2	3916.0	665.0	252.0
PRP group V2	23	596.3	637.1	2526.0	321.0	96.0
CG V0	23	880.3	540.9	2591.0	716.0	317.0
CG V1	23	783.3	520.7	2513.0	620.0	259.0
CG V2	23	582.8	505.3	2454.0	429.0	101.0

dressing, the ulcer was cleaned with saline and covered with silver sulfadiazine 1% cream (Dermazin crème®) every second day.

Control group (CG)

The VLU in the control group were treated according to their clinical stage with debridement, antiseptics and epithelotonic agents. The ulcers were first cleaned with Octenilin® (Aqua valde purificata, Glycerol, Ethylhexylglycerin, Octenidin HCl) with subsequent dressing with Dermazin® cream.

The size of the surface area of each VLU was assessed at baseline (visit 0), 15 days (visit 1) and 30 days after the procedure (visit 2).

Statistical analysis

Data was analyzed using correlation analysis (Person correlation coefficient).

RESULTS

Results showed a significant reduction of the size of the ulcer both in the group treated with PRP (mean surface 1368.2 mm² at visit 0 and 596.3 mm² at visit 2) and in the CG (mean surface 880.3 mm² at visit 0 and 582.8 mm² at visit 2) (Table I).

The reduction in the size of the VLU was more pronounced in the group treated with PRP compared to the CG (Fig. 1). Statistical analysis showed a significant change in the size of the ulcer between visit 0 and visit 2 in both groups ($p < 0.0001$).

Examples of the clinical results in both groups are shown in Figs. 2-3. There is a reduction of the size of the VLU and appearance of fresh granulations at V2 in both PRP and CG.

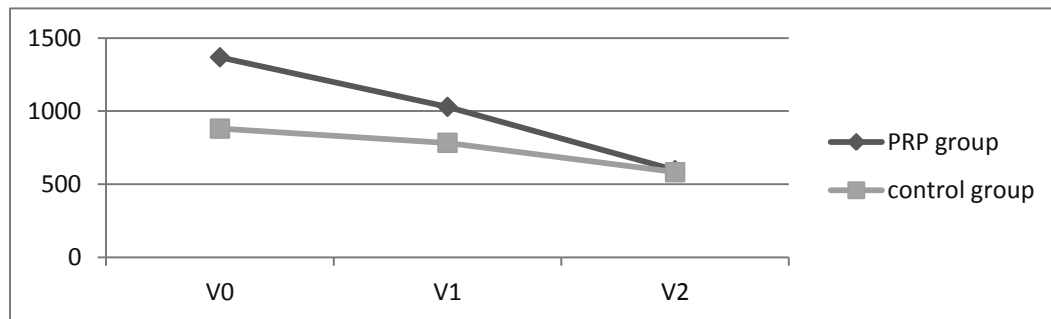


Fig. 1. Reduction of the size of the VLUs in the PRP group and the CG (mm²), V0-V2.

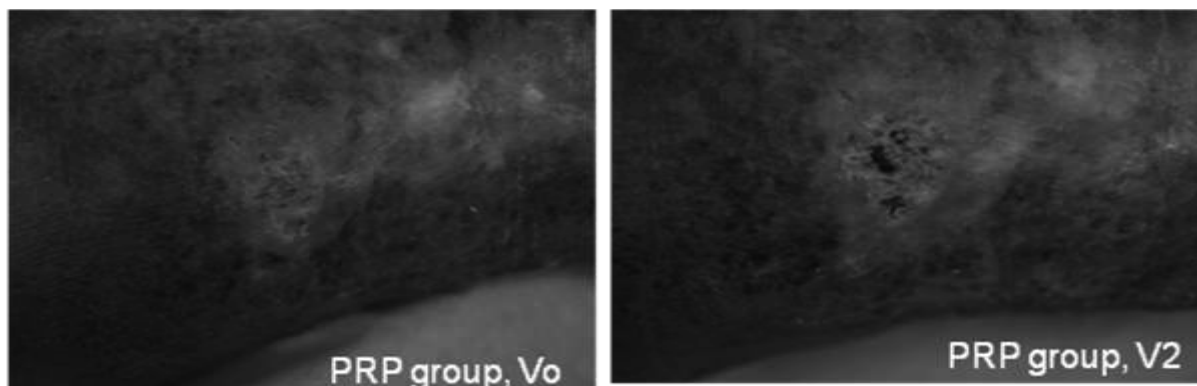


Fig. 2. Clinical results of the group treated with PRP (V0-V2).

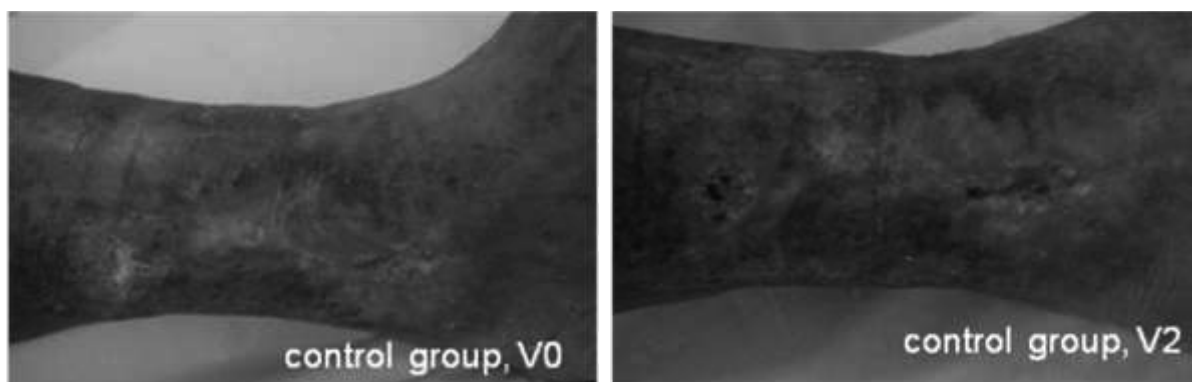


Fig. 3. *Clinical results of the control group (V0-V2).*

DISCUSSION

Systematic review of 18 clinical studies on the effectiveness of PRP on the healing process in chronic leg ulcers, conducted in the period between 1996 and 2006, proves that PRP could be considered a method of choice in these types of ulcers (8).

A study of Knighton et al. on 49 patients with difficult-to-treat chronic ulcers of various origins (venous stasis, arterial insufficiency, diabetes, posttransplantational, decubital wounds) demonstrates that a single application of platelet-derived wound healing factors accelerates the formation of granulation tissue and facilitates the reepithelisation of the ulcer (9).

Crovetti et al. (10) confirmed the beneficial effect of the administration of platelet-rich gel on the process of tissue reconstruction in 24 patients with chronic non-healing ulcers. In 9 of them the authors reported complete wound healing after 10 applications of the gel, and in other 9 a partial response to the treatment.

Mazucco et al. (11) report similar results by comparing 17 patients with chronic non-healing ulcers (postsurgical dehiscent sternal wounds and necrotic skin ulcers) treated with platelet-rich gel with 14 controls treated with conventional methods. Results show that the healing time in the investigated group was shortened by approximately 50%.

A number of subsequent studies confirm the positive effect of the platelet rich media on the

healing of chronic skin ulcers (12-16).

Nevertheless, despite the above mentioned existing data, which support the use of PRP in the treatment of chronic ulcers, a metaanalysis of 9 randomized controlled trials (RCTs) with 325 patients, failed to demonstrate a statistically significant difference between PRP and placebo in influencing the healing process (17).

Our study showed a statistically significant reduction of the size of the ulcer both in the group treated with PRP (mean surface 1368.2 mm² at visit 0 and 596.3mm² at visit 2) and in the control group (mean surface 880.3 mm² at visit 0 and 582.8 mm² at visit 2). The reduction was more pronounced in the group, treated with PRP. These findings are in accordance with the vast majority of the present reported data (9-15) and do not correspond to the conclusions of the metaanalysis of RCTs (17).

The difference in the reported outcomes may be due to the different design of the trials and the resulting difficulty in comparing the data.

A particular strength of this study is the selection of a control group of ulcers with comparable similar characteristics with the investigated group. A limitation of our study is the lack of randomization due to the insufficient number of patients and ulcers.

Our results confirm the beneficial effect of PRP on improving the treatment outcome of VLU, but further studies on larger cohorts are needed to demonstrate its probable superiority to the conventional methods. The application of PRP in

difficult-to-treat skin ulcers may be a promising new method for the therapy of this problem. The results of this study correlate with the data from the majority of previous studies and confirm the effectiveness of PRP in VLUs.

Nevertheless, further research in the area is needed to evaluate the therapeutic significance of the method and investigate its potential superiority to conventional treatments in larger cohorts.

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LETTER TO THE EDITOR

COMPARATIVE STUDY OF METHOTREXATE AND HUMAN UMBILICAL CORD MESENCHYMAL STEM CELL TRANSPLANTATION IN THE TREATMENT OF RHEUMATOID ARTHRITISYH. WANG¹, ZQ. YANG¹, SF. ZHU¹ and Y. GAO²

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Received January 15, 2018 – Accepted March 14, 2018

In this study, a collagen-induced arthritis (CIA) model was established to simulate rheumatoid arthritis (RA) using two intradermal injections of bovine type II collagen and Freund's complete adjuvant mixture given at two-week intervals. Subsequently, the transplantation of human umbilical cord mesenchymal stem cells (hUC-MSCs) was used to treat RA and the treatment efficacy, as well as the possible regulatory mechanism underlying hUC-MSC transplantation, was observed. During the study, forty rats were randomly divided into four groups and their blood samples were collected at different time points to measure levels of serum cartilage oligomeric matrix protein (COMP). Based on the symptoms and pathological features of the rats, a total success rate of 83% was achieved by the treatment. Furthermore, the improvement of joint symptoms was more obvious when methotrexate and MSC transplantation were used. In summary, it was concluded that MSC transplantation relieved the symptoms of arthritis by down-regulating the expression of COMP on the synovial membrane and in the serum of CIA rats.

To the Editor,

Rheumatoid arthritis (RA) is a highly disabling autoimmune disease characterized by progressive and aggressive arthritis (1). Since the destruction of bone and cartilage is involved in the development of RA, some studies have shown that cartilage oligomeric matrix protein (COMP) can also be used as an indicator for the efficacy of RA treatment (2).

Mesenchymal stem cells (MSCs) are pluripotent stem cells with the potential of self-replication and multi-directional differentiation. MSCs can migrate to injured and inflammatory sites, and differentiate into different types of cells (3). In

recent years, studies have found that MSCs have immunomodulatory effects (4) and hence MSC transplantation has become a hot research topic for the treatment of autoimmune diseases. For example, animal studies have found that the infusion of MSCs can significantly improve autoimmune cerebrospinal meningitis, systemic sclerosis, dermatomyositis and systemic lupus erythematosus (5). In this case, the immune regulation ability of MSCs can be used to treat RA patients with hyperthyroidism, while the ability of MSCs to undergo multi-directional differentiation can be harnessed at the same time to repair joint damage (6, 7).

Key words: rheumatoid arthritis, human umbilical cord mesenchymal stem cells, methotrexate, cartilage oligomeric matrix proteins, CIA rats

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0393-974X (2018)

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In this study, the efficacy of MTX and MSC treatments was studied using a collagen-induced arthritis (CIA) model. In addition, the therapeutic effects of MSCs on immunoregulation and injury repair were investigated by assessing the arthritis scores, imaging and pathological manifestations of the animals. Such a study provides an experimental basis as well as a theoretical basis for the clinical use of MSC transplantation in the treatment of RA.

MATERIALS AND METHODS

Animals

Thirty SD rats aged 45~50 days with the body weight of (160±20) g were purchased from Shanxi Medical University Animal Research Center. The rats were housed in an air conditioning controlled animal room of the Second Hospital of Shanxi Medical University. The room temperature was about 20°C and the relative humidity was 50%. The rats had access to free food and water, and the lighting time was 8h/day. The experiment was approved by the Ethics Committee of the Affiliated Hospital of Hebei University and all the procedures were carried out according to animal protection directives.

Materials

Natural cow type II collagen (CII), 5 mg/unit (Sigma company), complete Freund's adjuvant (Sigma company), acetic acid (analytical grade, Tianjin Second Chemical Reagent Factory), anhydrous ethanol (analytical grade, Tianjin Third Chemical Reagent Factory) and elhylene diamine tetraacetic acid (Tianjin Fuchen Chemical Reagent Factory) were purchased from different suppliers. In addition, the following main devices and equipment were also employed for the study: an imaging system (BI2000 medical image analysis system), a rat serum COMP test kit [Po biological engineering (Dalian) Co., Ltd.], and an enzyme labeling instrument (Willscan type MK-3, Finland).

Experimental methods

The first immunization was carried out at the tail, back and right foot of each rat using a multi-point injection method. The animals in the experimental group were injected with 0.5 mL type II collagen (containing 0.5 mg type II collagen). After 14 days, the immunization was

enhanced by an intraperitoneal injection of 0.2 ml immune complex (containing 0.2 mg type II collagen) in each rat. After immunization, the clinical, X-ray, pathological and other indicators for arthritis were regularly evaluated to ensure the success of modeling.

Rats in the control group were injected with the same dose of normal saline using the method described above.

Modeling of CIA rats

During clinical evaluation, the body weight of each rat was measured at each observation point (once before the first immunization and once a week afterwards) using an ordinary experimental balance.

The evaluation of RA was made based on the following arthritis index (AI):

0: no joint inflammation (no redness and swelling); 1: red spots or mild swelling; 2: moderate joint swelling; 3: severe joint swelling; 4: joint stiffness and even deformity or severe dysfunction.

The posterior ankle joint was mainly observed in this study. The joint was observed once before the first immunization. After the first immunization, the joint was observed once every 3 days until 6 weeks before the acute phase. Subsequently, the joint was observed once a week during the chronic phase.

The rats were anesthetized before each shot of immunization. Each rat was given 5 mL/kg of 5% chloral hydrate by intraperitoneal injection. After about 5 min, the muscle tone and the conjunctival reflex of the anesthetized rats both disappeared. The tail of each rat was then cut off to collect 2 mL of the whole blood sample. After incubation for 1 h at room temperature, the blood sample was centrifuged at 3,000 rpm for 5 min and excessive supernatant was discarded until about 1 mL of the supernatant was left. The cells were then placed in a centrifuge tube and stored at -70°C. The blood samples were collected at 0W, 1W, 2W and 3W after the first immunization. The serum content of COMP in the samples was measured by the ELISA method.

Statistical analysis

All measurement data were expressed using mean ± standard deviation. The normality test and variance homogeneity test were carried out to examine the data. The difference between the two groups was evaluated using one-way analysis of variance, while the LSD method

was used for multiple comparisons when the variance was uniform. When the variance was not uniform, the Dunnett's T3 test was used for multiple comparisons. Data analysis was carried out with SPSS 13 software. $p < 0.05$ was considered statistically significant.

RESULTS

Clinical manifestations of an animal model of collagen-induced arthritis

The success of CIA modeling was determined based on the following definition: chronic, progressive, symmetrical and peripheral polyarthritis with systemic symptoms. The pathological manifestations of RA are proliferative synovitis accompanied by cartilage and bone destruction. In addition, under imaging assessment, the CIA rats should display the onset of osteoporosis, multiple articular cartilage destruction, and bone destruction.

In this study, animals were immunized with multi-point intradermal injection. After the initial immunization, multiple small ulcers were formed at the site of intradermal injection. In about one week, the ulcers had healed by themselves. The

clinical presentations of the rats included weight loss, malaise, decreased appetite, reduced physical activities, a morbid status, slight hair loss, and loss of hair color. Compared with the rats in the control group, the weight of CIA rats was significantly reduced. The acute phase of arthritis was characterized by redness and swelling of the knee joint, interphalangeal joint, wrist joint, and interphalangeal joint. The joint symptoms generally started from the hind feet and gradually spread to the forefeet, indicating the progressive aggravation of the symptoms. The swelling in the hind limbs of CIA rats reached its peak within about 20 days after the first immunization and then subsided to a certain extent. After the second immunization of a cattle type II collagen emulsifier on day 14, the rats in the CIA group showed symmetrical and polyarticular swelling, which then progressively aggravated and reached the peak within about 35 days. Subsequently, the swelling gradually subsided and the CIA entered a chronic phase, which was associated with slowly appeared joint stiffness, deformity and even dysfunction. In the experimental group, 33 SD rats were successfully modeled.

Table I. Comparison of foot swelling and serum COMP level at four time points ($\pm s.$)

Group	0 week	1 week	2 weeks	3 weeks
Total foot swelling				
Control group	1.0 $\pm$ 0.1	1.22 $\pm$ 0.88	1.36 $\pm$ 0.11	1.4 $\pm$ 0.08
Control group	2.46 $\pm$ 0.42 [#]	2.3 $\pm$ 0.45 [#]	2.26 $\pm$ 0.18 [#]	1.72 $\pm$ 0.26 [#]
Serum COMP level				
Control group	6.47 $\pm$ 2.25	7.58 $\pm$ 1.88	6.56 $\pm$ 2.16	7.26 $\pm$ 3.51*
CIA group	25.38 $\pm$ 4.53 [#]	21.10 $\pm$ 4.83 [#]	17.99 $\pm$ 2.63 [#]	15.24 $\pm$ 2.69
MTX treatment group	24.65 $\pm$ 6.45 [#]	18.11 $\pm$ 2.58 [#]	13.62 $\pm$ 1.66 ^{**}	8.45 $\pm$ 2.54*
MSC treatment group	22.05 $\pm$ 4.01 [#]	15.39 $\pm$ 3.34 ^{**}	11.16 $\pm$ 2.31 ^{**}	9.36 $\pm$ 1.96*

[#] Compared with the control group, $p < 0.05$; * compared with the CIA group, $p < 0.05$.

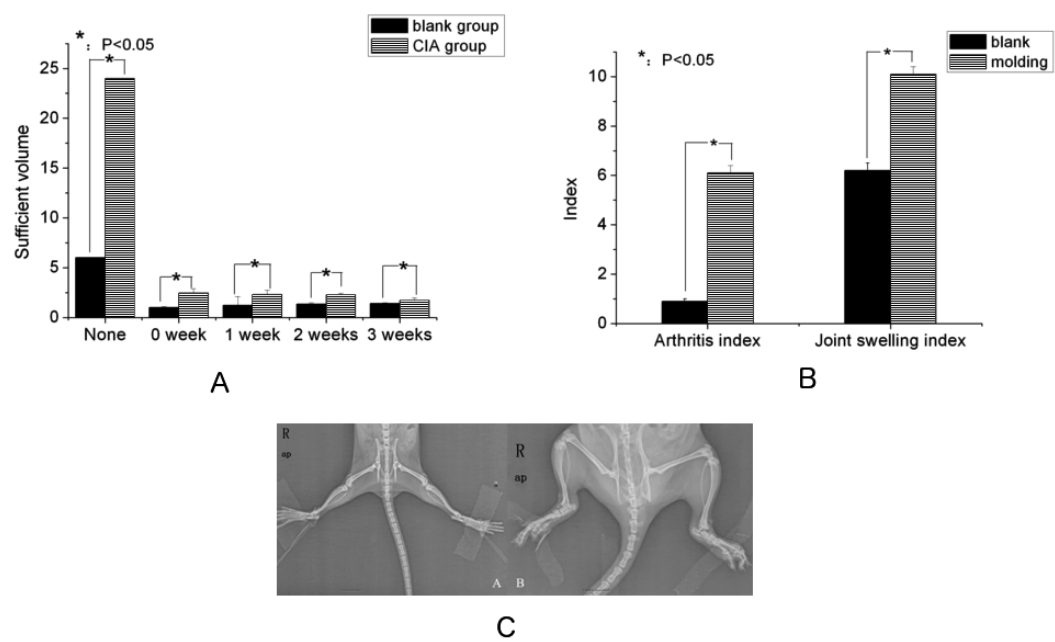


Fig. 1. Comparison of rats in blank and CIA group. **A)** Comparison between two groups at four time points. **B)** Comparison of arthritis index and joint swelling index. **C-A)** left foot of a normal rat; **C-B)** joint swelling and deformity in a CIA rat.

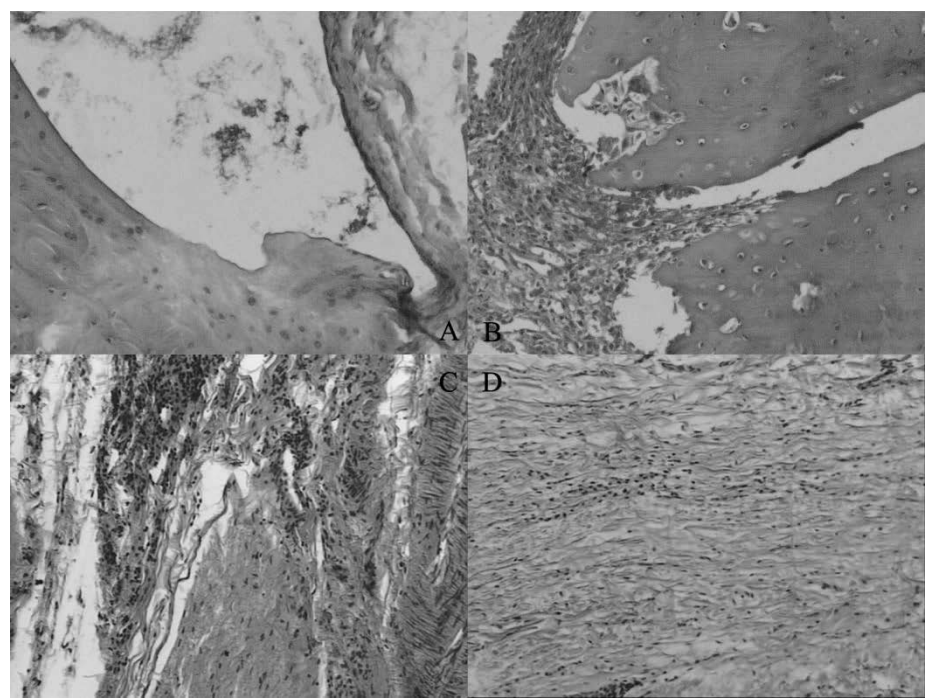


Fig. 2. HE staining of the rat joint in different groups. **A)** Control group, x100 magnification; **B)** inflammatory cell infiltration in the CIA group, x100 magnification; **C)** MTX treatment group, x100 magnification; **D)** MSC treatment group, x100 magnification)

Measurement standard

The swelling of both hind limbs was measured by a volumetric method (drainage method). In this study, the mean values of total swelling in both the control group and the CIA group were recorded and compared at four time points, and significant difference was found between these two groups ($P < 0.05$), as shown in Table I and Fig. 1.

At weeks 0, 1 and 2, the arthritis index and joint swelling index of the control group were significantly lower than those of the CIA group, as shown in Fig. 1.

The radiographs of the rats in this study mainly showed the modification of lower limbs in the following fashion: The double stepping joint in the rats of the control group did not change much throughout the entire experiment. However, in the CIA group, although the rats showed no obvious osteoporosis, no bone destruction and a clear joint space at week 0, the bone marrow became visible and the joint space became blurred at week 2. After the second immunization, the joint space became clearly blurred and narrowed at week 3, as shown in Fig. 1.

Table II. COMP expression in the synovial membranes in different groups.

Group	Mean $\pm$ standard deviation	F value	P value
CIA group	11.9 $\pm$ 2.1*	207	0.00
MTX treatment group	5.0 $\pm$ 0.11		
MSC treatment group	0.55 $\pm$ 0.19		

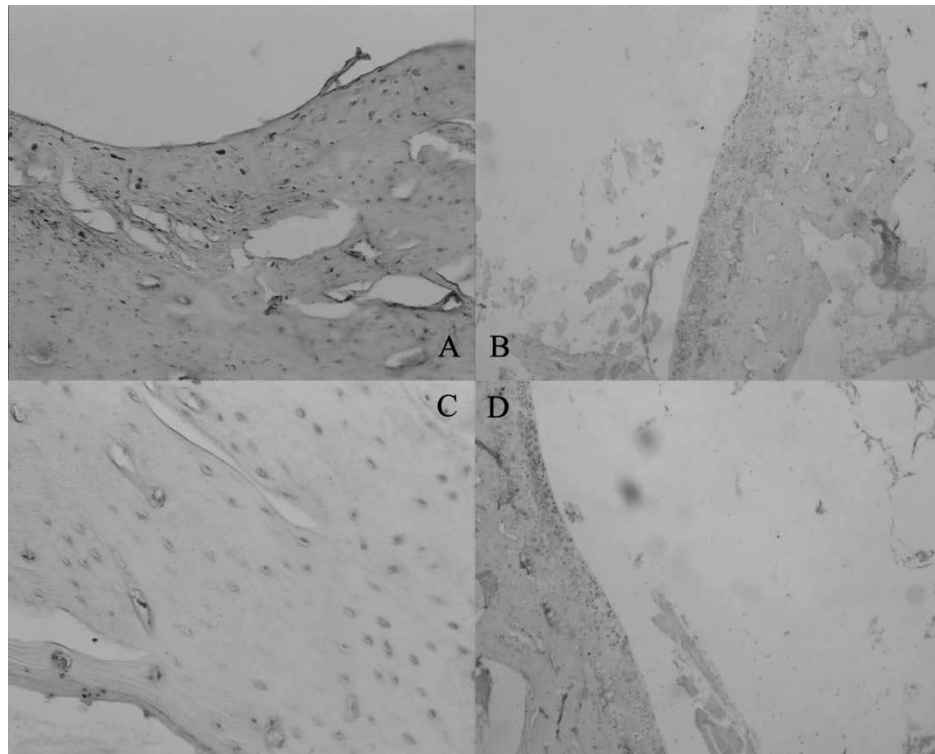


Fig. 3. COMP expression in the synovial membranes in different groups. **A)** Control group, x100 magnification; **B)** abnormal proliferation of synovial cells in the CIA group, x100 magnification; **C)** MTX treatment group, x100 magnification; **D)** MSC treatment group, x100 magnification

Morphological observation by HE staining

HE staining was used to observe the joint morphology of normal and CIA rats. Before the first immunization, HE staining of both groups showed a clear synovial structure of the joint cavity. In addition, there were no signs of hyperplasia, while the infiltration of lymphocytes and plasma cells in the synovial membrane was not obvious. After 3 weeks of modeling, the pathological sections of articular samples collected from CIA rats showed that the synovial structure of the joint cavity was still clear, while the number of lymphocytes and pulp cells in the synovial membrane was increased. In addition, the thickening of synovial cell layer was seen, and up to 4~6 layers of cells had formed. The cells were arranged disorderly and the synovial membrane began to show significant inflammatory cell infiltration. In addition, the blood vessels started to be generated around the articular cartilage and bone tissue, as shown in Fig. 2.

Expression of COMP in the synovial membranes of different groups

There was significant difference in the expression of COMP in the synovial membranes among the MTX treatment group, MSC treatment group and CIA group ($P < 0.01$). However, the difference between the MTX treatment group and the MSC treatment group was not statistically significant, as shown in Table II and Fig. 3.

Comparison of serum COMP levels in different groups

At week 0, there was no significant difference in serum COMP levels between the CIA group and the two treatment groups ($P > 0.05$). However, 3 weeks later, although the serum COMP levels in CIA and the two treatment groups all showed a downward trend, there was significant difference in the serum COMP levels between the two treatment groups and the CIA group ($P < 0.05$). In addition, the serum COMP levels of MTX and MSC treatment groups dropped to the same level, as shown in Table I and Fig. 4.

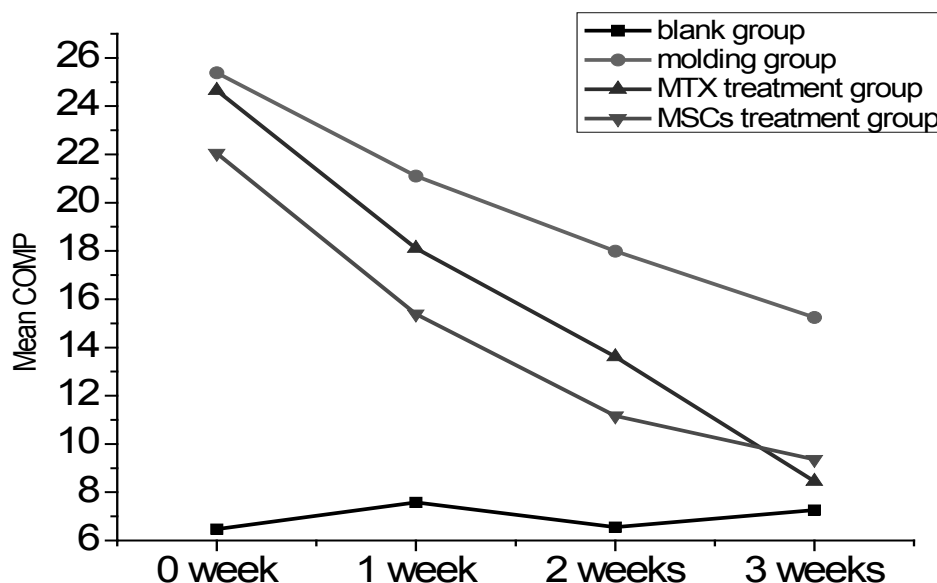


Fig. 4. Serum COMP levels in different groups.

DISCUSSION

Human umbilical-cord-derived mesenchymal stem cells (hUC-MSCs) constitute an attractive alternative to bone-marrow-derived MSCs for potential clinical applications because of easy preparation and lower risk of viral contamination (8). Study has shown that hUC-MSCs have the capability to differentiate into epithelial-like cells that are normally derived from the endoderm, implicating their potential applications in tissue repair and regeneration of many endoderm-derived internal organs (9). In this study, there was a significant positive correlation between the severity of CIA and the level of COMP in the synovial fluid, suggesting that COMP was associated with synovial invasion of the disease.

The hUC-MSCs could differentiate into cells with mature adipocyte structure and functions. In fact, hUC-MSCs not only differentiate into osteocytes and express osteopontin, sialin, osteoproteins and osteocalcin, but also form a globular bone needle with a diameter of 1-2 mm, similar to that of articular cartilage, on a substrate of polonium (11). Early treatment by hUC-MSCs can inhibit cartilage destruction and reduce the increase in COMP levels (12). Therefore, hUC-MSCs provide new hope for the treatment of destructive joint diseases (13). In this study, hUC-MSCs transplantation was used to treat CIA rats.

In summary, it was concluded that MSC transplantation relieved the symptoms of arthritis by down-regulating the expression of COMP on the synovial membrane and in the serum of CIA rats.

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LETTER TO THE EDITOR

EFFECTS OF HOUSEHOLD BLEACH ON SPUTUM SMEAR MICROSCOPY TO CONCENTRATE ACID FAST BACILLI FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS

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Received January 19, 2018 – Accepted March 22, 2018

Tuberculosis (TB) is one of the major public health problem among contagious diseases in Pakistan. TB diagnosis mainly depends on sputum smear microscopy. The main objective of this study was to evaluate the effects of household bleach on sputum smear microscopy to concentrate acid fast bacilli for the diagnosis of pulmonary tuberculosis. Sputum specimens of 200 suspected TB patients were collected for the study. Smears were prepared from the purulent part of sputum sample before and after bleach treatment, heat fixed and stained with the ZN technique. The obtained data were analyzed by chi-squared test using SPSS software. Out of 200 isolates, 22 (11%) patients had positive smears for acid fast bacilli (AFB) by direct ZN staining. After treatment with household bleach (NaOCL) and centrifugation, the number of AFB positive patients were increased from 22 (11%) to 37 (18.5%). The bleach-concentration method for sputum samples significantly increased the TB detection rate as compared to direct sputum smear microscopy. Thus, a shift from direct sputum microscopy to bleach-concentration technique should be considered a better method for detection of AFB in sputum through smear microscopy.

To the Editor,

Mycobacterium tuberculosis (MTB) causes tuberculosis, which is a worldwide problem, especially in developing countries (1). Tuberculosis (TB) remains a global public health issue in spite of the discovery of the TB causative agent more than 100 years ago (2). It is estimated that two billion people are infected with tubercle bacilli throughout the world (3). World Health Organization (WHO) reports indicated that the highest percentages of global TB patients are located in Asia (55%), Africa (30%), eastern Mediterranean region (7%), Europe

(4%), and in the Americas (3%) (4). In Pakistan, TB is a major community health problem. Pakistan is fifth among 30 highest TB burden countries in the world (5). MTB is transmitted via small airborne droplet nuclei produced by coughing and sneezing. Immunocompromised individuals are more prone to develop TB infection (6, 7).

Diagnosis of TB depends on clinical history, sputum smear microscopy, MTB culture, chest X-ray and PCR (8). The MTB culture method is the gold standard for MTB detection but takes longer due to the slow growth of bacilli. PCR-based methods are

Key words: sodium hypochlorite, sputum, *M. tuberculosis*

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0393-974X (2018)

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much more sensitive but are costly to be routinely used in developing countries (9). Thus, sputum smear microscopy remains the cornerstone of TB diagnosis for most laboratories in high endemic areas (8). Direct microscopy has high specificity but low sensitivity. The sputum concentration method using various liquefaction agents, such as sodium hypochlorite (NaOCl), has shown to improve the sensitivity of direct microscopy (10). Previous studies have shown that NaOCl digestion of sputum gives the best recovery of acid fast bacilli (AFB) in combination with concentration by centrifugation. Furthermore, NaOCl as disinfectant has the advantage of lower risk of infection to laboratory workers (11). The aim of this study was to investigate the usefulness of an alternative method for increased detection of AFB by concentration and digestion through sodium hypochlorite.

MATERIALS AND METHODS

Before specimen collection, the patients were informed about the nature of the study and gave written consent. All the study subjects were asked to submit two samples for two days, the first sample immediately and the second early next morning. Samples were collected in

wide necked, leak proof, sterile containers and properly labeled. The patients were requested to cough deeply to produce a sputum specimen avoiding saliva. All the procedures in the current study were conducted according to the guidelines according to the institutional research scrutiny/bioethics committee.

All samples were sent to the microbiology laboratory of the FMH College of Medicine & Dentistry, Lahore, and processed within 24 hours. A proforma was used to record all required data which included patient history, gender and age etc. Direct smears were prepared from the purulent part of the sputum, air dried, heat fixed and stained with the Ziehl-Neelson (ZN) method (12). The remaining specimen was digested by adding an equal volume of locally obtained household bleach (5% NaOCl). This bleach-filled container was left on the laboratory bench for half to one hour and then centrifuged for 15 min at 3000 rpm. Supernatant was discarded, smears were made from sediments and then stained using the hot ZN staining technique (13). Acid fast bacilli appeared red, straight or slightly curved rods, at times with a beaded appearance occurring singly or in small groups. After ZN staining, the smears were examined under microscope using oil immersion lens (100 x objective) and the smears were reported AFB negative when no AFB was seen in 100 microscopic fields. The positive smears were graded as 3+

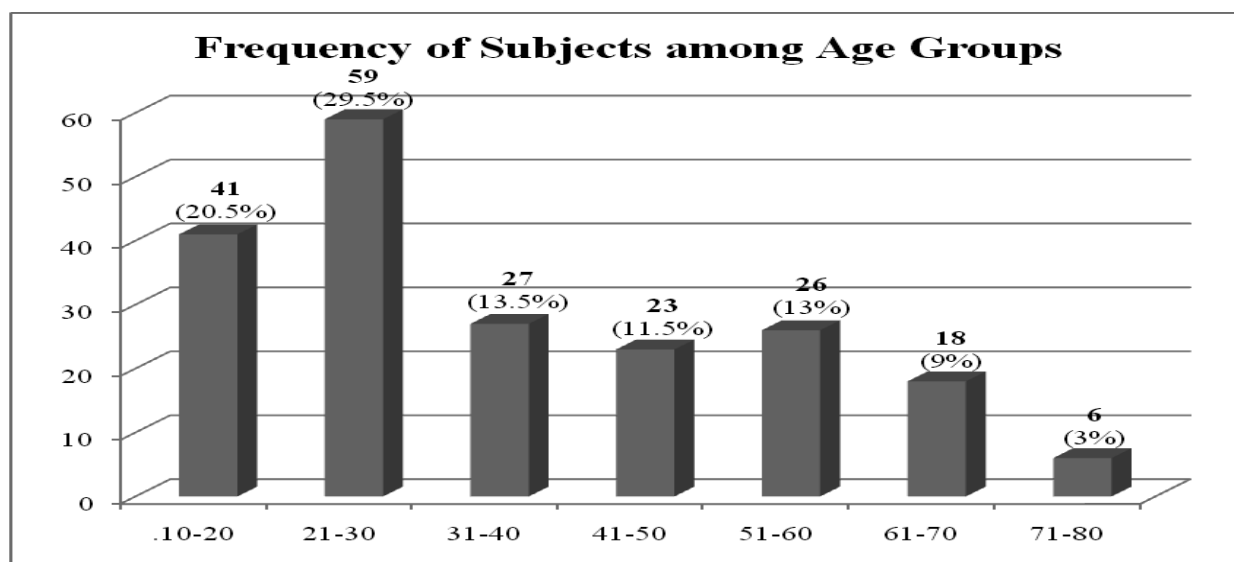


Fig. 1. Frequency percentage of study subjects among age groups.

(more than 10 AFB per field), 2+ (1-10 AFB per field), 1+ (10-100 AFB per 100 fields) and scanty or exact number (1-9 AFB per 100 fields). The obtained results were analyzed for statistical significance by applying the *chi*-squared test using the SPSS version 16.0 to compare the frequencies of both direct and concentration techniques.

RESULTS

In this study, 200 TB-suspected patients were enrolled, of whom 107 (53%) were male and 93 (47%) female. Considering the area wise distribution, 78 (39%) patients were from urban areas and 122 (61%) from rural areas. All the patients were over 10 years of age, but most of them were young adults of aged 21-30 years (29.5%) (Fig. 1). By using the direct Ziehl-Neelsen (ZN) staining, 22 (11%) patients had AFB positive smears, while 178 (89%) had AFB negative smear. Nevertheless, bleach (NaOCl) treatment increased the number of AFB positive smears from 22 (11%) to 37 (18.5%) and AFB negative smears reduced to 163 (81.5%). By using household bleach (NaOCl) treatment ZN staining, out of 37 positive cases, 22 were males and 15 were females. All the smears positive for AFB by

direct ZN method were confirmed positive by the bleach digestion method. However, 15 AFB negative samples by direct ZN method were found positive by the bleach concentration method (Fig. 2).

No AFB was seen in 178 (89%) samples by the direct method while 22 (12.5%) were positive by this method. In the smears prepared by direct method, 7 (4%) were scanty, 6 (3%) were 1+, 7 (3.5%) were 2+ and 2 (1%) samples were 3+. On the other hand, a significant increase in the number of AFB positive samples was found after household bleach treatment. Therefore, 15 (7.5%) false negative samples were found positive after the concentration method. In concentrated smears no AFB was seen in 163 (81.5%) smears while, 37 (18.5%) were found positive for AFB. In grading the positive smears after bleach treatment, 12 (6%) were found scanty, 15 (7.5%) were 1+, 7 (3.5%) were 2+ and 3 (1.5%) were 3+ (Table I).

Significant increase in AFB number was seen per microscopic field in smears prepared from bleach digested sputum sample. Smears which were graded scanty by direct method increased to 1+; similarly, sensitivity of smears graded 1+ or 2+ were increased to 2+ or 3+, respectively, by

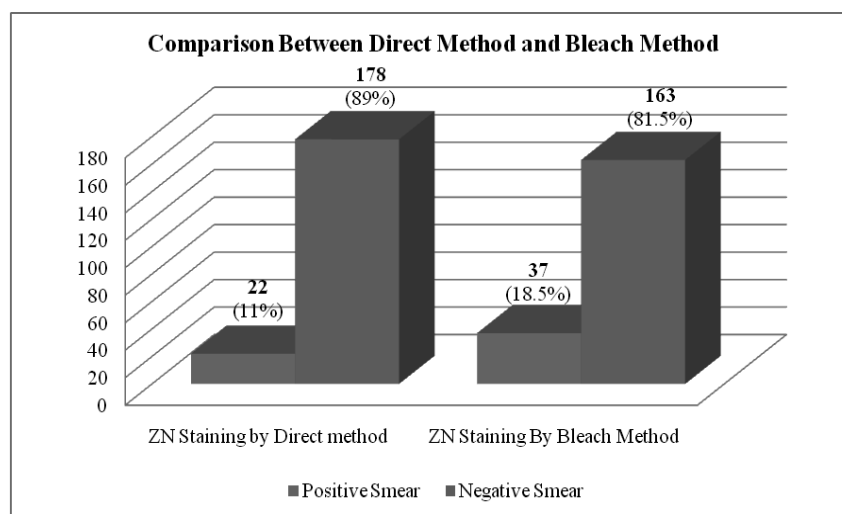


Fig. 2. Frequency percentage of sputum microscopy positive and negative TB patients comparing the direct and bleach concentration methods.

Table I. Grading distribution of ZN staining results comparing the AFB detection sensitivity of direct and bleach concentration methods.

Reporting of ZN Positive Smears	Grading of ZN smear by Direct Method	Grading of ZN smear by Bleach Method
1-9 AFB (Scanty)	7 (3.5%)	12 (6%)
1 ⁺	6 (3%)	15 (7.5%)
2 ⁺	7 (3.5%)	7 (3.5%)
3 ⁺	2 (1%)	3 (1.5%)

household bleach concentration. The microscopic background field appeared clearer as debris was reduced after bleach treatment and centrifugation. The increase in number of AFB positive smears by the bleach digestion method was significant ($p = 0.023$, $\chi^2=3.95$) by using the *chi*-squared test.

DISCUSSION

The continuously increasing TB cases are a major life threat to humans that requires rapid intervention (14). In developing countries, sputum smear microscopy is considered as rapid and the most reliable method for pulmonary TB diagnosis. A positive smear for AFB will alert the physician to possible TB infection, while a false negative smear may lead to delay in treatment and spread of the infection (15).

In the present study, the efficiency was evaluated of sputum smear microscopy through ZN staining technique for the detection of AFB before and after bleach treatment. Higher numbers of TB cases were detected on ZN-stained sputum smear under microscopy after NaOCl as compared to the direct ZN staining method. Anawag et al. in 2012 (10) observed

increased detection rate of AFB on bleach treated sputum smears. In our study, all smears positive for AFB by the direct ZN method were also positive by household bleach digestion method. Household bleach method significantly increased the number of AFB-positive samples as it was clear that 7.5% false negative sputum samples by direct ZN method were found positive after the bleach digestion method. In 2009, Srikanth et al. (16) reported a similar pattern of results in their study. There was also a marked increase in AFB seen per microscopic field in smears prepared from household bleach digested sputum samples. In another study conducted during 2013 by James (17) reported similar results to those observed in the present research.

The implementation of household bleach method clearly improves detection of cases of TB and can be a valuable tool for AFB detection in pulmonary TB suspects. In our study, after examining the bleached smears, we were able to detect MTB in 24.83% of cases which were not detected by the direct method. A similar pattern of result was reported in 2013 by Mindloi et al. (13), and Kandati et al. in 2017 (11) who conducted research studies on pulmonary tuberculosis.

In conclusion, the bleach concentration method significantly increased the sensitivity of sputum smear microscopy for the diagnosis of pulmonary tuberculosis. This method is very effective and could considerably increase the detection of smear negative pulmonary TB cases.

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LETTER TO THE EDITOR

COMPARISON OF GEFITINIB AND PLATINUM-BASED CHEMOTHERAPY AND ONLY PLATINUM-BASED CHEMOTHERAPY TO TREAT LUNG ADENOCARCINOMA

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Received January 24, 2018 – Accepted March 14, 2018

To study the curative effects and safety for patients who adopt both gefitinib and platinum-based chemotherapy or only platinum-based chemotherapy in the treatment of lung adenocarcinoma, 80 EGFR mutation-positive lung adenocarcinoma patients in stage IIIB/IV were divided into two groups. Half of them received both gefitinib and standard chemotherapy (group A), and the others (group B) received only standard chemotherapy. Overall response rate (ORR), disease control rate (DCR), progression-free survival (PFS), overall survival (OS) and the related toxicities of both groups were recorded in order to take certain nursing measures for a variety of toxicities. Next, statistical methods were used to analyze the curative effects and safety of the two treatments. The results showed that ORR, DCR and median progression-free (mPFS) survival of the two groups of patients showed no statistical difference ($P>0.05$). However, group A (18.56 months) had a longer median overall survival (mOS) than group B (14.87 months), which was of statistical significance ($P<0.05$). Nausea and loss of appetite were common mild adverse reactions, and anemia and leukocytopenia were moderate common adverse reactions. The difference between these two groups of patients regarding adverse reactions was not statistically significant ($P>0.05$). In conclusion, the two treatments have similar safety, but lung adenocarcinoma patients with drug resistance during stage IIIB/IV after using first-line gefitinib therapy have lower survival benefits than patients who take both gefitinib and platinum-based chemotherapy.

To the Editor,

Clinically, most lung adenocarcinoma patients are in the late stage when diagnosed, and the effective rate of chemotherapy for them is as low as 20-50%. The application of molecular-targeted drugs is limited by the development of drug resistance (1, 2). If late stage lung adenocarcinoma patients adopt chemotherapy,

the best median survival time (MST) is 4-5 months, but the one-year survival rate is lower than 10% (3). Currently, Pemetrexed combined with platinum is the first-line chemotherapy for lung adenocarcinoma patients, but treatment with platinum has a greater toxic effect (4). At present, molecular-targeted treatment of lung adenocarcinoma mainly includes

Key words: lung adenocarcinoma, gefitinib, platinum-based chemotherapy, epidermal growth factor, adverse reaction

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0393-974X (2018)

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therapy targeting the epidermal growth factor receptor (EGFR) and angiogenesis (5, 6). As EGFR protein tyrosine kinases (EGFR-TKIs), gefitinib is a composite micromolecularquinazoline derivative that can inhibit multiplication and metastasis of tumor cells and speed up cell apoptosis (7).

Research has found that, as a first-line treatment, EGFR-TKIs have a higher objective response rate and PFS for the treatment EGFR mutation-positive patients with non-small-cell lung cancer than for mutation-negative patients (8, 9). However, most patients will have acquired drug resistance after long-term drug therapy (10). Moreover, follow-up therapy should revert to existing drugs and methods after drug resistance. There are two main treatments: EGFR-TKIs combined with standard chemotherapy, and only standard chemotherapy. The main purpose of this research aims to study the curative effects and safety of the two treatments.

MATERIALS AND METHODS

One hundred forty-two EGFR mutation-positive patients with lung adenocarcinoma were selected who were treated in the Affiliated HongQi Hospital of Mudanjiang Medical University from August 1, 2014 to March 1, 2017. Of these, 107 patients received giftinib therapy and their disease progressed after drug resistance. Eighty patients met the inclusion criteria with intact follow-up data. Of the 80 patients, there were 48 males 32 females aged 34-79, and their ECOG score was 0-1. All of the selected patients received standard chemotherapy after progressive disease. And according to whether they continued to take gefitinib orally, they were divided into group A (gefitinib combined with standard chemotherapy) and group B (only standard chemotherapy). Before collecting cases, the research process and potential risks were explained to patients and their families. Informed consent rights of patients' families were fully respected, and their willingly-signed informed consent forms are stored with their case history. After discussion with the medical ethics committee of the hospital, all of the patients included decided to enroll in a clinical trial at the Affiliated HongQi Hospital of Mudanjiang Medical University. In addition, although there are some common mutations in the study, such as T790M mutation, the

mechanism of drug resistance through tissue specimens is not implemented in this experiment.

Inclusion criteria: lung adenocarcinoma patients were proved as EGFR mutation-positive by cytology and histopathology; the patients were in stage IIIB-IV per WHO; there was no less than one detectable lesion through CT inspection; ECOG score was 0-1; organ functions were basically normal through the evaluations including blood routine, liver and kidney functions and electrocardiography.

Exclusion criteria: patients who had suffered from interstitial lung disease (ILD) or other malignant tumors in their past history; patients who had received cytotoxicity, molecularly targeted or immunotherapy before gefitinib; ECOG score was greater than 1 or the patients could not tolerate treatment for other reasons.

Main drugs

Pemetrexed (Jiangsu Hansoh Pharmaceutical Group Co., Ltd, State Medical Permitment No.H20093996, 0.5g/bottle); cisplatin (Yunnan Biovalley Impatiens Balsamina Pharmaceutical Co., Ltd, State Medical Permitment No.H20043889, 2ml: 10mg); gefitinib (Britain AstraZeneca KK, Imported Medical Permitment No.H20090759, 0.25g/pc).

Therapeutic methods

Chemotherapy regimens that the patients received were standard: 500 mg / m² pemetrexed was injected iv on the first day; 75 mg / m² cisplatin was transfused on the second day; every three weeks was set as a cycle. Method for administering gefitinib: 250 mg gefitinib was taken orally once a day, and every three weeks was set as a cycle. All patients received standard chemotherapy for 2-6 cycles depending on their condition.

Nursing measures

Related nursing for adverse cutaneous reactions: if patients had itchy or dry skin, nurses were able to apply diphenhydramine ointment locally in order to avoid infection resulting from damage to the skin. Rashes were treated by dexamethasone ointment, hydrocortisone or erythromycin ointment, depending on the patient's condition. For severe rash, the dose of drugs was decreased, and appropriate antibiotics were used for concurrent infection.

Psychological counseling: because some patients were concerned for their lives, they were depressed, therefore psychological counseling was given to help alleviate their tension and despair. Medical workers' attitude, communication between doctors and nurses and patients, and therapeutic effects also affect the patients' mental health. Moreover, chemotherapy has many severe toxicities or adverse therapeutic reactions, including nausea, emesis and alopecia. These reactions can serve to aggravate patients' stressful emotional states, as well as influence the curative effects and quality of life of patients with lung cancer. Therefore, medical workers gave special attention to using therapeutic and comforting words in order to create a comfortable environment.

Health education: general knowledge about lung cancer, considerations of therapy and balanced diet were explained to patients. A good diet nursing can effectively improve the nutrition of lung cancer patients and their tolerance to toxicities of chemotherapy drugs.

Follow-up visits

Two weeks before the start of chemotherapy, CT scanning was taken as limited standard to evaluate disease severity. From the start of chemotherapy onwards, a laboratory examination and CT scan of the chest were conducted every two weeks. Response evaluation criteria in solid tumors were used to evaluate the patients' conditions. After the last round of chemotherapy, laboratory examinations and CT chest scans were carried out every two months, and follow-up visits for patients continued until their death or June 1, 2017. During this period, patients could undergo head MRI and whole-body bone imaging, depending on their physical conditions, which were taken as a comprehensive assessment.

Clinical assessment criteria

This research took two weeks of examinations for baseline criteria before grouping patients. Clinical effects were evaluated using the response evaluation criteria in solid tumors (RECIST) that were put forward by the US National Cancer Institute (11). ORR refers to the percentages of complete remission (CR) and partial remission (PR) of total evaluable cases after therapy; DCR refers to the percentages of CR+PR and stable disease (SD) of total evaluable cases after therapy; PFS refers to the time from the beginning of therapy to

secondary growth of tumor; OS is the duration from the beginning of randomization to the time of death from any cause. The main indexes for curative effects in this study were mPFS and mOS, and subordinated indexes included ORR and DCR. Adverse reactions were classified in 0-IV in accordance with the adverse anti-cancer drug reactions of the WHO.

Statistical analysis

All the data were statistically analyzed by SPSS 19.0 software. Curative effects and safety between subgroups were compared by *chi*-squared test; the Kaplan-Meier method was adopted to analyze survival time; and log-rank was used to make a significance test for the differences of survivorship curves. This study took $\alpha=0.05$ as test criteria, and the difference is of statistical significance when $P<0.05$.

RESULTS

Basic information of patients

Through the statistics of clinical data, the basic information (age, gender), ECOG score, smoking history, clinical stages and brain metastases) of the two groups had no statistically significant difference ($P>0.05$). In both EGFR mutation of groups, deficiency of 19 expressed region (Group A: 28 cases; and Group B: 25 cases) and L858R mutation (Group A: 12 cases; and Group B: 15 cases) also had no statistically significant difference $P=0.058$.

Evaluation of short-term curative effects

The median follow-up time was 13.2 months (ranging from 6 to 23 months). The median chemotherapy time for group A was 115.5 days (ranging from 80 to 129 days); and for group B was 123.5 days (ranging from 47 to 134.5 days). The median gefitinib therapy time for group A was 151 days (ranging from 93 to 217.5 days). The median number of chemotherapy cycles for the two groups was 5 (group A: 3-6 cycles; group B: 2-6 cycles).

Moreover, ORR (Group A: 11 cases; and Group B: 13 cases) difference comparison of the two groups showed no statistically significant difference ($\chi^2=0.265$, $P=0.761$); nor did their DCR (Group A is 34 cases and Group B is 32 cases) comparison

($\chi^2=0.283$, $P=0.734$). This showed that two treatments had similar curative effects in the short term.

PFS analysis

mPFS was 4.83 months for group A (95%CI, 3.24-5.74 months), and was 4.78 months for group B (95%CI, 3.05-6.14months). The difference in mPFS between the two groups was not statistically significant ($\chi^2=0.852$, $P=0.41$) (Fig. 1).

OS analysis

The 18.56-month mOS in group A (95%CI, 15.45-22.10 months) was longer than the 14.87-month mOS in the standard chemotherapy group (95%CI, 12.2-17.35 months), and the difference was statistically significant ($\chi^2=3.871$, $P=0.048$) (Fig. 2).

Toxicity

As seen in Table I, common toxicities in the two groups had no significant differences, mainly focusing on I-II. Of these, the occurrence rate for diarrhea, stomatitis, emesis and anemia was higher in group A than in group B. The two groups had no patients who died of severe adverse reactions.

DISCUSSION

The results of this study show that the two treatments have similar curative effects in the short term (from the beginning of the treatment to the end of the follow-up).

From this data, patients in group A had longer OS than those of group B ($P<0.05$). Moreover, during the therapy or a month after the final round of chemotherapy, the two groups had similar death rates. Therefore, after progressive disease, patients still may obtain benefits from gefitinib. In addition, mOS of patients in group A was longer than group B. However, analysis of mOS remains inaccurate. On one hand, the patients in group B had more brain metastases than group A, and lung adenocarcinoma patients with EGFR mutation-positive brain metastases had shorter median survival times than patients without brain metastases after first-line EGFR TKIs therapy (12); on the other hand, during the follow-up period, group A accepted more EGFR TKIs, combined dual-drug plans, local radiotherapy and other active treatments.-

Gefitinib combined with cisplatin + pemetrexed share the same tolerance and safety of other known

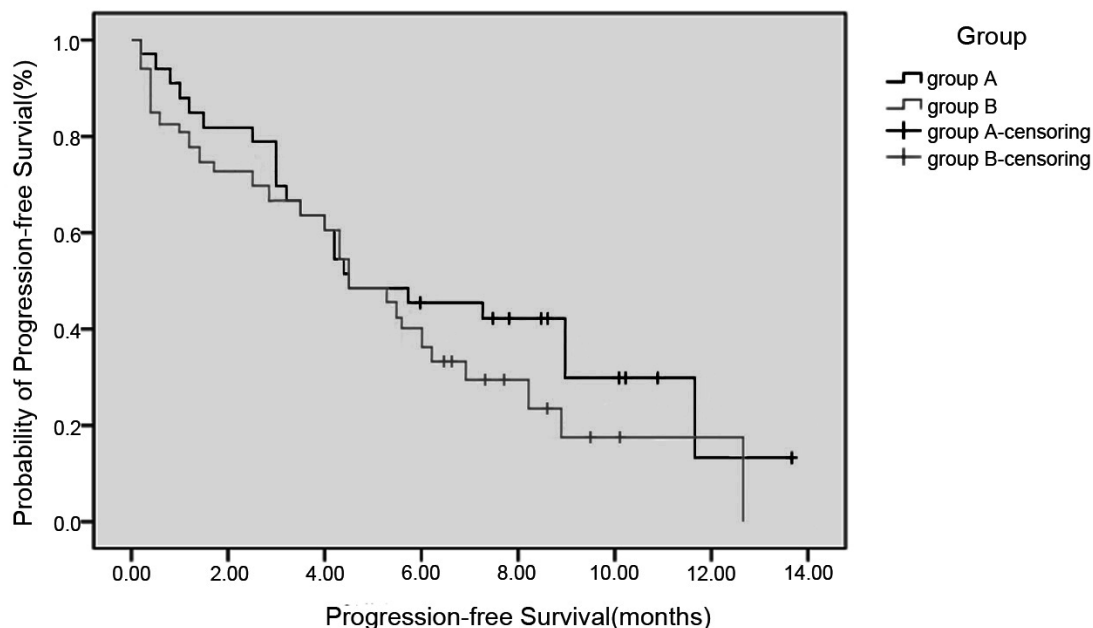
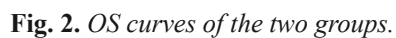


Fig. 1. PFS curves of the two groups.

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treatment plans. Occurrence rates of rashes between patients receiving each treatment are similar, and are much lower than in patients who first accept gefitinib therapy (13, 14). The occurrence rates of certain adverse reactions related to chemotherapy in group A was higher than in group B ($P>0.05$). It can thus be inferred that these two methods of treatments have similar safety.

In conclusion, lung adenocarcinoma patients with drug resistance after first-line gefitinib therapy during III/IV stage have lower survival rates than patients who take gefitinib combined with platinum-based chemotherapy.

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LETTER TO THE EDITOR

ANTI-TUMOR MECHANISM OF IL-21 USED ALONE AND IN COMBINATION WITH 5-FLUOROURACIL *IN VITRO* ON HUMAN GASTRIC CANCER CELLS

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To study the effect and related mechanism of IL-21 alone and in combination with 5-Fluorouracil on the proliferation and growth, transferability, and apoptosis of gastric cancer cells, we cultivated gastric cancer cell SGC-7901 and created four experimental groups with varying concentrations of IL-21 and 5-Fluorouracil: IL-21 group (IL-21 100ng/ml), semi-combination group (5-Fluorouracil 25µg/ml+IL-21 100ng/ml), 5-Fluorouracil group (5-Fluorouracil 50µg/ml), and combination group (5-Fluorouracil 50µg/ml+IL-21 100ng/ml). The MTT (3-(4, 5)-dimethylthiazolyl-2-yl)-5-phenyltetrazolium bromide assay was used to detect the inhibitory effect of each group on the proliferation and growth of gastric cancer cells. A scratch-wound assay was carried out to detect the inhibitory effect of each group on transferability. TUNEL assay was used to detect the effect of each group on the apoptosis of the gastric cancer cells, and Western blot was used to detect the expression of caspase-3, caspase-8, bcl-2, and c-myc, which are the proteins related to apoptosis, after the drug effect in each group. The results show that, compared to the 5-Fluorouracil group, the inhibitory effects after 24 h of the IL-21 group and the semi-combination group on SGC-7901 were weaker ($P<0.001$). However, if the effect lasted 48 h, then the inhibition in the semi-combination group had no significant difference compared to the 5-Fluorouracil group ($P>0.05$). The scarification test showed that all groups could inhibit the transferability of SGC-7901 and that the effect increased successively from the IL-21 group, the semi-combination group, the 5-Fluorouracil group, to the combination group. The TUNEL assay indicated that all groups could promote the apoptosis of SGC-7901. The percentage of cell apoptosis increased, and the Western blot showed that the expression of caspase-3, caspase-8, and c-myc, respectively, in the semi-combination group, the 5-Fluorouracil group, and the combination group increased successively and that the successive increasing of c-myc was the most significant. The expression of bcl-2 tended to decrease. In conclusion, IL-21 used alone and in combination with 5-Fluorouracil are anti-tumor mechanisms in SGC-7901.

To the Editor,

IL-21 is a newly discovered cell factor that can regulate the immune function of an organism. *In vivo* and *in vitro* experiments have demonstrated that when IL-21 is used alone, it has a definite anti-tumor effect (1-2), but its effect mechanism is unclear. The current animal models and clinical

experiments show that the use of IL-21 is safe and reliable with few side effects (3-5). Currently, there are multiple chemotherapeutic drugs, among which 5-Fluorouracil is a standard treatment for malignant tumors related to the digestive system (6). The effect rate of 5-Fluorouracil used alone in treating gastric cancer is 20% (7, 8). Studies have

Key words: IL-21, 5-Fluorouracil, gastric cancer cell, anti-tumor mechanisms

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0393-974X (2018)

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shown that if two or three drugs are combined, the effect can never surpass the effect produced by using 5-Fluorouracil alone (9-11). Despite 5-Fluorouracil being the standard chemotherapeutic for tumors in the digestive system, its overall treatment effect is limited due to patients' resistance to it. During treatment with 5-Fluorouracil, many patients experience nausea, vomiting, diarrhea, leukocyte and thrombocytopenia, mucositis, dermatitis, and other related adverse reactions. Some individual cases have resulted in fatal gastrointestinal reactions and myelo-suppression. Given that 5-Fluorouracil has such a low effect rate, we studied the influence of IL-21 used alone and in combination with 5-Fluorouracil to determine their influence on the proliferation and growth, transferability, and apoptosis of SGC-7901 and related mechanisms, as well as to evaluate the IL-21 combination for treating gastric cancers.

MATERIALS AND METHODS

Materials

Human gastric cancer cells SGC-7901 were purchased from Shanghai Zeye Biotech Co., Ltd. The main reagents were: IL-21 (Shanghai Kexin Biotech Co., Ltd.); 5-Fluorouracil (Hainan Zhonghua Lianhe Pharmaceutical Industry Co., Ltd.); RPMI1640 culture medium (Nanjing Bianzhen Biotech Co., Ltd.); FCS (Langfang Pengcai Fine Chemical Co., Ltd.); 0.25% trypsin (Trypsin-EDTA, Shanghai Danniyue Biotech Co., Ltd.); double-antibody (mycillin, Beijing Saiyintan Science Co., Ltd.); glutamine (Shanxi Scipher Natural Product Co., Ltd.); TUNEL test kits (Roche); total protein extraction kits (Beijing Solarbio Co., Ltd.); rabbit caspase-3 monoclonal antibody, rabbit caspase-8 monoclonal antibody, rabbit bcl-2 monoclonal antibody, and rabbit c-myc monoclonal antibody (Abiocenter Beijing Biotech Co., Ltd.); and developing and fixing kits (Shanghai Huzhen Biotech Co., Ltd.).

Instruments

An inverted microscope [Techlod Precision Instrument (Shenzhen) Co., Ltd.]; a microplate reader (Shandong Biobase Industry Co., Ltd. a CO₂ incubator (Shanghai Shengke Instrument Co., Ltd.);

high-speed centrifuge, refrigerated centrifuge, and ordinary centrifuge (Taizhou Tongyang Washer Manufacturing Co., Ltd.); an electronic balance with type JA2003N (Shanghai Pingxuan Scientific Instrument Co., Ltd.); horizontal gel electrophoresis system (Jinan Xinbeixi Biotech Co., Ltd.); E-Gel Imager (Shanghai Shangtian Precision Instrument Co., Ltd.); and ELISA Reader (96 tiny hole board, Wuxi Hua Lang Weide Instrument Co. Ltd.).

MTT assay

The sub-culturing of cells was carried out first, with SGC-7901 cells reaching the logarithmic phase. The number of cells was counted under the microscope, and the cell concentration was adjusted to $5 \sim 10 \times 10^4/\text{mL}$ using the culture medium. The appropriate inoculum concentration of tumor cells was used to fill the marginal pores of the board with 5000-8000 faces with sterile PBS. After the cell suspension was mixed, it was used to fill the board. To each pore, 100 μL cell suspension was added to maintain cell density of 5000-10000 per pore. Thirty-two pores were left around the board, with 96 pores remaining blank, to create the blank control group without added cells. The inoculated culture board was hatched in the incubator at a temperature of 37°C and 5% CO₂ until the cell monolayer paved all pores. The drugs were injected at the bottom of the 96 pores. In each group (the IL-21 group, semi-combination group, 5-Fluorouracil group, and the combination group), each pore was 100 μL , and there were 5 compound pores in every group. A zero-adjusting pore (zero-adjusting, MTT, and dimethyl sulfoxide) and control pore (cell, drug dissolution medium of the same concentration, inoculum, and inoculum and dimethyl sulfoxide) were also set. The termination of the culture was between 24 h and 48 h. The MTT solution (5mg/mL, namely 0.5% MTT) was made during the experiment. To each pore, 20 μL MTT solution was added, and the culture was timed for 4 h. When the culture period ended, the 96-pore board was removed, and the culture medium in each pore was carefully extracted, to which dimethyl sulfoxide was added and 100 μL were injected into the pores. Then, the board was oscillated at low speed so that the crystals would fully dissolve. The ELISA

Reader detected the absorbance (A) of all pores at OD 490 nm. The cell inhibition rate was calculated according to the formula: cell inhibition rate = $(A_{CG} - A_{EG})/A_{CG} * 100\%$.

Wound scratch assay

SGC-7901 cells at logarithmic phase were chosen to culture, and the number of cells was counted under an inverted microscope. The cell concentration was adjusted to $2 \sim 3 \times 10^6/\text{mL}$ by using the culture medium. One mL cell was used to pave the board with 6 pores, and 2 mL inoculum containing 10% RMPI1640 were added into each pore. The cell was cultured for 24 h to form a monolayer tumor cell. A 10 uL gun tip was used to scratch lines, which were then washed three times with a PBS buffer solution. The cultures were observed under the inverted microscope and photographed. After that, the following drugs in each group were injected: IL-21 group (IL-21 100ng/mL), semi-combination group (5-Fluorouracil 25ug/mL+IL-21 100ng/mL), 5-Fluorouracil group (5-Fluorouracil 50ug/mL), and the combination group (5-Fluorouracil 50ug/mL+IL-21 100ng/mL). Two parallel samples were chosen in each group, and the culture process continued for 24 h and 48 h. The inoculum was extracted three times: at the beginning of continuous culture, after 24 h and after 48 h. PBS buffer solution was used on all groups, and the cell transference was observed and photographed. The distance of the scratch edges was processed at different times using ImageJ and compared between the different groups [the distance: migration distance (nh) = edge distance (0h) - edge distance (nh)].

TUNEL assay

The cultured cells perfused with the treatment drugs were collected, the culture medium removed, and the cells washed 2 to 3 times with PBS before removing as much PBS solution as possible. The cells later were fixed 30 to 60 min with 4% paraformaldehyde and washed with PBS once or twice. Next, cell-penetrating fluid was applied to the cells, and PBS was used again for washing once or twice. The cells were then bathed in 4% paraformaldehyde for 5 min and then washed with

PBS once or twice. Next, 100 uL equilibrium mixture was added to the cells, which were then put in a wet box 10 min. Then, 100 uL TUNEL mixture solution was applied to the samples, which were put in a dark wet box for reaction with a cover glass or parafilm at the temperature of 37°C for 1 h. The reaction terminated when samples were immersed in $2 \times \text{SSC}$ for 15 min, and PBS was used for washing them 2 to 3 times. The samples were immersed in 0.3% H₂O₂ for 15 min to cover the POD. The PBS was used for washing, and the enzyme-labeling reaction was promoted by adding 100 uL HRP (diluted by PBS with 1:500) tagged by streptavidin lasting 30 min. Then, 100 uL DAB intermixture (50 uL DAB+50 uL DAB substrate buffer+50 uL H₂O₂+950 uL distilled water for three times) was added and colored for 10 min. When it was observed under the microscope that the background had turned to light brown, ionized water was used to wash the samples several times. Samples were re-dyed by hematoxylin for 3 s and running water was used to wash them. They were dehydrated using graded alcohol, including 50%, 70%, 85%, 95%, and 100% for one min each. Dimethylbenzene transparency was carried out twice, lasting one min each time. The last step was mounting, which was done by neutralbalsam. The apoptosis was observed by light microscopy and photographed.

Western blot

The total protein was extracted and its concentration detected in sample cells using the BCA method. Then SDS-PAGE glue was made, followed by pre-running. The glue channel was dredged, and the denaturation and electrophoresis of the protein samples were carried out. After electrophoresis, the device was removed and the gel transmembrane carried out. Next, the membrane was taken out and placed in a plate containing sealing fluid. The primary antibody (diluted to 1; 1000 by TBST kept in a fresh-keeping bag) and secondary antibody (diluted to 1; 2500 by TBST kept in a fresh-keeping bag) were adopted to make an immunological reaction, during which the reaction time was fixed. When washing the membrane, the primary and secondary antibodies were discarded, and a $1 \times$ developing and fixing

solution used. The fixing solution was washed away, and the film was restored to the normal temperature for drying. Gel electrophoresis was used to analyze the target strips.

Statistical analysis

Data in the study were expressed by mean $\pm$ standard deviation ($\bar{x} \pm s$). SPSS19.0 software was used to perform one-way ANOVA analysis, and the *t*-test determined that the difference between groups is $P < 0.05$, indicating that the difference is of statistical significance.

RESULTS

Multiplication capability and apoptosis condition

Table I shows that the inhibition rate of drugs on gastric cell SGC-7901 proliferation increased successively from the IL-21 group, the semi-combination group, the 5-Fluorouracil group, to the combination group, as results from the four experimental groups show. The rate of inhibition on proliferation in 48 h was significantly higher than

in 24 h ($P < 0.001$). Compared to the 5-Fluorouracil group, the inhibition rate of the IL-21 group and the semi-combination group was relatively low ($P < 0.001$) within 24 h, and the inhibition rate of the IL-21 group was still low with no significant difference between the semi-combination and 5-Fluorouracil groups ($P > 0.05$) within 48 h. When the experiment lasted 24 h and 48 h, the inhibition rate of the combination group was higher than that of the 5-Fluorouracil group ($P < 0.001$).

Data from Table I show that in the TUNEL experiment, the drugs used in the four groups can promote the apoptosis of SGC-7901. The percentage of apoptotic cells increased successively from the IL-21 group, the semi-combination group, the 5-Fluorouracil group, to the combination group. At 48 h, the percentage of apoptotic cells compared to that in 24 h is of statistical difference ($P < 0.001$). Compared to the 5-Fluorouracil group, the percentage of apoptotic cells in the combination group notably increased from 24 h to 48 h ($P < 0.001$); and compared to the 5-Fluorouracil group, the percentage of apoptotic cells in the semi-combination group was

Table I. Growth inhibition reaction and apoptosis data ($\bar{x} \pm s$).

Group	IL-21 group	Semi-combina-tion group	5-Fluorouracil group	Combination group
24-h inhibition rate (%)	19.32 $\pm$ 0.69**	39.46 $\pm$ 0.51**	50.26 $\pm$ 0.31	64.68 $\pm$ 0.49**
48-h inhibition rate (%)	33.36 $\pm$ 0.60**aa	59.28 $\pm$ 0.81 ^{aa}	60.38 $\pm$ 0.72 ^{aa}	76.33 $\pm$ 0.68**aa
24-h apoptotic cells (%)	19.33 $\pm$ 0.78 ^{##}	35.80 $\pm$ 0.92 ^{##}	46.54 $\pm$ 1.24	63.09 $\pm$ 1.52 ^{##}
48-h apoptotic cells (%)	29.24 $\pm$ 0.83 ^{##cc}	44.96 $\pm$ 1.21 ^{##cc}	51.13 $\pm$ 1.23 ^{cc}	71.35 $\pm$ 2.35 ^{##cc}

** indicates that compared to 5-Fluorouracil, $P < 0.001$ in each group, and aa indicates that $P < 0.001$ when comparison were made at 48 and 24 h. # $P < 0.05$, compared to the 5-Fluorouracil group; ^{##} $P < 0.001$, compared to the 5-Fluorouracil group; ^{cc} means at 48 h $P < 0.001$ compared to that at 24 h.

of significant difference in 24 h ($P<0.001$). In 48 h, the percentage in both groups was of statistical significance ($P<0.05$).

Transferability

Table II and Fig. 1 show that in the scratch-wound assay, the drugs in the four experimental groups all had inhibitory effect on the transferability of SGC-7901 and that the transfer distance decreased successively from the IL-21 group, the semi-combination group, the 5-Fluorouracil group, to the combination group. At the same time, the transfer distance in the four groups was of statistical difference compared to the blank control group ($P<0.001$); compared to the combination group, the inhibitory effect of the 5-Fluorouracil group clearly strengthened as the

duration increased from 24 h to 48 h ($P<0.001$). Compared to the 5-Fluorouracil group, the transfer distance was of significant difference ($P<0.001$) after 24 h. After 48 h, the transfer distance of the two groups was still of statistical significance ($P<0.05$).

Protein expression related to apoptosis

Table III shows that the expression of protein caspase-3, caspase-8, and c-myc of the semi-combination group, the 5-Fluorouracil group, and the combination group tended to increase ($P<0.001$), respectively, and the increasing tendency of c-myc was the most obvious; the expression of protein bcl-2 tended to decrease by degrees ($P<0.001$). In the same group, the rate of detected protein and internal reference at 48 h was of statistical difference

Table II. Transfer inhibition data ($\bar{x} \pm s$).

Group	Blank control group	IL-21 group	Semi-combination group	5-Fluorouracil group	Combination group
24-h transfer distance (um)	36.48±0.61	25.12±1.43 ^{@@bb}	21.45±0.82 ^{@@bb}	15.54±0.59 ^{@@}	8.98±0.35 ^{@@bb}
48-h transfer distance (um)	58.54±0.63	38.05±1.08 ^{@@bb}	31.93±0.77 ^{@@b}	28.24±1.23 ^{@@}	16.55±0.97 ^{@@bb}

^{@@} $P<0.001$ compared to the blank control group; ^b $P<0.05$ compared to the 5-Fluorouracil group; ^{bb} $P<0.001$ compared to the 5-Fluorouracil group.

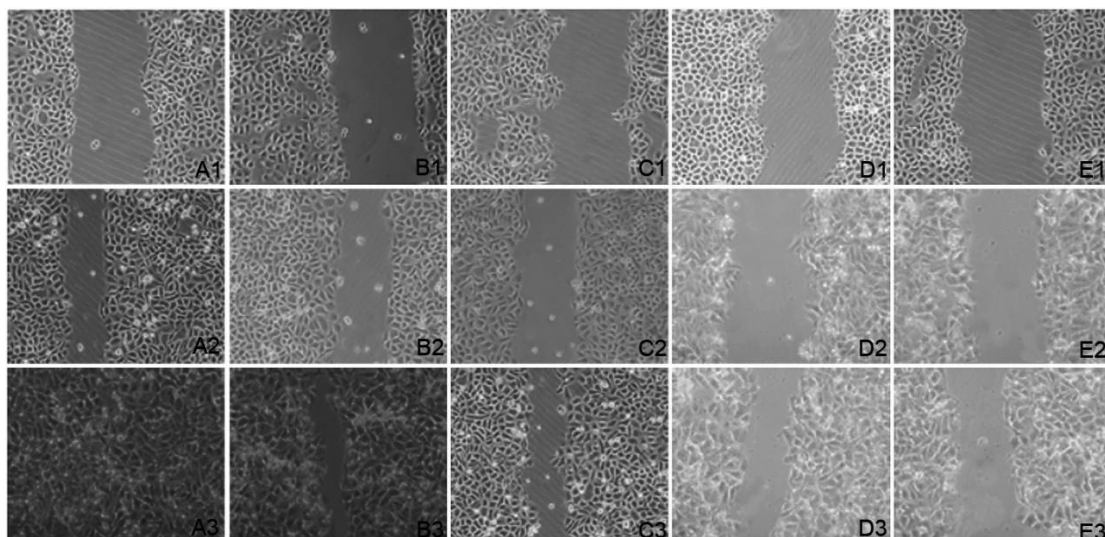


Fig. 1. Observation images of the cell transferability. 1 = 0 h, 2 = 24 h, and 3 = 48 h; **B**) IL-21 group; **C**) semi-combination group; **D**) 5-Fluorouracil group; **E**) combination group. Magnification 10×.

compared to that at 24 h ($P<0.001$). Compared to the 5-Fluorouracil group, the expression of apoptosis proteins at 24 h and 48 h in the semi-combination group was of significant difference ($P<0.001$).

DISCUSSION

Results from the MTT assay showed that IL-21 had an inhibitory effect on the hyperplasia of tumor cells *in vitro* and that the duration of treatment was significant. The scratch-wound assay supported that both the use of IL-21 alone or in combination with 5-Fluorouracil can notably inhibit the proliferation and migration of SGC-7901, but the combination was more effective. The results of the TUNEL assay indicated that all groups in the experiment could promote the apoptosis of gastric cell tumors and that the apoptotic cells increased successively from the IL-21 group, the semi-combination group, the 5-Fluorouracil group, to the combination group. Furthermore, the promoting effect of IL-21 alone on cell apoptosis was far weaker than that of 5-Fluorouracil, a standard chemotherapeutic. Thus, in our experiment, the effect of the combined used of IL-21 and 5-Fluorouracil and its mechanism were primarily explored.

To further explore how the combined effect of IL-21 and 5-Fluorouracil promotes the apoptosis of SGC-7901, Western blot was performed. Cell apoptosis is regulated by several types of genes, specifically p-53, bcl-2, and c-myc (12). Therefore, bcl-2 is the gene inhibiting cell apoptosis, c-myc is the gene promoting cell apoptosis, and the combined effect of c-myc and bcl-2 can prevent p-53 protein from entering the nucleus and further halt the apoptosis of cells induced by p-53 (13, 14). We found that the use of IL-21 and its combination with 5-Fluorouracil were effective in promoting the apoptosis of SGC-7901, and the expression of protein caspase-3 and caspase-8 related to the apoptosis of gastric cancer cells was also improved, the expression of bcl-2 was decreased, and the expression of c-myc was significantly strengthened. Compared to 5-Fluorouracil used alone, the combination of IL-21 and 5-Fluorouracil had synergistic action on the occurrence of tumor cell apoptosis. It should be noted that the significant increase of c-myc expression was likely due to the key step when IL-21 induces SGC-7901 apoptosis.

In conclusion, IL-21 has an effect on the proliferation and migration of SGC-7901 and can also promote the apoptosis of the tumor cells.

Table III. Protein expression related to apoptosis.

Group	Time	caspase-3/ internal reference	caspase-8/ internal reference	bcl-2/ internal reference	c-myc/ internal reference
Semi-combination group	24h	19.56±0.65 ^{&&}	16.79±0.74 ^{&&}	34.42±0.68 ^{&&}	24.92±0.72 ^{&&}
	48h	23.04±1.28 ^{&&dd}	22.83±1.02 ^{&&dd}	51.12±0.76 ^{&&dd}	53.45±0.91 ^{&&dd}
5-Fluorouracil group	24h	25.43±0.23	23.66±0.60	20.99±0.70	43.27±1.02
	48h	41.97±0.57 ^{dd}	38.56±1.11 ^{dd}	33.33±0.56 ^{dd}	63.62±0.86 ^{dd}
Combination group	24h	49.24±0.75 ^{&&}	48.23±0.66 ^{&&}	16.03±0.43 ^{&&}	54.73±1.81 ^{&&}
	48h	59.34±1.31 ^{&&dd}	58.12±0.76 ^{&&dd}	19.02±0.69 ^{&&dd}	79.45±0.37 ^{&&dd}

^{&&} $P<0.001$, compared with the 5-Fluorouracil group; ^{dd} in 48 h $P<0.001$, compared to that in 24 h.

When IL-21 is combined with 5-Fluorouracil, the synergistic effect is enhanced and is more effective than using 5-Fluorouracil alone. When IL-21 and 5-Fluorouracil are semi-combined, the effect on inhibiting the proliferation of tumor cells exists, but is weaker than that of 5-Fluorouracil used alone in standard chemotherapy. However, the use of 5-Fluorouracil in the proposal is reduced, which contributes to alleviating the poisonous effect of chemotherapy.

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LETTER TO THE EDITOR

EFFECT OF MANNOSE ON THE LUNG FUNCTION OF RATS WITH ACUTE PANCREATITIS

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Received February 1, 2018 – Accepted March 27, 2018

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The present study aimed to investigate the mechanisms by which mannose protects the lung injury induced in rats with acute pancreatitis (AP). An AP combined with Acute Lung Injury (ALI) model was established. A total of 90 healthy adult male Sprague-Dawley rats (300±50g weight) were randomly divided into three groups: sham operation group (SO group), severe acute pancreatitis lung injury group (SAP group), and mannose intervention group (MT group). Subsequently, each group was divided into two subgroups based on the time passed from intervention, namely 6 and 12 h. Each subgroup comprised 15 rats. The ratio of wet/dry weight of the lung tissue exhibited no significant change at different time points in the SO group. This parameter was significantly increased in the SAP group compared with the SO group at each time point of the treatment ($P < 0.05$) and it was significantly lower in the MT group than that in the AP group ($P < 0.05$) and it was significantly increased in the AP group at each time ($P < 0.05$) compared with the SO group. The levels of TNF- α in the lung tissue in the SO group exhibited no significant change at different time points, but they were significantly decreased in the MT group at each time point ($P < 0.05$) compared with the SAP group. The mannose receptor (MR) mRNA and protein levels in the lung tissues exhibited no significant change at different time points. The mRNA and protein levels of MR in the SAP group were significantly decreased at each time point ($P < 0.05$) compared with the SO group. The mRNA and protein levels of MR, in the lung tissue of the MT group were significantly increased at each time point compared with the SAP group ($P < 0.05$). Mannose could reduce the injury caused to the lung tissue of rats with severe acute pancreatitis by up-regulation of the expression of MR mRNA and protein.

To the Editor,

Severe acute pancreatitis (SAP) is one of the most common acute abdominal diseases encountered in the clinic (1). The incidence of SAP has increased with the improvement of the standard of living (2, 3). The disease exhibits complex complications,

high mortality and poor prognosis. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are the most common and most serious complications of SAP. These complications include the most important cause of SAP-related death (4-7). SAP often induces systemic inflammatory

Key words: acute pancreatitis, lung injury, mannose receptors (MR), endotoxemia, tumor necrosis factor- α (TNF- α)

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0393-974X (2018)

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response syndrome (SIRS). The lung is the main organ that is affected. Mannose derivatives have anti-inflammatory effects on peritonitis and adjuvant arthritis. However, the ability of mannose to inhibit chemotaxis and activation of PMN, thereby decreasing the inflammation in the lung, has not been fully investigated (8). Several factors affecting this process, such as the release of inflammatory mediators in macrophages and the association of mannose receptors (MR) with macrophages, are still unclear (9). Therefore, in order to investigate the mechanism by which mannose protects from lung injury, we established an acute pancreatitis(AP)-related acute lung injury (ALI) model in rats.

MATERIALS AND METHODS

Animals

A total of 90 healthy adult male Sprague-Dawley rats were provided by the Medical Animal Experimental Center of the 1st Affiliated Hospital of the Zhejiang University hospital. The mean body weight was 300±50g. The rats were kept under 12 light/12 dark/light cycle and temperatures of 18-23°C with 40-60% of humidity. The experimental protocol was approved by the Ethics Committee of the 1st Affiliated Hospital of the Zhejiang University hospital and all the procedures were conducted according to the animal protection directives.

Reagents

Sodium taurocholate and D-mannose were purchased from Sigma Corporation (USA), paraformaldehyde from Union Branch Biological Limited, L-polylysine from Huarui Innovation (China) and TNF- α EIA Kit from Union Branch Biological Limited (Russia). The rabbit anti-rat MR polyclonal antibody was purchased from Abcam (UK), the anti-rabbit SP Detection Kit from Beijing Zhongshan biological Co., Ltd. (China) and the DAB (diaminobenzidine) color kit from Beijing Zhongshan biological Co., Ltd. (China). The primer design and synthesis was carried out from the Beijing Parkson Gene Company Limited (China).

AP with ALI model

Rats were fasted 12 h prior to experimentation with free access to water. The rats were anesthetized with an

intraperitoneal injection of 10% chloral hydrate (3ml/kg). The duodenum was removed and the pancreatic duct was exposed. Subsequently, the hepatic duct was clamped by the arterial clip. In the vicinity of the duodenal nipple, the syringe needle was retrograde inserted into the pancreatic duct. 5% of sodium Taurocholate (1ml/kg) was injected at a rate of 0.1ml/min. When the injection was complete, the needle was removed. Following surgery, glucose and sodium chloride solution (5%) was injected subcutaneously in the back of each animal as a replacement fluid. A dose of 25ml/kg body weight was injected.

Animal Groups

A total of 90 healthy adult male Sprague-Dawley rats (300±50 g weight) were randomly divided into three groups: sham operation group (SO group); severe acute pancreatitis lung injury group (SAP group); and mannose intervention group (MT group). Subsequently, each group was divided into two subgroups of 6 and 12 h, with a total of 15 rats in each subgroup.

Primer design

The primers were designed by a computer software purchased from Beijing Parkson Gene Company Limited. The primer sequences were as follows: 5' to 3' Rat GAPDH F: TGGAGTCTACTGGCGTCTT. Rat GAPDH R: TGTCATATTTCTCGTGGTTCA. Rat Mrc1 F: TATGCCCTTATGAAATTGAAGTCGC. Rat Mrc1 R: ACCGCACCCTCCATTATCTGTC.

The above primers were then used in PCR amplification.

Statistical analysis

The experimental data were expressed as mean $\pm$ SD, and SPSS15.0 statistical software was used for statistical analysis. The comparison between multiple sets of data was based on one-way ANOVA. A pairwise comparison was performed using the LSD method and/or the Student-Newman-Keuls method. A P value of less than 0.05 ($P < 0.05$) was considered statistically significant.

RESULTS

Assessment of animal vital signs and behavior

In the sham operation group (A6, A12), all

animals survived. They had glossy fur and smooth respiration. In the model group (B6, B12) the reaction of the animals was sluggish. Their activities were significantly reduced. Their fur was rough. One rat died within 6 h, whereas three rats died within 12 h of treatment. In the treatment group (C6, C12), the reactions of the animals were slow and their activity was reduced. The fur was rough, and the degree of bloating was slightly less than that of the model group. One rat died within 6 h, and two rats died within 12 h of treatment.

Pathological changes of pancreatic tissue in rats

The pancreatic tissue structure was normal in the SO group and no ascites were observed by visual investigation of the animals. Scattered necrosis and

saponification spots were noted in the periphery of the pancreas and in the retinas.

In the SO group, the structure of the pancreatic lobule was complete, whereas the outline of the acinus was clear, and no inflammatory cell infiltration was present, as determined by light microscopy. A great number of inflammatory cell infiltrations could be seen in the necrotic area. The lobule was destroyed at different extents. The histological appearance of the pancreas in the different groups (H&E $\times$ 200) is shown in Fig. 1.

Pathological changes of the lung tissues in rats

The lung tissue structure of the SO group was approximately normal as determined by visual investigation. The splinter hemorrhage could be

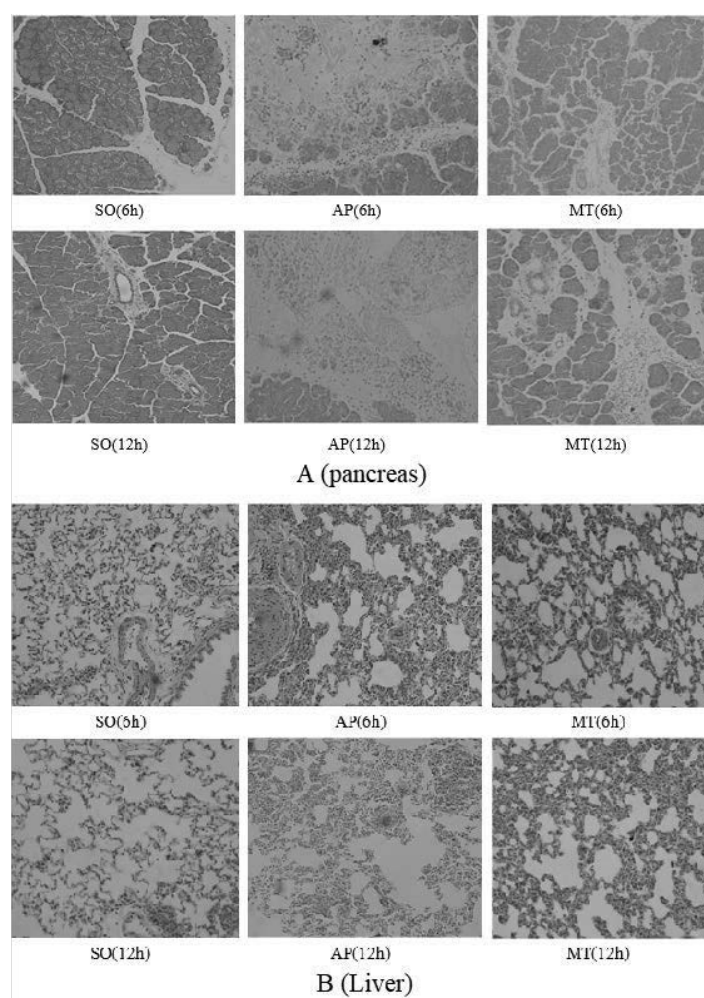


Fig. 1. The histological appearance of the pancreas and liver in the different groups (H&E $\times$ 200).

observed. The levels of congestion, edema and hemorrhage in the lung tissue of the MT group were less than those in the SAP group.

In the SO group, the pulmonary alveolar contour was clear, and edema, hemorrhage, necrosis and neutrophil infiltration were not observed, according to investigation by light microscopy. The lung tissue was infiltrated with large amounts of inflammatory cells. The partial alveolar normal structure disappeared. However, the development of edema and hemorrhage in the MT group was less than that of the SAP group. The same result was noted for the widening of the interstitial space and the infiltration of the inflammatory cells between the two groups. The histological appearance of the lung in the different groups (H&E×200) is shown in Fig. 1.

Changes of wet/dry weight at different time points

No significant difference was observed in the SO group between time points. The wet/dry (W/D) weight of the SAP and the MT groups increased significantly

at the 6 and 12 h time points compared with the SO group (3.85 ± 0.26 , 4.13 ± 0.24) ($p < 0.05$), whereas the wet/dry weight of the MT group was significantly decreased (4.53 ± 0.34 , 4.72 ± 0.25) at the same time points compared with the SAP group (4.93 ± 0.32 , 5.16 ± 0.24) ($P < 0.05$). The level of W/D weight in the rat lungs (Mean±SD) is shown in Table I and Fig. 2A.

TNF- α expression levels at different time points

No significant difference was noted with regard to TNF- α expression levels in the SO group at each time point. The content of TNF- α in the lung tissue was significantly increased in SAP and MT groups at the 6 and 12 h time points compared with the SO group (62.95 ± 13.23 , 71.84 ± 14.13) ($p < 0.05$). The expression of TNF- α in the lung tissue of the MT group was significantly decreased at the 6 and 12 h time points (98.65 ± 14.82 , 104.7 ± 13.45) compared with the SAP group (121.73 ± 19.84 , 134.04 ± 22.33) ($P < 0.05$). The expression levels of TNF- α in the rat lungs are shown in Table I and Fig. 2B.

Table I. Comparison between groups at the 6 and 12 h time points.

Group	6h	12h
Level of W/D in the rat lungs		
SO	3.85 ± 0.26	4.13 ± 0.24
SAP	$4.93\pm0.32 \Delta$	$5.16\pm0.24 \Delta$
MT	$4.53\pm0.34 \blacktriangle \blacksquare$	$4.72\pm0.25 \blacktriangle \blacksquare$
The content of TNF- α in the rat lungs		
SO	62.95 ± 13.23	71.84 ± 14.13
SAP	$121.73\pm19.84 \Delta$	$134.04\pm22.33 \Delta$
MT	$98.65\pm14.82 \blacktriangle \blacksquare$	$104.7\pm13.45 \blacktriangle \blacksquare$
The expression of MR protein in the rat lungs		
SO	0.77 ± 0.33	0.87 ± 0.32
SAP	$1.32\pm0.16 \Delta$	$1.48\pm0.34 \Delta$
MT	$1.87\pm0.32 \blacktriangle \blacksquare$	$2.06\pm0.38 \blacktriangle \blacksquare$
The expression of MR mRNA in the rat lungs		
SO	1.02 ± 0.16	1.13 ± 0.15
SAP	$1.54\pm0.42 \Delta$	$1.63\pm0.42 \Delta$
MT	$1.93\pm0.31 \blacktriangle \blacksquare$	$2.05\pm0.29 \blacktriangle \blacksquare$

(Mean ± SD); $\Delta P < 0.05$ vs SO group, $\blacktriangle P < 0.05$ vs SAP group, $\blacksquare P < 0.01$ vs SO group.

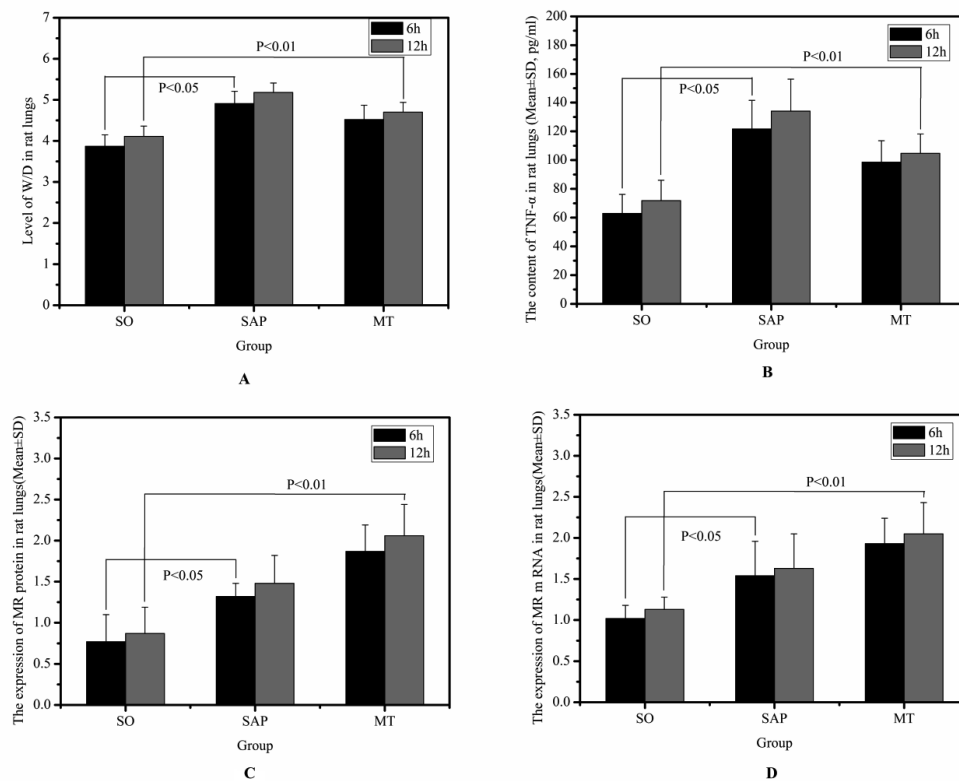


Fig. 2. Comparison of the two groups. **A)** Level of W/D in rat lungs. **B)** Content of TNF- α in the rat lungs. **C)** Expression of MR protein in the rat lungs **D)** Expression of MR mRNA in the rat lungs).

MR protein expression levels in lung tissue

The positive staining indicated the expression of the MR protein in the lung tissue. MR expression in the SO group was lower compared to the other two groups, and the color was lighter. In the MT group, the lung interstitial and alveolar cavities at the 6 and 12 h time points demonstrated strong immune staining with diffuse distribution, and their color was notably brown. The immunohistochemical results indicated no significant difference in the SO group between time points. The MR expression levels in the lung tissue at the 6 and 12 h time points were significantly increased (1.32 ± 0.16 , 1.48 ± 0.34) compared with the SO group (0.77 ± 0.33 , 0.87 ± 0.32) ($P < 0.05$). In addition, the expression of MR protein in the lung tissue at the 6 and 12 h time points was further increased (1.87 ± 0.32 , 2.06 ± 0.38) compared with the SAP group ($P < 0.05$). The immunohistochemical expression of the mannose receptor protein in the lung (x 400) is shown in Fig. 3. The expression of

the MR protein in the rat lungs (Mean $\pm$ SD) is shown in Table I and Fig. 2C.

MR mRNA expression at different time points

No significant difference with regard to the mRNA levels of MR in the SO group was noted at each time point, whereas MR expression increased significantly in the lung tissue at the 6 and 12 h time points (1.54 ± 0.42 , 1.63 ± 0.42) compared with the SO group (1.02 ± 0.16 , 1.13 ± 0.15) ($P < 0.05$). The mRNA expression levels of MR in the lung tissue at the 6 and 12 h time points were further increased (1.93 ± 0.31 , 2.05 ± 0.29) in the MT group compared with the SAP group ($P < 0.05$). The mRNA expression levels of MR in the rat lungs (Mean $\pm$ SD) are shown in Table I and Fig. 2D.

DISCUSSION

TNF- α plays an important role in maintaining

the stability of the cellular environment and in the resistance against various pathogenic factors (10). It is further involved in toxic shock, inflammation, tumor development, cytotoxicity and antiviral and bacterial infections (11). During the induction of acute lung injury by acute pancreatitis, alveolar macrophages activate and release $\text{TNF-}\alpha$, which induces neutrophil activation and aggregation, and releases a great number of inflammatory mediators such as $\text{TNF-}\alpha$, IL-13, IL-6, intercellular adhesion molecule-1 and SP responsible for lung injury (12). Mannose receptors can modulate the phagocytosis of AM against *Candida albicans*, *Pneumocystis carinii*,

Leishmania lucidum, *Mycobacterium tuberculosis* and HIV by binding to a variety of sugar molecules on the cell wall of the pathogen (13). MR can initiate a proinflammatory response that may be due to the complex molecular structure of mannose glycans on the pathogenic organisms, whereas the different mannan-like structures may determine the biological effects according to the types of cytokine production. These mannose molecules may be associated with both Toll-like receptors (TLRs) and C-type lectin-like receptors (CLR). Upon binding of the mannose glycans with the TLR and CLR, the activation of the proinflammatory and antiinflammatory

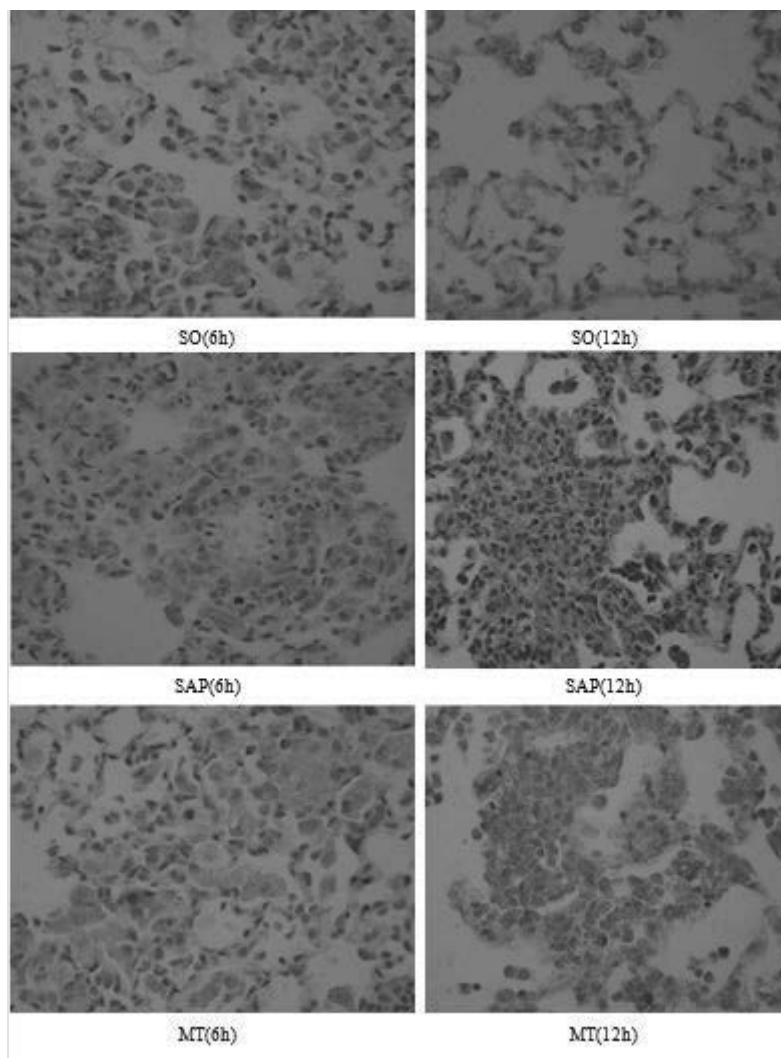


Fig. 3. Immunohistochemical expression of the mannose receptor protein in the lung ($\times 400$).

signaling pathways is facilitated, which in turn regulates the production of the proinflammatory and antiinflammatory cytokines (14).

In conclusion, mannose can reduce the injury of the lung tissue induced in rats with severe acute pancreatitis by up-regulation of the expression of the mannose receptor mRNA and proteins.

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LETTER TO THE EDITOR

SALINE CONDITIONS ALTER MORPHO-PHYSIOLOGICAL INTENSIFICATION IN PURSLANE (*PORTULACA OLERACEA* L.)S. ZAMAN¹, S-B. SHAH², Y-T. JIANG³ and S-Q. CHE¹

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Received November 16-2017 – Accepted March 1, 2018

In this study, primary investigations of selected cultivar of purslane named as Tall Green under particular salinity stress were evaluated to understand the basic concept of different mechanisms of physiological attributes which will play an important role for molecular and proteomic level research. The evaluation of morphological and physiological attributes under 0 mM (without salt addition) 100 mM and 200 mM salt stress changed dramatically. The results showed high salt stress at 200 mM significantly decreasing the morphological attributes and performance of leaves, stems, and roots. At moderate salt stress levels, 100 mM, the ratio of Fv/Fm slightly increased compared to high stress. In addition, salt stress significantly decreased the total chlorophyll content (chl a+b) at 200 mM. The relative water content percentage was high at 0 mM. Moreover, the electrolyte leakage (EL) significantly increased with increasing salinity stress compared to control 0 mM.

To the Editor,

Salinity is the most important environmental factor that limits plant growth and productivity (1-2). It can diminish substrate water potential in plants, which develop an assortment of physiological and biochemical procedures in response to stress conditions, e.g. relative water content, solute accumulation, chlorophyll contents, and the development of enzymatic antioxidant systems (3). Salinity effects show reduction in plant growth due to the osmotic effects (4-5). Salt tolerance in plants is a multifarious observable fact that might engage in growth as well as in physiological and biochemical processes (6).

Purslane (*Portulaca oleracea* L.), also well-known as pigweed, fatweed, pusle, and little hogweed, is an annual succulent herb in the family

Portulacaceae, found in most places around the world from sea level up to 3835 m above sea level (7). Purslane encompasses approximately 150 species, of which 40 species are habitant to tropical and warm climates. At present, substantial attention is being paid to purslane by agriculturists and nutritionists. WHO listed purslane as an important medicinal plant, and scientifically it is termed as 'Global Panacea' (8-9).

Salt treatment led to significant reduction of plant height, number of leaves, and number of flowers and physiological attributes of the purslane accessions (10). The effect of salinity reduced the growth of two sorghum varieties (11). The effect of salinity on the plant might be due to the intervention of nutrient assimilation and physiological water stress created by high salt concentrations in the root zone,

Key words. chlorophyll, electrolyte leakage, photochemical efficiency, relative water content, salt stress

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0393-974X (2018)

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reduction in plant height under saline conditions reported in many crops, for instance, rice, jute, and *amaranthus* (7-10).

Therefore, the objective of this study was to evaluate the morphological and physiological attributes of selected purslane genotypes under varied saline conditions to promote their cultivation in salinity areas as a vegetable crop and to use them for human consumption.

MATERIALS AND METHODS

A glasshouse experiment was carried out at Shanghai Jiao Tong University, Shanghai, to evaluate the effect of salinity on growth, photochemical efficiency measurement, (Fv/Fm) and chlorophyll content (Chl) electrolyte leakage.

Morphological parameters analysis

Morphological parameters, such as true number of leaves and number of roots, were observed naturally. Main stem and root length were measured by using a scientific ruler. Moreover, the stem diameter was calculated by digital screw meter in fresh samples.

Chlorophyll fluorescence measurement (Fv/Fm)

For measurement of Fv/Fm, a layer of leaves was kept in darkness for 30 min using leaf clips, and the Fv/Fm ratio was recorded with the fluorescence meter (Fim 1500; Dynamax, Houston, TX, USA) according to Li, et al. (10).

Chlorophyll content (Chl)

For chlorophyll measurement, 0.1 g leaves were cut into pieces and kept in aluminum foil. The leaf samples were then put into small centrifuge tubes with 10 ml dimethyl sulfoxide (DMSO) and kept in the dark for 2~3 days. Measurements were taken of six samples under OD663 and OD645 for one time and then taken again for another six samples (including an empty sample) with only DMSO.

Content of chlorophyll *a*, *b* and *t* (total) calculated according to the formula:

$$\text{Chl } a \text{ (mg l}^{-1}\text{)} = 12.7 \text{ OD}_{663} - 2.69 \text{ OD}_{645}$$

$$\text{Chl } b \text{ (mg l}^{-1}\text{)} = 22.9 \text{ OD}_{645} - 4.68 \text{ OD}_{663}$$

$$\text{Chl } t \text{ (mg l}^{-1}\text{)} = 20.2 \text{ OD}_{645} + 8.02 \text{ OD}_{663}$$

Relative water content

For leaf relative water content (RWC), 10-15 first and second fully expanded leaves per pot (at least 4 replications) were taken, clipped and weighed [fresh weight (FW)] and put into tea bags and placed into small Petri dishes. The excess moisture was removed with paper towels [turgid weight (TW)], and weighed immediately. Later, leaves were dried in an oven at 80°C for 48-72 h to determine the DW.

Leaf RWC, was calculated as $(FW - DW) / (TW - DW) \times 100$.

Electrolyte leakage

Leaf electrolyte leakage, was calculated as the percentage of $C_{\text{initial}}/C_{\text{max}}$. Approximately, 10 first and second fully expanded leaves (0.1g) per pot were carefully removed, cut into 1 cm pieces, washed three times with distilled water and placed into 50-ml vials containing 30 mL distilled water. After shaking for 24 h, initial conductivity (C_i) of leaves was calculated with a conductivity meter, leaves were destroyed in an autoclave at 100°C for 30 min, placed on a shaker for 24 h and final conductivity (C_f) of the bathing solution was measured. The electrolyte leakage (EL) were calculated as $C_i/C_f \times 100$

Experimental design and statistical analysis

Genotype and treatments were set as a randomized block design. Genotype and salt stress were measured by the analysis of variance using the Statistics Analysis System (SAS) (SAS 8.1.SAS Institute Inc., Cary, NC). Differences among species and treatment means were assessed by Fisher's protected least significance difference test (LSD) at $P = 0.05$ probability level.

RESULTS

Effects of salt stress on purslane and morphological characteristics

Morphological indexes significantly changed under different salinity concentrations. True number of leaves increased (12.6%) at 0 mM and (6.13%) at 100 mM and decreased under 200 mM. Main stem (29.3%) at control and (14.3%) at 100 mM were increased significantly compared to 200 mM NaCl stress. Diameter of stem marginally increased under

all conditions. Root was (7 cm) at control and (4 cm) at 100 mM grew significantly under 200 mM salt concentration. Number of roots (21.8%) at control and (10.3%) at 100 mM and (5.9%) at 200 mM were increased significantly as shown in Fig. 1a. Chlorophyll fluorescence measurement is an important index to determine the changes of the active photosynthetic pigments in leaves. The photochemical efficiency (F_v/F_m) augmented at 0 mM and declined at 200 mM, as depicted in Fig. 1b. The amount of chlorophyll content

in purslane leaves (PL) treated under different saline conditions is shown in Fig. 1c. Considerable variations existed between control and NaCl concentrations. At 0 mM, the chlorophyll content was not affected as compared to 100 mM. Furthermore, relative water contents of leaves declined at 200 mM compared to 100 mM. The relative water content significantly increased at 0 mM as exhibited in Fig. 1d. The EL enhanced at 200 mM and declined at 100 mM salt stress as documented in Fig. 1e.

Table I. Physical and chemical properties of soil.

Sand %	Silt %	Clay %	OM %	Na mg/kg	K mg/kg	P mg/kg	pH	EC(dsm ⁻¹)
14	80	10	0.28	114	290.1	46	7.7	0.6

OM: organic matter; EC: electric conductivity

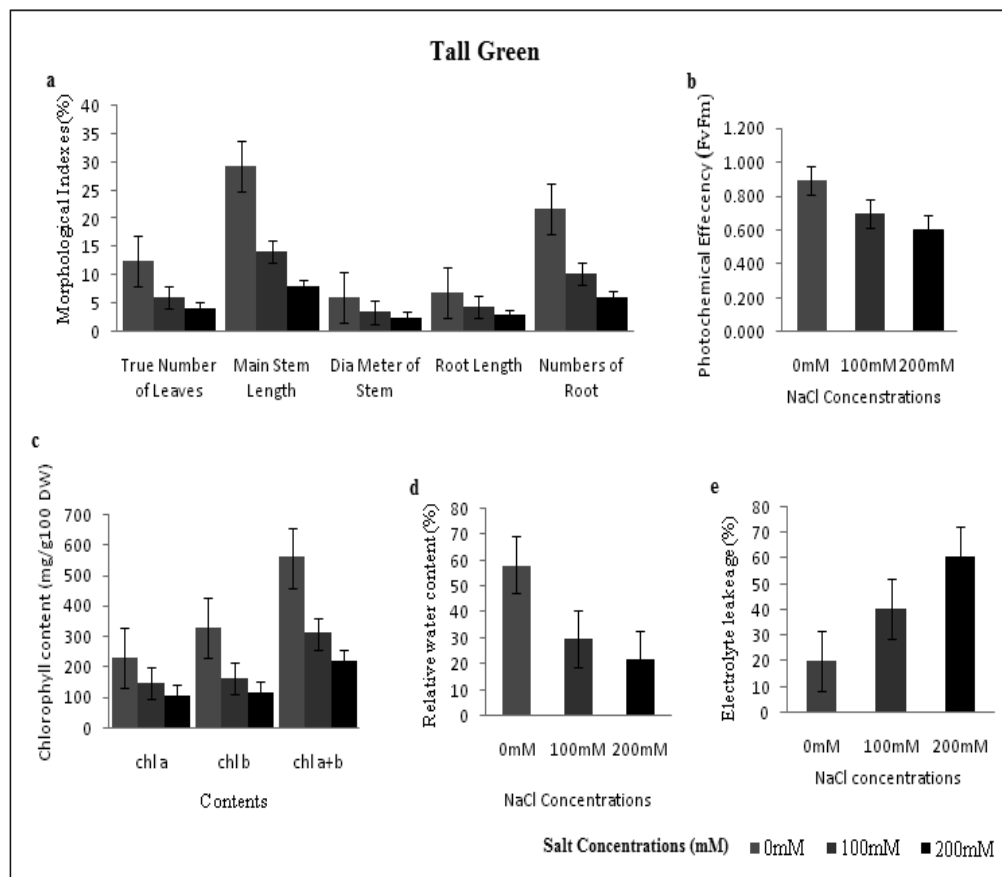


Fig 1. a) Morphological parameters. b) Chlorophyll fluorescence measurement. c) Contents of chlorophyll. d) Relative water content. e) Electrolyte leakage in leaves of purslane (*T. Green*) at 28 d of NaCl treatment with concentrations of 0, 100 and 200 mM. Vertical bars indicate standard error of each mean. Columns indicate the significant differences among 0, 100 and 200 mM NaCl for a given genotype based on LSD test ($P = 0.05$).

DISCUSSION

Plants have varying levels of tolerance for salt stress. Purslane is a susceptible, moderate, and tolerant medicinal plant and responds differently under several environmental factors. At present, little information is available regarding the different effects of salt stress on purslane growth and development. The present study was determined to examine the effect of salt stress on purslane (6). The results showed that salinity reduced the morphological parameters such as number of true leaves, stem length, diameter of stem, root length and number of root, as shown in Fig. 1a. This is a common attribute of plants growing under salt stress. This decrease under salt stress may be due to the reduction in turgor potential that is essential for cell elongation in plants (5). In addition, reduction in chlorophyll content (Chl) and photochemical efficiency (Fv/Fm) was observed. The contents of chlorophyll level is affected under varied salt concentrations, which is due to biosynthesis resulting from activation of the chlorophylls delayed in leaf senescence during stresses (10). On the other hand, effects of salinity reduced the physiological attributes such as relative water content (RWC) shown in Fig. 1d. Through salinity stress, plant organs undergo drought stress, which is the main reason for reduction in RWC (11). Electrolyte leakage results are demonstrated in Fig. 1e. 'EL' is the most important physiological attribute in salinity levels, and the increase in electrolyte leakage is mainly related to K^+ efflux from plant cells, which is mediated by plasma membrane (12).

The results of the present study demonstrated a decline in overall growth of plants due to high salinity as dry root length, stem height, number of leaves, and stem diameter reduced. This significant effect of salt stress on purslane root vs stem may be due to more water loss from the root than shoot during salt stress. The effect of salts on shoot length and diameter may be due to stomata closure, which controls the water loss in different tissues. In fact, salt stress treatment causes osmotic changes in the leaf tissue or affect CO_2 concentration on leaf that leads towards stomata closure (6). Our results have similarities with few previous studies on most crops (7-10).

This study concluded that high salt stress declined the overall growth of selected cultivar in our pot experiment, but selected cultivar showed satisfactory performance at control and moderate salt conditions. In this experiment, we should consider the purslane as a moderate tolerant cultivar. Based on above results, we hope that these findings will be very helpful for future molecular and proteomic research on particular cultivar, which will be used for commercial cultivation to fulfill the increased demands for fresh vegetables and be beneficial to food industries as well as for sustainable agriculture, especially for saline areas around the world.

ACKNOWLEDGEMENTS

The authors express sincere thanks to the School of Agriculture and Biology; Shanghai Jiao Tong University, China and The China Scholarship Council (CSC) for awarding a Chinese Government Scholarship.

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LETTER TO THE EDITOR

CONTINUOUS FEMORAL NERVE BLOCK AND PATIENT-CONTROLLED INTRAVENOUS POSTOPERATIVE ANALGESIA ON Th1/Th2 IN PATIENTS UNDERGOING TOTAL KNEE ARTHROPLASTY

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The purpose of this study is to observe and compare the effects of continuous femoral nerve block (cFNB) and patient-controlled intravenous analgesia (PCIA) on postoperative analgesia and Th1/Th2 in patients undergoing total knee arthroplasty (TKA). Forty-six TKA were selected and randomly divided into two groups: the cFNB group and PCIA group. Patients in the two groups all underwent general anesthesia using a laryngeal mask. In the cFNB group, the femoral nerve block and catheterization were performed after induction of general anesthesia: 0.375% ropivacaine hydrochloride with a 20 mL loading dose was provided. After the end of the operation, the electronically controlled analgesia pump was connected. In the PCIA group, fentanyl with a 0.05 mg loading dose was provided and the electronic controlled analgesia pump was connected at the end of the operation. Venous blood was collected before anesthesia (T0), 1 h postoperatively (T1), 24 h postoperatively (T2) and 48 h postoperatively (T3). Th1/Th2 was calculated and analyzed by flow cytometry, and other indexes of these time points were recorded. The results show that there was no significant difference between the two groups regarding changes in blood pressure, heart rate and postoperative sedation Ramsay score. There was no significant difference in Th1 percentages (Th1%), Th2 percentages (Th2%) and ratios of Th1-to-Th2 (Th1/Th2) between the two groups at T0, T1 and T2 ($P>0.05$), while the Th1%, Th2% and Th1/Th2 of the PCIA group were lower than those of the cFNB group at T3 ($P<0.05$). It was concluded that cFNB represents a better postoperative analgesia for patients than PCIA, and has a lesser effect on Th1/Th2 balance, which can improve the outcome of patients.

To the Editor,

Not only can the severe pain after total knee arthroplasty (TKA) affect the early functional recovery of patients, but it can also have adverse effects on expected surgical results (1). Patient-controlled intravenous analgesia (PCIA) is a traditional, classic postoperative method that is both effective and reliable (2). However, due to individual differences among patients, adverse reactions, such as incomplete analgesia and respiratory depression,

may occur in the use of PCIA (3). The effect of continuous femoral nerve block (cFNB) is more specific than PCIA, and the incidence of adverse reactions is significantly lower (4).

T lymphocytes have various subtypes with different functions. T helper cells (Th cells) are among the cells that express CD3 and CD4 molecules on the surface of T lymphocytes simultaneously (5). Th cells can be divided into three subtypes, Th0, Th1 and Th2, according to the different kinds of cytokines

Key words: continuous femoral nerve block analgesia, PCIA, TKA, Th1/Th2

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0393-974X (2018)

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that they secrete. Th1 cells participate in the cellular immune response by secreting cytokines such as interleukin (IL)-2 and IFN- γ (6). However, Th2 cells participate in the antibody-mediated immune response by secreting cytokines such as IL-4, IL-5, IL-10 (7). Th1 and Th2 cells are in a state of dynamic equilibrium in the normal human body. Disturbances to this balance will have an adverse effect on the body (8). This study observed the effects of two kinds of analgesia on TKA postoperative analgesia. By detecting Th1/Th2, it can be determined which of the two methods is better able to maintain this balance, as well as which of the two is more sensitive to the patient's cell immunosuppression, in order to find an optimal means for patient analgesia.

MATERIALS AND METHODS

Study subjects

In this study, 46 patients with unilateral TKA in the Taizhou Hospital of Zhejiang Province were selected between September 2016 and May 2017. The patients were randomly divided into two groups of 23 patients each: a continuous femoral nerve block analgesia group (cFNB group), and a patient-controlled intravenous analgesia group (PCIA group). All patients gave informed consent after the approval of the medical ethics committee.

Main drugs, reagents and instruments

Main drugs: etomidate fat emulsion injection (20mg/10mL injection) (Jiangsu Enhua pharmaceutical Co., Ltd., China); suxamethonium chloride injection (0.1g/2 mL injection) (Shanghai Xudong Haipu Pharmaceutical Co., Ltd., China); sevoflurane (150mL/bottle) (Shanghai Hengrui Medicine Co., Ltd., China); cis atracurium besilate for injection (10mg/branch) (Zhejiang Xianju pharmaceutical Co., Ltd., China); ropivacaine hydrochloride injection (75mg/10mL injection) (Yichang humanwell pharmaceutical Co., Ltd., China); fentanyl citrate Injection (0.5mg/10mL injection) (National Drug Group Industrial Co., Ltd. Langfang branch, China); remifentanyl hydrochloride for injection (1 mg powder) (National Drug Group Industrial Co., Ltd. Langfang branch, China).

Main reagents: RPMI 1640 (Shanghai Haoran Biotechnology Co., Ltd., China); Anti-Human CD3 APC,

Anti-Human CD8a Per CP-e Fluor 710, Anti-Human IL-4 PE, Anti-Human IFN gamma Alexe Fluor488, Mouse Ig G1 K Isotype Control PE, Mouse Ig G1 K Isotype Control Alexe Fluor488, PMA/Ionomycin mixture (250x), BFA/Monensin Mixture(250x), flow buffer solution, Fix & Perm Medium A, Fix &Perm Medium B (Hangzhou Lianshi Biologic technology Co., Ltd., China); buffer solution (Thermo Fisher Scientific (China) Co., Ltd., China).

Main instruments: multifunctional anesthesia machine (Beijing Yian medical system Co., Ltd., China); disposable laryngeal mask, multi-parameter monitor (Shanghai Yuqi medical equipment Co., Ltd., China); portable two-dimensional ultrasound, ultrasonic probe (Shenzhen Hongyuanbo science and technology Co., Ltd., China); continuous nerve plexus block suite (B. Brain Melsungen AG, Germany); electronically-controlled analgesia pump (Taizhou Pinqing medical equipment Co., Ltd., China); CO₂ incubator (Thermo Fisher Scientific S/N :300019366), BY-160C-type medical centrifuge (Beijing Baiyang medical devices Co., Ltd., China) ; Haimen Qi Lin-Beier Vortex-5 vortex mixer (Beijing open source countries create science and technology Co., Ltd., China); flow cytometry (American BD FACS Calibur).

Medication

The cFNB group was given 0.2% ropivacaine hydrochloride, 240 mL normal saline.

The PCIA group was given 20ug/kg fentanyl, 100 mL normal saline.

Anesthesia methods

All patients were required to fast for 6-8 hours and were not allowed to drink 2 hours before the operation. Noninvasive blood pressure (NBP), electrocardiogram (ECG), heart rate (HR) and SpO₂ were continuously monitored. All patients were treated with general anesthesia using a laryngeal mask. Anesthesia induction process: 23 ug / kg fentanyl, 0.2~0.6 mg / kg etomidate, 1~1.5mg/kg suxamethonium. The laryngeal mask was placed after 1 min, and 0.15-0.2mg/kg cis atracurium was provided. The anesthesia machine was connected (Mechanical ventilation with volume control mode, 2L/min Oxygen flow rate, 8-10mL/kg tidal volume, 10-12 times/min breathing rate, 1:2 inspiratory-to-expiratory ratio). Anesthesia maintenance: static suction composite

mode combined with sevoflurane and remifentanyl was used. The exhaled concentration of sevoflurane was maintained at 1MAC, and the exhaled concentration of remifentanyl was maintained at 0.05-0.15 mg/kg/hr. The dose of remifentanyl was adjusted according to blood pressure and heart rate, and the end-tidal carbon dioxide partial pressure (EtCO_2) was maintained between 35-45 mmHg. Mean arterial pressure (MAP) and HR fluctuation did not exceed 20% of the baseline state. When the operation time exceeds each more than one hour, additional 0.05-0.1 mg / kg cis atracurium was added. Drugs were discontinued 30 min before surgery, while sevoflurane and remifentanyl were discontinued during the suturing. After the operation, the patients were asked to open their eyes and once muscle strength was recovered, the laryngeal mask was removed and the patient was sent to PICU for further observation.

Postoperative analgesia

cFNB: After the laryngeal mask was inserted with rapid general anesthesia induction, the affected limb was slightly extended, the femoral nerve was located by ultrasound of the groin and its position marked. After skin disinfection, the puncture needle of the continuous nerve plexus block kit was inserted through the skin under the guidance of ultrasound, and the puncture process was gradually deepened. When the puncture needle was near the femoral nerve, 0.375% 20 mL ropivacaine hydrochloride was injected around the femoral nerve, with the injection and withdrawal performed simultaneously. After injection, the needle core of the puncture needle was removed, and the 5-10 cm catheter was inserted

through the puncture needle. A small amount of saline was injected to confirm the location of the catheter around the femoral nerve under ultrasound. Finally, the catheter was properly fixed to complete the operation. The 240 mL electronically-controlled analgesia pump was connected before the patient was sent into the resuscitation room after the operation. The analgesic pump was set to a 5mL/hr baseline dose, and 5 mL dose PCA was adopted every time. The lock time was 30 min.

PCIA: 0.05 mg fentanyl was injected intravenously as the loading dose during the surgical suture. The 100 mL electronically-controlled analgesia pump was connected after the operation. The analgesic pump was set with a 2mL/hr background dose. Two mL PCA was adopted each time, and the lock time was 15 min. The analgesia pump was also configured by the same anesthesiologist.

Th1/Th2 determination

1-2 mL of venous blood was collected from before the operation anesthesia (T0), 1 hour postoperatively (T1), 24 h postoperatively (T2) and 48 h postoperatively (T3). A heparin anticoagulant tube was then placed. 100 uL blood samples were taken for cell culturing, and Th1/Th2 was detected by flow cytometry. The prerequisite for using flow cytometry to detect Th1/Th2 was to screen the Th cells through a CD4^+ T cell gating. However, CD4^+ proteins are not only expressed in Th cells, but also in monocytes. It can thus also be assumed that CD3^+ can screen Th cells, and the PMA can decrease the expression of CD4^+ , but CD8 will not be affected. The majority of $\text{CD3}^+\text{CD8}^+$ cells are $\text{CD3}^+\text{CD4}^+$ cells. Therefore, using flow cytometry to determine Th1/Th2 is to circle $\text{CD3}^+\text{CD8}^+$ cell as Th cell.

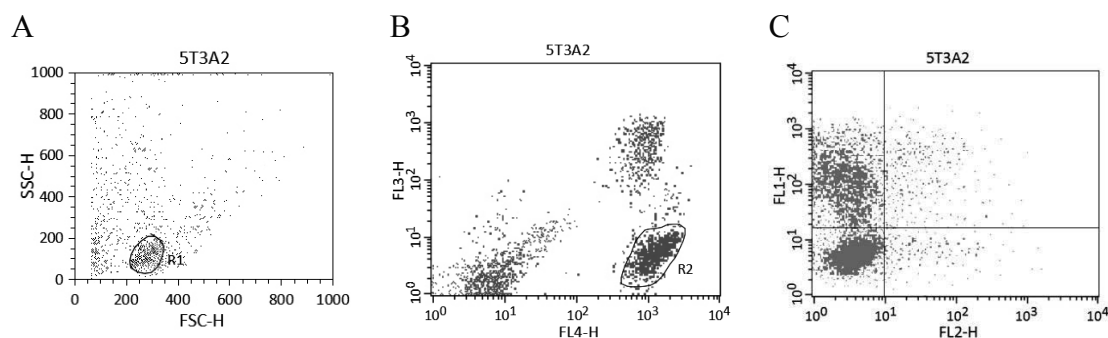


Fig. 1. Detection chart of flow cytometry. *A)* R1 is the lymphocyte portal and encircles the lymphocytes in whole blood samples; *B)* R2 is the $\text{CD3}^+\text{CD4}^+$ helper T cell portal and encircles the R2 porta, that is the $\text{CD3}^+\text{CD4}^+$ helper T cells in the lymphocyte portal; *C)* Further analysis in the R2 portal reveals that the cells in the left upper quadrant are Th1 cell and the cells in the right lower quadrant are Th2 cells. Th1/Th2 is the ratio of two types of cells.

Positive expression of IFN- γ corresponds to a Th1 cell, and positive expression of IL-4 to a Th2 cell, while Th1/Th2 is the ratio of two groups (shown in Fig. 1).

Observation index

The general situation, operation time, anesthesia time and the changes in non-invasive blood pressure, heart rate and SpO₂ before anesthesia, 1 hour postoperatively, 24 h postoperatively and 48 h postoperatively were observed and recorded.

Sedation scores (SS) were carried out at 1 hour, 24 hours and 48 hours after general anesthesia, respectively. A score of 1 corresponds to an irritable and non-quiet state; 2 corresponds to a state of quiet cooperation; 3 to sleepiness, with the patient able to obey instructions; 4 corresponds to a sleep from which the patient can be awoken; 5 to a slow respiratory response; 6 to a state of deep sleep and inability to be awoken. A score of 1 implies a low degree of sedation; 2-4 a satisfactory effect; and 5-6 oversedation.

Pain score: in accordance with the visual analogue scale (VAS), the VAS score was recorded at 1 h, 24 h and 48 h after the operation under resting and active-passive flexion and extension. VAS pain score standard (0-10 points): a score of 0 corresponds to painless; a score below 3 corresponds to mild pain that can be endured; 4-6 corresponds to pain that can affect the patient's sleeping state; 7-10 corresponds to powerful pain that cannot be endured, where the pain will affect the patient's appetite and sleep.

Adverse reactions such as nausea, vomiting, headache, dizziness, respiratory depression, local anesthetic poisoning, and the number of adverse reactions recorded

specifically for those using the electronically-controlled pump were recorded.

Percentages of Th1 and Th2 of venous blood before anesthesia was calculated 1 h, 24 h and 48 h postoperatively and were detected by flow cytometry in the two groups of patients.

Statistical analysis

The experimental data was analyzed by State12.0 software, and measurement data was expressed by $\bar{x} \pm s$. If the data corresponds to a normal distribution and variance homogeneity, *t*-test and one-way analysis of variance were used. If not, the rank-sum test and Kruskal-Wallis test were used. Enumeration data were analyzed using the *chi*-squared test or Fisher exact probability, and $P < 0.05$ corresponded to a statistically significant difference.

RESULTS

General clinical data

As shown in Table I, there was no significant difference in the general situation between the two groups ($P > 0.05$).

Vital signs, postoperative pain and sedation scores

There was no significant difference in HR, SpO₂, SBP, DBP, MAP between the two groups of patients at T0, T1, T2 and T3 ($P > 0.05$). There was no statistically significant difference in the above indexes between the two groups at T1, T2 and T3 when compared with T0 ($P > 0.05$). There was no statistically significant difference in Ramsay scores

Table I. General situation.

Type	cFNB group	PCIA group
Case number (male / female)	9/14	6/17
Age	70.7 $\pm$ 11.32	65.2 $\pm$ 7.5
BMI(Kg/cm ²)	25.6 $\pm$ 3.1	25.2 $\pm$ 4.2
Operation time (min)	88.3 $\pm$ 10.8	99.2 $\pm$ 25.9
Anesthesia time (min)	120.4 $\pm$ 12.3	133.7 $\pm$ 18.6

($\bar{x} \pm s$)

between the two groups at T1, T2 and T3, nor in VAS score (static) at T1. However, the VAS scores (static) of the cFNB group at T2 and T3 were lower than that of PCIA group. The VAS scores (dynamic) of cFNB group after the operation were lower than PCIA group at each time point ($P < 0.05$) (Table II).

Th1/Th2

Comparison between the two groups: the differences in Th1%, Th2% and Th1/Th2 at T0, T1 and T2 between the two groups were not statistically significant ($P > 0.05$). However, at T3, the Th1% and Th2% of the PCIA group were less than that of the cFNB group, and Th1/Th2 was also lower than that of the cFNB group. There was a statistically significant difference between the two groups ($P < 0.05$).

Intra group comparison: at T3, the Th1%, Th2% and Th1/Th2 of the PCIA group were lower than those at T0 ($P < 0.05$). There was no statistically

significant difference in Th1%, Th2%, Th1/Th2 at T1 and T2 compared with T0 ($P > 0.05$). However, there was no statistically significant difference in Th1%, Th2%, Th1/Th2 of the cFNB group at T1, T2 and T3 compared with T0 ($P > 0.05$) (Table III).

Postoperative condition

In this study, the adverse reactions in the PCIA group (11 cases) were significantly higher than in the cFNB group (1 case), and the number of auto-control keys for the analgesia pump (cFNB group was 1.08 ± 0.34 , PCIA was 1.72 ± 0.91) was significantly more than for the cFNB group. There was a statistically significant difference between the two groups ($P < 0.05$).

DISCUSSION

Tang et al. showed that the blood pressure and heart rates of the patients in their cFNB group were

Table II. Vital signs, postoperative pain and sedation scores of the two groups at different times.

Groups		T0	T1	T2	T3
HR(time/min)	cFNB group	78.3±16.5	79.2±10.6	76.3±7.2	72.8±8.2
	PCIA group	74.1±13.5	81.7±11.3	73.2±10.2	74.5±12.6
SpO ₂ (%)	cFNB group	95.24±1.37	93.57±1.12	94.08±1.14	94.16±1.32
	PCIA group	95.42±1.26	94.36±1.05	94.72±1.35	94.88±0.92
DBP(mmHg)	cFNB group	83.5±8.2	85.2±6.1	79.6±9.4	74.7±6.8
	PCIA group	77.3±10.9	83.2±9.0	74.2±9.2	73.2±9.8
SBP(mmHg)	cFNB group	152.5±28.2	160.8±14.7	149.3±18.2	141.2±12.3
	PCIA group	144.6±23.6	155.1±21.3	141.8±24.2	138.3±21.2
MAP(mmHg)	cFNB group	104.8±14.3	110.7±8.1	102.3±11.6	96.9±7.3
	PCIA group	98.5±13.7	107.7±10.6	97.3±13.2	95.2±13.1
Ramsay score	cFNB group	-	2.20±0.35	2.25±0.26	2.16±0.15
	PCIA group	-	1.52±0.50	1.73±0.36	1.91±0.34
VAS sore (static)	cFNB group	-	2.65±0.95	1.50±1.45	1.25±0.90
	PCIA group	-	3.34±0.92	4.65±1.70*	2.52±0.51*
VAS score (dynamic)	cFNB group	-	3.22±1.25	2.54±1.54	2.38±1.31
	PCIA group	-	4.33±0.72*	6.15±1.43*	4.08±0.51*

($\bar{x} \pm s$). Comparison between the two groups: * $P < 0.05$.

Table III. Statistics of Th1%, Th2%, Th1/Th2 after surgery.

Groups		T0	T1	T2	T3
Th1(%)	cFNB group	25.88±11.26	37.03±16.92	31.03±12.02	25.99±9.26
	PCIA group	19.65±8.23	26.83±9.14	21.26±7.58	2.92±1.89*#
Th2(%)	cFNB group	3.12±1.14	2.74±0.56	3.21±1.23	3.52±1.33
	PCIA group	2.15±0.83	1.92±1.11	2.12±0.73	1.32±0.43*#
Th1/Th2	cFNB group	9.58±6.17	13.22±4.68	9.82±5.45	9.63±5.75
	PCIA group	11.43±6.92	23.05±15.45	13.54±11.18	1.39±0.84*#

($\bar{x} \pm s$). When PCIA group is compared with and cFNB group, * $P < 0.05$. When intra-group (T1, T2, T3) is compared to T0, # $P < 0.05$.

more stable than those of the PCIA group (9). In this study, the VAS scores of the two groups were lower than the PCIA group ($P < 0.05$) when the pain score was assessed in patients on the first and second day post-surgery, respectively. However, the pain score at 1 hour postoperatively and the score of cFNB group in a static state were lower than that of the PCIA group ($P > 0.05$). cFNB was better than PCIA under the active and passive motion ($P < 0.05$). Continuous femoral nerve block is similar to epidural anesthesia in its mechanism action, and both are local block analgesias. Therefore, the authors believe that cFNB may also play a protective role in immunity, and this is also in line with the results of this study.

Theoretically, postoperative analgesia can improve the postoperative Th1/Th2 balance by reducing postoperative pain and reducing the postoperative stress response (10, 11). But in practice, the analgesic drugs used in postoperative analgesia may also directly or indirectly inhibit the immune system (12). In this experiment, the main analgesic drug used in the PCIA group was fentanyl. It was also found that the number of Th1 and Th2 cells decreased significantly at 48 hours post-operative, and Th1/Th2 decreased significantly compared with that before surgery. Therefore, the inhibition by fentanyl of the immune system is far

more consequential than its protective effect on the immune system. The results of the cFNB group showed that the number of Th1 and Th2 cells did not decrease significantly after the operation, and Th1/Th2 after surgery had no obvious change compared with that before the operation. The Th1/Th2 balance of the PCIA group after surgery was conspicuously shifted in favor of Th2. Therefore, cFNB had a lesser inhibitory effect on Th cells and cell immune function than did PCIA.

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LETTER TO THE EDITOR

SACCHAROMYCES CEREVISIAE, KEY ROLE OF MIG1 GENE IN METABOLIC SWITCHING: PUTATIVE FERMENTATION/OXIDATION

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Received December 27, 2017 – Accepted April 12, 2017

Saccharomyces cerevisiae can utilize a wide range of carbon sources; however, in the presence of glucose the use of alternate carbon sources would be repressed. Several genes involved in the metabolic pathways exert these effects. Among them, the zinc finger protein, Mig1 (multicopy inhibitor of GAL gene expression) plays important roles in glucose repression of *Saccharomyces cerevisiae*. To investigate whether the alleviation of glucose effect would result in a switch to oxidative production pathway, MIG1 were disrupted in a haploid laboratory strain (2805) of *S. cerevisiae*. The impact of this disruption was studied under fully aerobic conditions when glucose was the sole carbon source. Our results showed that glucose repression was partly alleviated; i.e., ethanol, as a significant fermentation marker, and acetate productions were respectively decreased by 14.13% and 43.71% compared to the wild type. In Δ MIG1 strain, the metabolic shifting on the aerobic pathway and a significant increase in pyruvate and glycerol production suggested it as an optimally productive industrial yeast strain. However, further studies are needed to confirm these findings.

To the Editor,

One of the most experienced eukaryotic model organisms for the study of metabolic engineering is the yeast *Saccharomyces cerevisiae* (1). The advantages in cultivation and the similarity of expression system to humans have remarkably indicated *S. cerevisiae* for industrial scale of recombinant products (2). Recently, substantial attention has been given to redirect the metabolic

process of *S. cerevisiae* away from alcoholic fermentation towards other preferred chemicals (3). Moreover, a large number of genes involved in the Krebs cycle, the glyoxylate cycle, and in oxidative phosphorylation are repressed in *S. cerevisiae* by a regulatory mechanism which is controlled by a glucose availability system known as catabolite or glucose repression (4-5). Different practical mechanisms have been considered for glucose

Key words: yeast, ethanol, metabolic engineering, glucose repression, aerobic pathway

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0393-974X (2018)

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control such as transcriptional glucose repression, increased decay of mRNA, decreased translational efficiency, carbon catabolite inactivation of which the most thoroughly investigated mechanism is glucose repression (6). Mig1 has been recognized as one of the main effectors in glucose repression. It is a Cys2His2 zinc-finger protein, which binds to the promoters of several genes and represses their transcription while glucose is added to the medium (5, 7-8). The Mig1p-binding consensus sequence on promoters of genes which are involved in glucose repression was identified (9, 10). Deletion/disruption of MIG1 gene can de-repress associated genes to alleviate the glucose repressing effect and increase the respiratory activity of *S. cerevisiae* (6). We explored whether the disruption of MIG1 would improve metabolic switching to oxidative pathway in *S. cerevisiae* under aerobic conditions. In the present study, we constructed an MIG1-negative *S. cerevisiae* strain (Δ MIG1) by deleting MIG1 gene from a haploid wild strain 2805 in order to remove the effect of glucose repression. Then, the metabolic characteristics of this genetically engineered strain were compared with the wild type to investigate the impact of MIG1 on the aerobic switching metabolism.

MATERIALS AND METHODS

Upstream and downstream fragments of MIG1 gene cloning

The yeast strains were maintained and grown on yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% Bacto peptone, and 2% glucose; solid media contained 2% agar) at 30°C. Total genomic yeast was extracted from the industrial *Saccharomyces* strain 2805 (11). Homologous up and down stream fragments (N and C) of the MIG1 gene were amplified by PCR using 2805 strain genomic DNA as template with F1-N and R1-N primers for upstream (N) and similarly F2-C and R2-C primers for downstream (C) fragments. Then, the PCR products were digested using the appropriate endonucleases and cloned to pBluescript II cloning vector at EcoR I and BamH I sites, and BamH I and Xba I sites, respectively, to construct plasmid pSK-NtCt. The URA3 nutrient selection marker, which was amplified by PCR and digested with BamH I

(9), was inserted between N and C fragments to construct the final plasmid, which was designated as pMIG1. Based on the aforementioned strategy, the pMIG1 plasmid was constructed by inserting the upstream homologous fragment, URA3 marker, and downstream homologous fragment into the pSK II cloning vector. pMIG1 preserved and amplified in *Escherichia coli* DH5 α , which was grown at 37°C in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 100 μ g/mL ampicillin. The plasmid was obtained using a Plasmid Mini Kit II (D6945, Omega, USA). Accuracy of the cloning and transformation were verified through PCR and gel electrophoresis at each step.

MIG1 disruption and recombinant screening

Transformation pMIG1 to *S. cerevisiae* was achieved through lithium acetate/PEG method (12). The disruption of MIG1 gene was achieved through integration of N and C fragments into the chromosome at the MIG1 locus of strain 2805 by homologous recombination to construct the disrupted (Δ MIG1) strain. The replacement of selection marker URA3 in MIG1 locus enables Δ MIG1 recombinant yeasts to synthesize required uracil for growth on the uracil-excluded selective medium (SC-URA).

The selection of MIG1 disrupted strain was performed based on the growth of the yeast colonies on the selective SC-URA medium. The substitution of MIG1 was verified by PCR analysis of total DNA isolated from the wild and Δ MIG1 transformant strains, respectively.

Batch cultivation procedure

The batch cultivation medium consisted of glucose (100 mM) (NH₄)₂SO₄ (7.5 g/liter), KH₂PO₄ (3.5 g/liter), MgSO₄·7H₂O (2.7 g/liter), trace metal solution (20 ml/liter), vitamin solution (2 ml/liter), and antifoam 289 (50 ml/liter). The preparation of the inoculum and the conditions of batch cultivation were carried out according to a previously described method (7).

Metabolic analysis

The amount of glucose was analyzed by an HPLC system with a refractive index detector and an Aminex® HPX-87H column at 65°C with 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 mL/min (13). Acetate, pyruvate, and succinate were determined by HPLC system Aminex® HPX-87H column. Glycerol was analyzed

by the HPLC system equipped with a charged aerosol detector (ESA, USA), using NH2P-50 4E column eluted with 75% (v/v) acetonitrile at a flow rate of 1 mLmin⁻¹ at 25°C (7). For the measurement of sugars and extracellular metabolites, cultivation media were sampled at intervals for 4 h and immediately filtered through a 0.45 mm-pore cellulose.

Assessment of alcohol productivity, acidification and invertase activity

The produced ethanol in medium was detected spectrophotometrically. A sample of batch culture from wild and MIG1-disrupted *S. cerevisiae* were measured separately every 6 h by a pH meter. The samples were taken out of both strain bioreactors and were thawed on ice. To prepare 1-ml sample, 0.5 ml of glass beads (0.75 to 1 mm in diameter) was added. The cells were disrupted in a bead mill for 30 min and centrifuged at 20,000g for 20 min. A 250-ml volume of the supernatant was mixed with 1.75 ml of sucrose solution (0.34 M in 0.1 M sodium citrate buffer, pH 5.0) and incubated at 30°C for 180 min. Placing the samples in the boiling water for 5 min and subsequently cooling them on ice stopped the enzymatic reaction. The background was subtracted by using samples which were not incubated at 30°C for 180 min but otherwise prepared in the same manner. The amount of formed glucose was determined as micromoles of glucose released per minute per gram of the cell mass and expressed as intracellular invertase activity.

CO₂ production

The assessment method of leavening ability was used for measurement of CO₂ production (13). Both strains of

wild 2805 and ΔMIG1 were inoculated separately to Lean dough consisting of 280 g of flour, 150 mL of water, 4 g of salt, and 8 g of fresh yeast. The dough was quickly mixed for 5 min at 30±0.2°C and placed inside the box of a fermentograph (Type JM451, Sweden) to monitor the rate of CO₂ production.

O₂ consumption

The consumption of oxygen was assayed as previously described (7). The Oxygen consumption rates of the samples from cultivations were evaluated with a Clark-type oxygen electrode with 100 mM glucose as substrate, while the value of the cell density at 600 nm was 20 units per milliliter and expressed as micromoles of O₂ consumed per minute per gram of cell biomass.

Statistical analysis

In order to identify the significant differences between the wild and mutant strains, Student's *t* test was carried out by SPSS 21.0 (Armonk, NY: IBM Corp. USA). Statistical significance was set with priori at p=0.05 level of probability.

RESULTS

Results were demonstrated as mean value and standard deviation of at least three independent experiments. During the exponential growth phase, the specific growth rate of ΔMIG1 strain (0.19±0.02 h⁻¹) was increased by 12% as compared to the wild type strain (0.17±0.01 h⁻¹) (Fig. 1a). The glucose consumption rates corresponded to the growth characteristics in each strain (Fig. 1b).

Table I. The concentration of metabolites produced by wild and ΔMIG1 strains under aerobic conditions.

Strain	Metabolite concentration (mM) ^a				CO ₂ (ml/cell*10 ⁻¹⁰)	Final pH
	Ethanol	Acetate	Pyruvate	Glycerol		
Wild	143±7	2.48±0.25	1.38±0.1	26.2±0.2	2.7±0.3	4.7±0.5
ΔMIG1	122.79±4	1.39±0.12	1.51±0.05	29.28±0.15	3.37±0.13	3.8±0.6

^a The concentrations of metabolites were determined after glucose was completely exhausted. For wild and ΔMIG1 engineered strains, the samples for metabolite measurement were taken at 16 h after starting batch cultivations. Values are given as means ± standard deviations in at least three independent experiments.

As expected in Δ MIG1 strain, the concentration of glucose in medium diminished faster within the time unit. In addition, the concentrations of acetate, pyruvate, glycerol, ethanol, and CO_2 in the media were measured when the glucose was exhausted. Compared to strain 2805, the acetate production in Δ MIG1 was reduced by 43.71%, while the concentration of pyruvate and glycerol produced by Δ MIG1 was increased by 9.5% and 11.75%, respectively, as compared to the wild type (Table I).

Moreover, Δ MIG1 strain showed a 14.13% decrease in the ethanol production in comparison with the wild type (Table I). The decline in the production of acidic intermediates in Δ MIG1 strain led to an increasing pH of the cultivation (Fig. 2a). The enzymatic invertase levels were found [65 ± 6.7 U/g (dry weight)] in wild strain, while the level was double [138 ± 5.8 U/g (dry weight)] for Δ MIG1 in the exponential growth phase and imposed glucose repression condition (Fig. 2b).

Throughout cultivation, the carbon dioxide formation increased linearly with time, and the specific CO_2 production rate of the Δ MIG1 was 10–20% higher than 2805 strain (data not shown). The

rate of carbon flux directed to CO_2 formation was about 24% (C-mol/C-mol). In batch cultivation, the rate of carbon flux directed to ethanol production was about 44% (C-mol/C-mol). To investigate whether the deletion of MIG1 would result in an increase of respiratory capacity, the oxygen consumption rates of the cell cultivation to mid-logarithmic phase were measured. The oxygen consumption rate of Δ MIG1 was not significantly affected by deletion of MIG1.

DISCUSSION

The yeast cells are capable of combining transformed DNA to their chromosome through homologous recombination process. During the metabolism of *S.cerevisiae*, glucose utilization exerts down-regulating effects on the metabolic genes of other sugars. In this study, the metabolic gene MIG1 was disrupted by homologous recombination through antisense technique using upward and downward N and C fragments of MIG1 in order to demonstrate the effects on the metabolic production of *S.cerevisiae*. It was observed that Δ MIG1 strain utilized more glucose in comparison with the wild

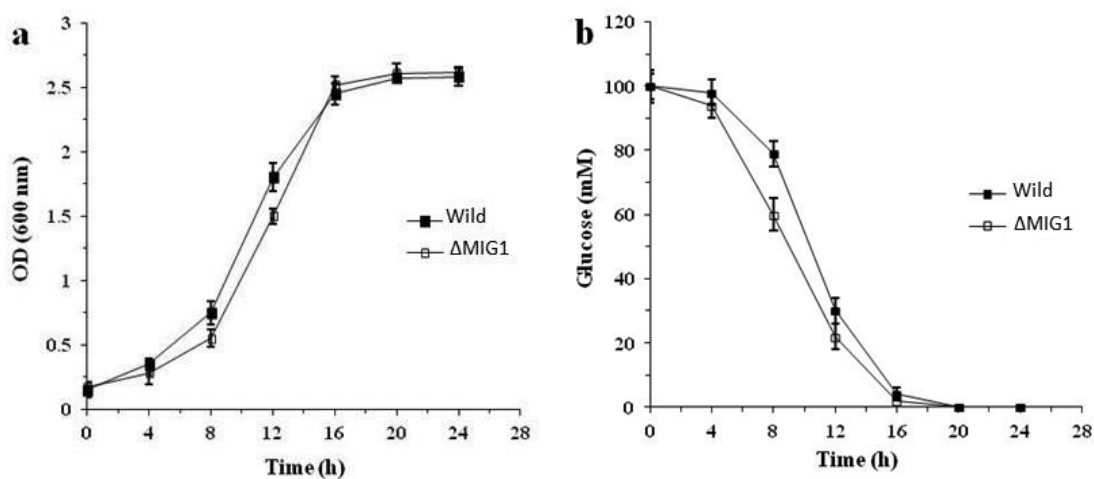


Fig. 1. *a)* The growth curves of the strains wild and Δ MIG1. *b)* The glucose consumption curves of the wild and Δ MIG1 strains. The strains were grown in mineral salt medium in 5 L bioreactors with a working volume of 3 L. The agitation rate was maintained at 400 rpm. The aeration rate was set at 3.5 L/min and maintained the dissolved oxygen tension above 50% of air saturation. The pH was controlled at 4.5. Closed squares represent wild strain; open squares represent Δ MIG1 strain. Mean values and standard deviations of at least three independent experiments are shown.

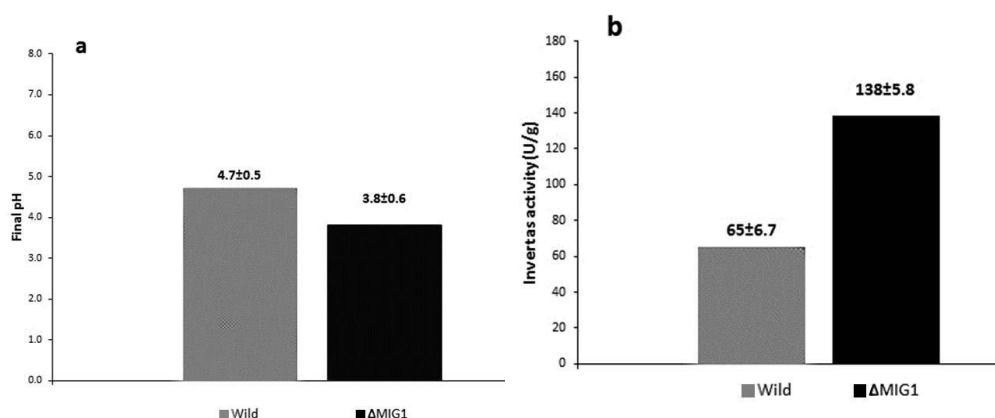


Fig. 2. a) The determined final pH of medium for wild and Δ MIG1 strains. **b)** The invertase activity of wild and Δ MIG1 strains measured at high glucose concentration (repressed conditions) during exponential growth phase of batch cultivations.

type strain within first 18 hours. Deletion of MIG1 is able to eliminate glucose repression partially from peripheral functions, rather completely from central metabolic functions (9). The invertase level in the wild type strain under equal glucose repressive conditions was found to be 65 ± 6.7 U/g (dry weight) and disruption of the target MIG1 gene, on the other hand, appeared to have affected translation as the invertase level was more than two times higher than that of the wild type under the same repressive conditions. Deletion of MIG1 gene can shift fermentative metabolism to aerobic pathway resulting in less ethanol production and promotion of TCA cycle leading to higher production of acidic derivative products (such as pyruvate) and CO_2 . The production of ethanol as an essential putative respiratory/fermentative compound explored by the HPLC method in mutant and wild strain cultivations determined 14.13% less production of Δ MIG1 strains. The other conducting metabolites, such as succinic acid, are an essential flavor component produced by *S. cerevisiae* in sakè fermentation. The deletion of MIG1 accompanied by Tup1 and Ssn6 corepressors in *S. cerevisiae* did not lead to any significant changes in its growth properties and had no meaningful effects on metabolism (13-14). Therefore, in single MIG1 deletion, the sufficient differences of metabolic parameters, i.e. sugar utilization and CO_2 production, support the strong correlations of MIG1 gene with metabolic switching

regulation of *S. cerevisiae*. Therefore, an effective alleviation of glucose control would help to achieve a better economic process for the cultivation of *S. cerevisiae* yeast, alcohol fermentation, bread-making and recombinant protein production (9, 15).

ACKNOWLEDGEMENTS

The Authors thank Mrs Eva Storey, president of Sign Shares International Interpretation Company, Houston, TX, USA for grammatical review.

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LETTER TO THE EDITOR

PHARMACOKINETIC CHANGES INDUCED BY FOCUSED ULTRASOUND IN GLIOMA-BEARING RATS USING DYNAMIC CONTRAST-ENHANCED MRI MEASUREMENT

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Received February 7, 2018 – Accepted march 14, 2018

It has been shown that focused ultrasound (FUS) can be effectively used for brain tumor therapy, and as a noninvasive, targeted drug delivery technique combined with microbubbles. In this study, magnetic resonance imaging (MRI) was used to measure the kinetics of Gadolinium diethylenetriamine Penta acetic acid (Gd-DTPA) in glioma-bearing rats. Ten glioma-bearing rats (9-12 weeks, 290-340 g weight) were used in the study. After the use of dynamic contrast enhancement (DCE)-MRI, the spatial permeability of FUS-induced blood-brain barrier disruption (BBB-D) was evaluated, and the kinetic parameters by the general kinetic model (GKM) were calculated. The results show that mean K_{trans} of the contralateral tumor was significantly lower than the mean K_{trans} of sonicated tumor. Moreover, the transfer constant K_{trans} in ultrasound tumors was closely related to tissue extravasation ($R = 0.95$), suggesting that DCE-MRI can determine drug accumulation in the brain. Except for a few small red blood cell extravasations, there was no macroscopic damage according to the histologic analysis. The study showed that the DCE-MRI can be a valuable tool in quantifying BBB permeability in tumors and monitor the kinetics of FUS-induced BBB-D processes.

To the Editor,

The blood-brain barrier (BBB) can block molecules of molecular weight above 180 Dalton due to its physiological and anatomical properties (1, 2). Therefore, even the most powerful therapeutic agents may be ineffective if they fail to reach the tumor cells outside the BBB. BBB has a low integrity in the vicinity of brain tumors, and selective permeability limits the therapeutic agents to reach the target so they are almost ineffective for the treatment of brain tumors (3). Despite several efforts using nanoparticle formulations, there are limitations due to side effects, especially on the

immune system (4, 5). Currently, in the presence of ultrasound contrast agent (UCA), focused ultrasound (FUS) can temporarily increase the permeability of BBB (6). The effect of FUS is reversible and it sets a time window for chemotherapy to maximize delivery to the target site of the brain tumor (7). In previous studies, FUS was reported to increase the ratio of tumor-to-normal tissue targeted by the drug, and the therapeutic effect for brain tumors was improved (8). In addition, FUS can be applied repeatedly to achieve high-dose chemotherapy and treat brain disease with minimal systemic toxicity. DCE-MRI is a method used in a non-intrusive way, which can

Key words: dynamic contrast enhancement, focused ultrasound, brain tumors, blood-brain barrier

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0393-974X (2018)

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be used to calculate the pharmacokinetic parameters of contrast agents within the tissue (9). In previous studies, DCE-MRI was frequently used to evaluate the kinetics of tumor-bearing rats (10).

In recent studies, some researchers described the kinetics of BBB permeability of normal brain after FUS exposure, using DCE-MRI. However, further research is warranted to establish the best theoretical model applicable in various conditions. It is also necessary to study the evaluation of brain tumor models. We also need to consider whether the kinetic model can be transformed into a vascular system. In the present study, we used DCE-MRI to detect changes in permeability after BBB-D as a function of time, in ultrasound-treated F98 glioma-bearing rats. Furthermore, the connection between transfer constant K_{trans} and tissue extravasation of Evans blue (EB) was estimated in the ultrasonic treated tumors.

MATERIALS AND METHODS

Production of human glioma model and experimental animals

Twenty-four rats (4-6 weeks, 20~30g weight) provided by the laboratory animal center of Mudanjiang Medical University were selected for the study. Glioma cell line SHG-44 was provided by Mudanjiang Medical University. 1640 medium (with 10% sera of newborn calves, 100g/L penicillin G, 100 g/L streptomycin was used to culture the glioma cell line at 37°C with 5% CO₂. The logarithmic growth phase cells of same generation were selected and resuspended in medium and the cell density was adjusted to 0.2 mL. Cell suspension was injected to the subcutaneous left and the right on the back of nude rats under the condition of local disinfection. After the establishment of human glioma nude rat model, 24 nude rats all presented a tumor. The tumor volume was (166.56±9.23) mm³ in 20d. The 24-subcutaneous tumor-burdened nude rats were divided into a control group and an irradiation group, 12 in each. Each group was divided into 6 subgroups of 2 rats each: 1d, 3d, 7d, 10d and 30d according to the time of animal death. This experiment was approved by the Ethics Committee of Affiliated Hongqi Hospital of Mudanjiang Medical University.

HIFU therapeutic apparatus and treatment parameter

CZF-U ultrasound therapeutic apparatus was developed by Chongqing HIFU Technology Co., LTD and provided by the Ultrasonic Engineering Research Institute of Chongqing Medical University. The apparatus was composed of three parts including power source, treatment probe and circulation degassed water cooling system. In the present study, the frequency of treatment probe was 9.7 MHZ with diameter of 9.5 mm, the focal length of 4.5 mm and the pulse of 1 MHZ. The maximum power output was 5w and the sound intensity at focus was 2500w/cm².

HIFU treatment methods

The nude rats were fixed on a special board. The tumor surface was coated with ultrasonic coupling agent, then, the parameters were input. In the control group, the probe was placed on the tumor surface coated with ultrasonic coupling agent but did not emit ultrasound. In the experimental group, the probe was placed to the tumor surface coated with ultrasonic coupling agent. The above treatment parameters were used for irradiation.

Preparation of the samples

Samples were prepared immediately after irradiation. The right side of the tumor sample was selected for *in vivo* electron microscopy and then the animals were sacrificed. The tumor sample on the left side was removed immediately and cut in the middle of the irradiated region. Some samples were cultured in a bath box at 37°C and dyed with 235 TTC. The rest of the samples were fixed with 4% paraformaldehyde. Paraffin sections were prepared using standard methods and observed by HE (haematoxylin and eosin) staining.

Data calculation and statistical analysis

A vernier caliper was used to measure the length (a), width (b) and depth (c) of nude rat tumors every day. The approximate volume of tumor was obtained according to the cubature (mm²) formula $V=abc\pi$ (π , ratio of a circle's circumference to its diameter). The doubling time was estimated using the formula $TD=t*\lg(V_t/V_0)$. In this formula, V_0 and V_t represent the approximate volume of tumor before the HIFU action and after the end of experiment respectively, and t represents the time elapsed during the start of HIFU action and the end of experiment.

If the extension of doubling time shows that HIFU can weaken the multiplication capacity of tumor cells and restrain the growth of tumor, then the cubature formula of necrotic tissue is calculated by $L \cdot W^2$. L represents the longest diameter (mm) of necrotic tissue, and W is the maximum transverse diameter (mm). The data is expressed by mean $\pm$ standard deviation, and the data processing was made using SAS 8.2 statistical package. The statistical analysis was calculated by variance used in repeated measurement data.

RESULTS

After GdDTPA administration, a series of DCE-MRI were performed to assess the kinetics of BBB permeability in the tumor. This process lasted for 1 h. Representative examples of DCE-MRI scans at 20 min and 24 h after FUS treatment of two fibrous rats are depicted in Fig. 1. Before Gd-DTPA injection, the signal enhancement was not found in T1-weighted MR images. BBB permeability was significantly increased in the right ultrasound treated tumors compared with the control tumors, especially 20 min after sonication. This study demonstrated that BBB permeability gradually decreased with the passage of FUS-induced BBB-D.

The middle cerebral artery (MCA) was selected

to measure the plasma Gd-DTPA concentration and the subsequent apoptosis inducing factor (AIF) parameters. The AIF was adjusted to the double exponential equation to produce an average of the amplitude constant and the decay rate, that is, $(A1, m1, A2, m2) = (0.168, 0.011, 0, 2, 0.521)$, which can be used to fit ectopic endocrine syndrome (EES) concentration. FUS treatment was divided into groups of brain tumors: 20-min FUS tumors (treated for 20 min); 24-h FUS tumors (treated for 24 h.) The 20-min tumor group was the corresponding heterolateral control tumors of the 24-h tumor in the tumor group. The estimation of mean changes in GdDTPA concentration in the sonicated tumor, the contralateral tumor and the ipsilateral brain can be seen in Fig. 2. In the tumor site, the 20-min FUS tumor group had the highest Gd-DTPA concentration. Differences in Gd-DTPA concentration curves in the 20-min FUS tumor group and 24-h FUS tumor group were higher compared to the contralateral tumor of the 20-min tumor and 24-h tumors. As shown in Fig. 2, the data set of the lowest Gd-DTPA concentration included the left contralateral brain and ipsilateral brain in 20-min FUS tumors and 24-h FUS tumors. No significant differences were detected among them.

Compared to the control tumors in the left hemisphere, rats 1 and 2 which located in the right

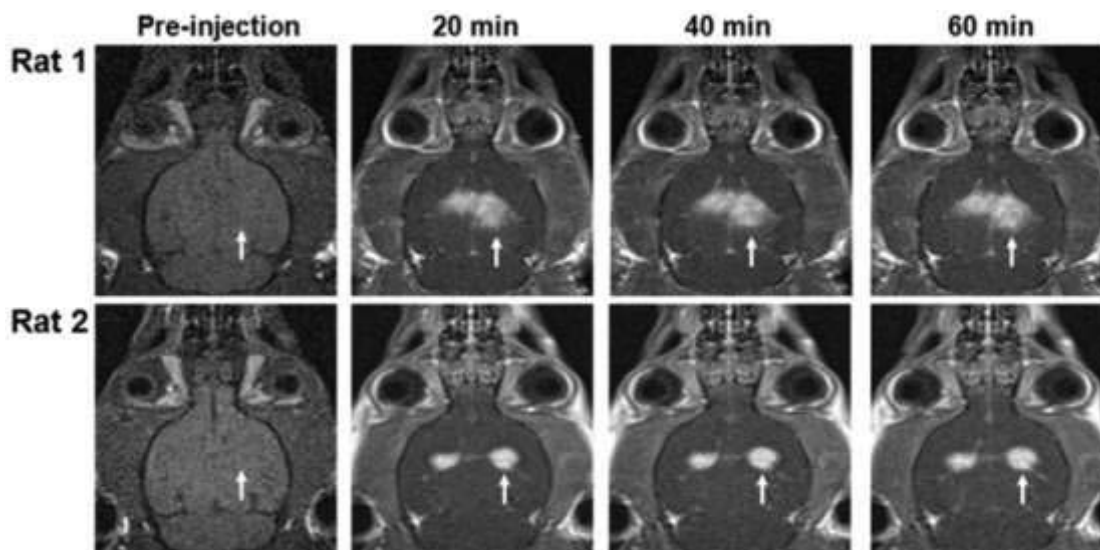


Fig. 1. Axial DCE-MRI in time series used for FUS-induced BBB-D in brain tumors around Gd-DTPA management.

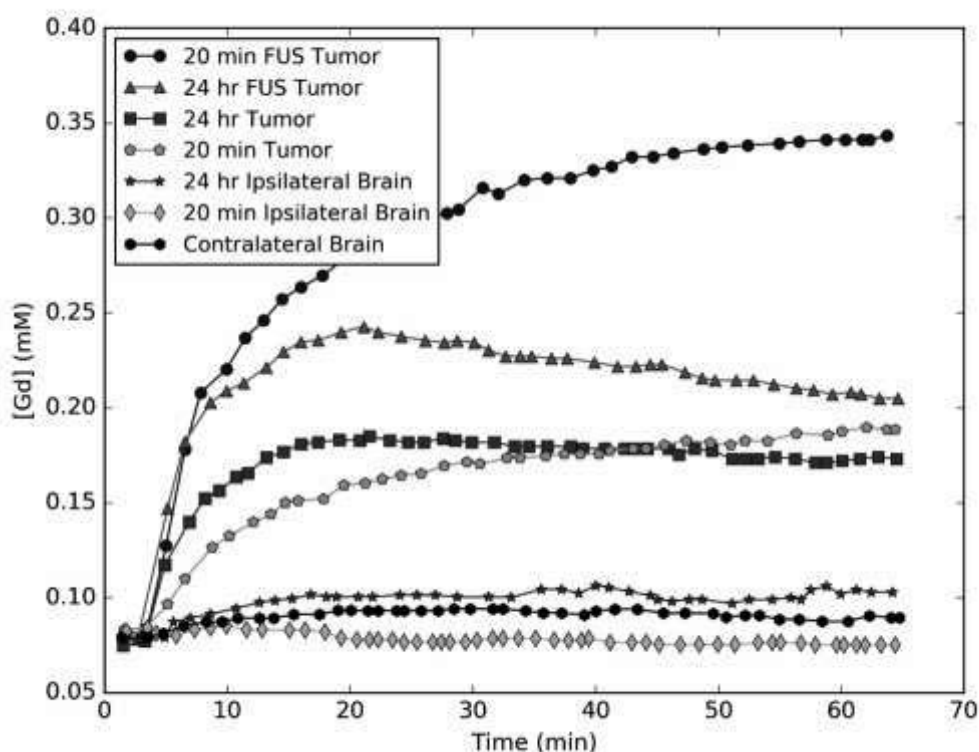


Fig. 2. The approximate calculation of Gd-DTPA concentration using ROI in the brain tumor, ipsilateral brain and contralateral normal brain after sonication between 20 min and 24 h.

ultrasound treated brain (arrows), show continuous contrast changes at 20 mins and 24 h after sonication, respectively.

EB entered the brain tumor in both ultrasound treated tumors and non-ultrasound treated tumors, as shown in Fig. 3. Analysis of typical brain slices showed that the size and color intensity of EB in the right ultrasound-treated brain was significantly greater compared to the size and color intensity of the left control tumor (Fig. 3A). As shown in Fig. 3B, the mean extravasation of EB in the brain (in ug/g tissue) after sonication was supported by the intravenous injection of EB for 3 hours and 27 hours of tumor support. Compared with these two extravasations, the degree of EB extravasation in the ultrasound-treated tumor had a higher advantage. There are significant differences between the two groups of ultrasound treated tumors because of the EB administration. Compared to the 3-h EB-injected tumor after sonication, the BBB integrity had been in part rebuilt at 27 hours. In the region of interest

of the ultrasonic tumor, contralateral tumor and the ipsilateral brain, the average K_{trans} and K_{ep} values were originated from DCE-MRI. The average permeability coefficients K_{trans} and K_{ep} using GKM and $V_e = K_{trans} / K_{ep}$ are shown in Table I. Moreover, the correlation coefficients related to EB extravasation are also presented. The mean K_{trans} was significantly lower in the contralateral tumor (0.05260.019) than in the 20-min FUS Tumor (0.12860.019). However, since BBB was partially recovered 24 hours after FUS exposure, there was no significant difference in mean K_{trans} between 24-h FUS tumors (0.10360.023) and contralateral tumors (0.05860.012). Additionally, the mean K_{trans} in the ipsilateral brains of 20-min and 24-h were significantly lower compared to the respective mean K_{trans} in the opposite tumor. Furthermore, compared to the mean V_e (0.85), the mean EB extravasation values and K_{trans} in brain tumors have a higher correlation coefficient (0.95).

Fig. 3A shows the distribution of BBB-D in the

right brain tumors after 3 and 27 hours of sonication (arrows). Left brain tumors did not receive ultrasound and were used as control tumors. Fig. 3B shows the number of EB in tumors and ultrasound-treated tumors after 3 and 27 hours of FUS exposure.

In Table I, R represents the correlation coefficient between the parameter and EB extravasation. In addition, * and # represent the divergence compared with the contralateral tumor and ipsilateral brain (* $P < 0.05$, # $P < 0.01$).

After sonication between 20-min and 24-h, MR images were selected as tumor-bearing rats for noninvasive monitoring of tumor growth, and then it was used as a function of time (the left column in Fig. 4). From 20 mins to 24 hours after sonication, histological observations of the brain were obtained.

Red blood cell mild effuses were observed in the treatment of tumor tissue and the surrounding focus area (the middle and right columns in Fig. 4).

Rat 1 demonstrated contrast changes and histology in the right sonicated brain tumors 20 min after sonication. Rat 2 demonstrated contrast changes and histology in the right sonicated brain tumors 24 h after sonication. The right column demonstrates the amplification of the rectangular region in the H & E staining of the whole brain slice. The arrow indicates the brain tumor area.

DISCUSSION

The biological focal region (BFR) was first

Table I. Quantitative kinetic parameters estimated from DCE-MRI.

DCE-MRI	Parameters	n	K_{trans}	K_{ep}	V_e
FUS	20 min	5	$0.137 \pm 0.018^*$	0.023 ± 0.021	3.01
Tumor	24 h	5	0.124 ± 0.214	0.098 ± 0.241	1.08
Contralateral	20 min	5	$0.521 \pm 0.127^*$	0.128 ± 0.182	2.47
Tumor	24 h	5	$0.057 \pm 0.021\#$	0.073 ± 0.041	0.76
Ipsilateral	20 min	5	0.087 ± 0.043	0.049 ± 0.034	0.18
Brain	24 h	5	0.018 ± 0.048	0.089 ± 0.024	0.13
EB	R		0.86		0.86

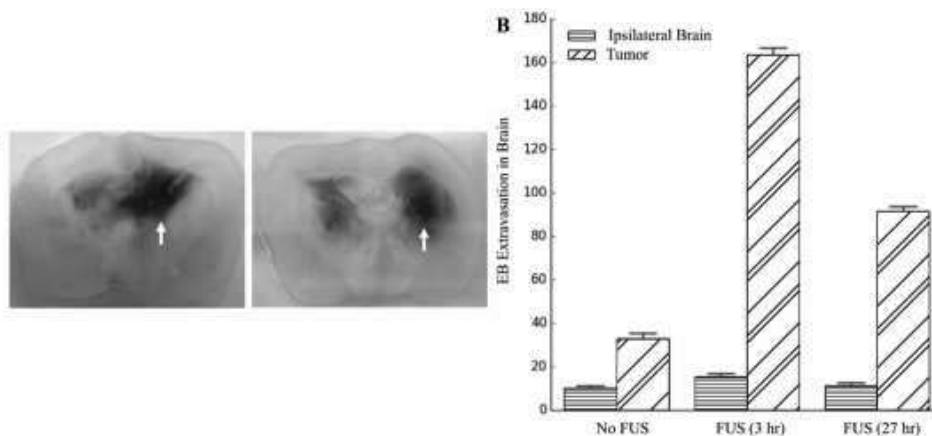


Fig. 3. EB extravasation of brain tumors based on sonication and without sonication.

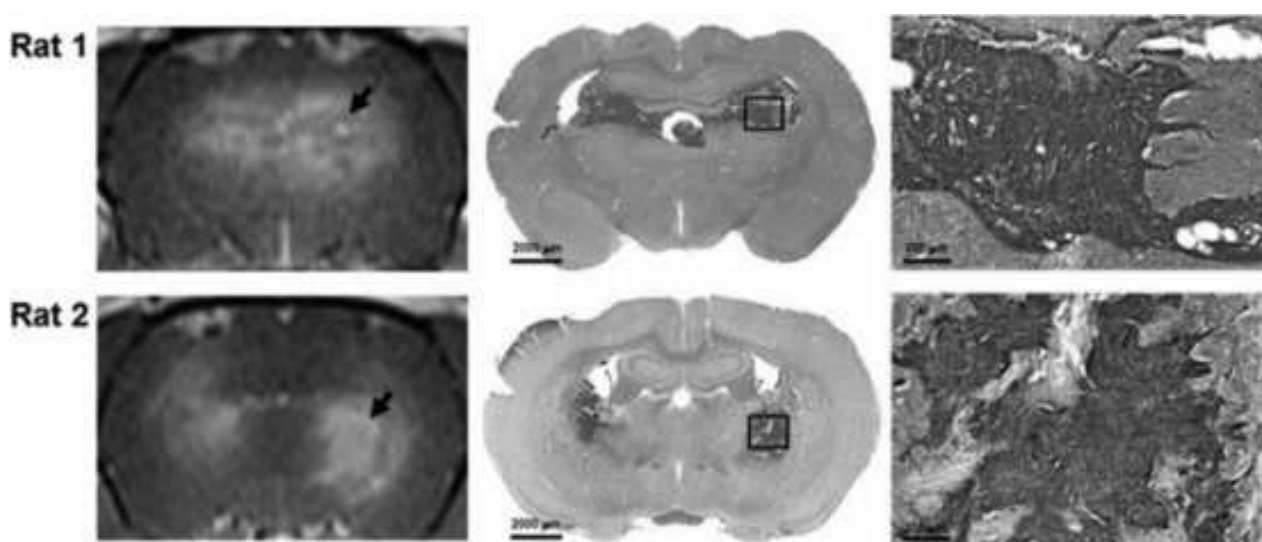


Fig. 4. The tumor sample with and without FUS exposure based on T1 weighted MR images and hematoxylin-eosin stained sections after 8 days of implantation.

proposed by Wang Shutao (11). It refers to the range of coagulation necrosis formed by energy accumulation in HIFU, which is the result of the interaction between acoustic focal field and acoustic dose and organizational structure and function. Triphenyltetrazolium chloride (TTC) staining is a method of assessing the activity of dehydrogenase in tissues. It can distinguish necrotic tissue from normal tissues. The normal tissue is dyed red and the necrotic tissue is not colored, therefore can be clearly seen (12). It was found that TTC staining is accurate, reliable and quick to observe the high intensity ultrasound biological focal fields. It improves the detection rate of the focal region of the naked eye (13). The frequency of the experiment was 9.7 MHz, the diameter was 9.5 mm and the focal length was 4.5 mm. The pulse was 1 MHz, the maximum output power was 5 W and the focal point was the sound intensity of 2500 W/cm² irradiation parameters. Nude rats subcutaneous gliomas were treated for 20 s, TTC staining showed significant necrotic areas, and there was a sharp boundary between the necrotic area and the normal area. The size of the necrosis was calculated as (66.12±2.52) mm³. The necrosis size was generated by HIFU after the treatment

parameters and dose were applied. Therefore, it could be regarded as the biological focal domain of glioma. Different tissues have different biological focal fields, and the study of tissue focal region is of great importance to the effectiveness and safety of HIFU therapy.

In this paper, DCE-MRI was used to describe BBB permeability in the rat brain tumor model, which is related to the tissue accumulation of measured EB in the ultrasound-treated tumor. In the brain tumor, we can see a good linear correlation between the transfer constant K_{trans} and the EB accumulations. To our knowledge this is the first study that investigated the application of DCE-MRI in the quantitative evaluation of BBB permeability in brain tumors after sonication. In future studies, pharmacokinetic models needs to be developed to assess the permeability of the capillaries and improve drug delivery by FUS-induced BBB-D.

ACKNOWLEDGEMENTS

This work was supported by Mudanjiang City Science and Technology Plan Projects (NO. Z2016s0101).

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LETTER TO THE EDITOR

EVALUATION OF SAFETY AND EFFICACY OF DIFFERENT CONTINUOUS BLOOD PURIFICATION METHODS IN TREATING INFANTILE SEPSIS

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The aim of this study was to compare the safety and the efficacy of two methods of continuous blood purification (CBP), continuous veno-venous hemofiltration (CVVH) and high volume hemofiltration (HVHF), for treatment of infantile sepsis. Eighty-six children with sepsis were enrolled in this study and randomly divided into two groups with 47 cases in the CVVH group and 39 cases in the HVHF group. Survival rate, duration of blood filtration, mean arterial pressure (MAP), mean heart rate and SaO_2 , APACHE II score, procalcitonin, hs-CRP and TXB2 were compared between the two groups. Results showed that survival rate, MAP, mean heart rate and SaO_2 in the two groups did not have any significant differences. Duration of blood filtration and APACHE II score in the HVHF group was significantly shorter than that in the CVVH group. After therapy, levels of procalcitonin, hs-CRP and TXB2 declined dramatically in both groups, however this reduction was more significant in the HVHF group. We conclude that HVHF is a safer and more effective method as it produced stable hemodynamics, shorter filtration time, better APACHE II scores and better results in alleviating inflammatory reactions.

To the Editor,

Infantile sepsis is a leading cause of infant death after severe infection. Morbidity rate ranges from 1.5-3.6% while the mortality rate is 50-75% (1). Sepsis is regarded as a consequence of imbalanced systemic inflammatory response (2). Researchers hypothesized that continuous blood purification (CBP) could effectively eliminate a variety of inflammatory mediators, adjust the immune imbalance, increase the therapeutic time and rapidly clear toxic material in circulation (3). Using therapeutic CBP could decrease the mortality rate by 30-60%, which symbolizes a vital progress in sepsis therapy (4). Since different characteristics of biological membranes had different efficacies in eliminating various mediators, different types of hemofiltration

models had different therapeutic efficiencies (5). To date, there have been several methods for CBP such as CVVH, continuous veno-venous hemodiafiltration (CVVHDF), HVHF and continuous plasma filtration adsorption (CPFA). According to previous studies, using a combination of different methods may reasonably increase their therapeutic effects (6). Successful use of hemoperfusion, double hemofiltration and molecular absorbent recycling system has been reported (7). The applied safety and efficacy of CVVH and HVHF in the treatment of infantile sepsis were compared in this trial in order to provide evidence for clinical therapy.

From January 2013 to January 2016, 86 children diagnosed with sepsis in our hospital were enrolled. All cases were subjected to continuous blood purification

Key words: CBP, CVVH, HVHF, infantile sepsis, survival rate, APACHE II score, procalcitonin, hs-CRP; TXB2

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0393-974X (2018)

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(CBP). Diagnosis was verified with standards of "International guidelines for the treatment of sepsis and septic shock in 2008" (8). The subjects with severe sepsis, expected survival less than one month, multi-resistance bacterial infection, futile treatment of primary diseases, and voluntary interruption of CBP treatment were excluded. The trial was approved by the Ethics Committee of the hospital and informed consent was obtained from parents of the children. The patients were randomly divided into two groups: 47 cases in the CVVH group and 39 cases in the HVHF group. In the CVVH group, there were 28 males and 19 females, aged 2-10 years (average 6.2 ± 3.6). The body weight was between 9.5 and 25 kg (average 16.3 ± 5.5) with an on-set duration of sepsis from 1 h to 2 d (mean = 20.6 ± 7.8 h) and the APACHE II score from 42 to 63 points (mean = 50.2 ± 6.7). Twenty-six cases of severe pneumonia, 13 cases of intestinal infection, 6 cases of post-operation and 2 infections caused by other bacteria were included in the CVVH group. Twenty-two cases and 25 cases were infected with gram-positive and -negative bacteria, respectively, and amongst them 6 cases were suffering from complicated single-organ dysfunction, 2 cases with two-organ dysfunction and 1 case with multi-organ dysfunction. In the HVHF group, 20 males and 19 females with an age range from 1.5 to 9 years (mean = 6.0 ± 3.9 years) were included. The body weight was from 9 to 26 kg (with an average of 16.2 ± 5.6 kg). The on-set time of sepsis lasted from 1.5 h to 1.5 days (with an average of 18.6 ± 7.5 h) and the APACHE II score ranged from 40 to 62 points (with an average of 50.0 ± 6.5). In the HVHF group, 17 cases suffered from severe pneumonia, 10 cases with intestinal infection, 10 cases with post-operation infections, and 2 cases with other infections. Twenty cases were infected with gram-positive bacteria and 19 cases with gram-negative bacteria and amongst them 4 cases suffered from complicated single-organ dysfunction, 2 cases had two-organ dysfunction while 1 case had multi-organ dysfunction. The basic demographic data between the two groups were comparable.

Regular therapy included treatment of primary disease, anti-infection, applying vasoactive drugs, maintaining circulation volume and stable internal environment, nutrition and assistant mechanical ventilation on occasion. 6.5–8 F double cavity femoral

or external jugular vein catheter was used in the CVVH group accompanied by Baxter BM25 hemofiltration apparatus and children-type pipeline (MFp for weights under 5 kg and AV400s for weights above 5 kg). Retrograde autologous priming through extracorporeal circulation and heparin were used for anticoagulation with initial doses of 0.25-0.5 mg/kg and maintaining doses of 0.125-0.5 mg/kg/h, and which were adjusted according to monitoring activated clotting time (ACT). Modified solution for replacement was prepared using the following protocol: mix A: normal saline (3000-3250 ml), 5% glucose (250- 500 ml), water (500-750 ml), 1- 1.5 g calcium chloride, 25% magnesium sulfate (3 ml), 10% potassium chloride (up to 12 ml) and mix B: 5% sodium bicarbonate (250-375 ml). Mix A and mix B were synchronously introduced into pipeline with 1~1.5 L/kg of total amount of solution for replacement in 24 h. The initial treatment lasted for 24-48 h and continued for 3-12 days with an interval of 12- 24 h. Prismaflex hemofiltration apparatus (Gambro AB) was used in the HVHF group. The pipeline, anticoagulation and displacement liquid utilized were same as the CVVH group. Parameters were set as follows: 50 ml/min for blood flow velocity, 10 ml/kg/h for replacement liquid velocity, >15 ml/kg/h for flow of replacement liquid which could be modified as 50-200 ml/h while dehydration, >10 L/d for replacement volume, 80% for pre-dilution. We continued treatment every 12 h or 24 h for 3 to 12 days in total.

Survival rate, MAP, mean heart rate, SaO_2 , duration of hemofiltration, APACHE II score, procalcitonin, hs-CRP and TXB2 of the two groups were compared. Hs-CRP was measured regularly by automatic biochemical analyzer (Hitachi 7170, Japan) and procalcitonin and TXB2 were assessed with ELISA kits (ZSGB-BIO) according to the manufacturer's instructions. Quantitative data were expressed as cases or percentages (mean $\pm$ standard deviation) and analyzed with SPSS19.0 software. The comparisons between groups were conducted with *t*-test and intra-group with paired *t*-test. The comparisons among groups were accessed with χ^2 test. $P < 0.05$ was used to indicate significant difference.

In the CVVH group, 9 deaths (19.1%) occurred: 4 caused by respiratory failure, 2 by circulatory failure, and 2 by renal failure, while one was attributed to other

Table I. Comparison of MAP, mean heart rate and mean SaO₂.

Groups	MAP (mmHg)		Mean heart rate (b/min)		Mean SaO ₂ (%)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
CVVH	30.4±3.5	35.2±3.7*	85.6±6.4	81.3±6.3*	85.6±5.2	95.7±5.9*
HVHF	31.2±3.6	33.6±3.8*	84.2±6.5	82.2±6.6*	84.7±5.5	96.0±5.3*
t	0.623	0.326	0.421	0.257	0.429	0.120
P	0.587	0.795	0.630	0.863	0.632	0.869

P*<0.05, relative to before treatmentTable II.** Comparison of duration of hemofiltration and APACHE II score.

Groups	Duration of hemofiltration	APACHE II score after treatment
CVVH	4.6±1.0	32.6±7.2
HVHF	2.3±1.2	25.7±5.6
t	6.328	6.754
P	0.035	0.030

Table III. Comparison of procalcitonin, hs-CRP and TXB₂.

Groups	Procalcitonin, (ng/mL)		hs-CRP (mg/L)		TXB ₂ (ng/L)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
CVVH	15.6±3.4	8.3±2.7	12.5±3.9	7.4±2.0	84.2±21.3	56.9±15.7
HVHF	16.0±3.5	5.7±2.5	12.8±3.6	4.9±2.2	85.3±25.2	35.2±13.6
t	0.625	6.203	0.632	6.325	0.459	6.425
P	0.721	0.030	0.864	0.026	0.632	0.023

causes. In the HVHF group, 8 children (20.5%) died: 4 caused by respiratory failure, 1 by circulatory failure, 1 by renal failure, and 1 because of other causes. The survival rate in the CVVH group and in the HVHF group had no statistical difference. ($\chi^2=0.025$, $P=0.874$).

Between the two groups, MAP, mean heart rate and mean SaO₂ were compared prior to and after treatment and showed no statistical significance ($P>0.05$, Table I). For intra-group comparison, MAP and mean SaO₂ were significantly increased and mean heart rate significantly decreased in both groups after treatment ($P<0.05$). (Table I). Duration of hemofiltration in the HVHF group was considerably shorter than that in the CVVH group. Also, APACHE II score decreased considerably in the CVVH group. All differences were statistically significant ($P<0.05$) (Table II). Procalcitonin, hs-CRP and TXB₂ in both groups were all significantly decreased after treatment, particularly in the HVHF group ($P<0.05$) (Table III).

Treatment of infantile sepsis with CBP can effectively eliminate circulating inflammatory mediators to block inflammatory cascade reaction and undermine the progress of systemic inflammatory reaction, which can improve the prognosis. Through plasma filtration and adsorption during CBP, endotoxins can be eliminated and microcirculation, oxygen intake and utilisation in tissue can be enhanced by interstitial water removal. Azotemia, imbalance of water, electrolyte and acid-base can be efficiently corrected with the treatment to create an ideal environment for nutrition and metabolism.

ISCCN (9) has suggested to classify the hemofiltration according to different replacement volume in adults: <35 ml/kg/h as very low volume hemofiltration (VLVH), 35-50 ml/kg/h as low volume hemofiltration (LVH), 50-100 ml/kg/h as high volume hemofiltration (HVH) and >100 ml/kg/h as very high volume hemofiltration (VHVH). In terms of intensive care unit (ICU) dosage range for adults, it was suggested VLVH to be insufficient dose, LVH for renal dose and HVH for dose of sepsis. So far, it is not yet proven whether therapy with replacement volume of $<45\sim 50$ ml/kg/h can work on sepsis cases. Using pig sepsis model, Grootendorst et al. (10) discovered that HVH improved the low blood pressure and cardiac function induced by endotoxins. Clinical trials showed that after HVH, hemodynamics of sepsis patient was improved

with a reduction in vasopressin medication while the survival time apparently extended (11).

Since the immunity and ability of renal removal in children were not as strong as adults, HVH has been not considered as a priority choice in infantile sepsis. Alternatively, regular replacement volume through HVHF was suggested to be safer with good outcomes on toxin elimination (12). Our study showed no significant differences for survival rate, MAP, mean heart rate and mean SaO₂ in the two groups (CVVH and HVHF), which indicated that HVHF stabilized hemodynamics without raising the mortality. Shortened duration of hemofiltration and improved APACHE II score also reflected that HVHF could be much safer and could gain better benefit compared to CVVH.

Procalcitonin, hs-CRP and TXB₂ in both groups decreased after treatment, especially more obviously in the HVHF group. Procalcitonin, which is a non-steroid anti-inflammatory mediator, usually plays an important role in regulating the cytokine network. The International Sepsis Definitions Conference in 2001 mentioned procalcitonin as an important diagnostic indicator (13). Hs-CRP is widely acknowledged as sensitive indicator of early stage inflammatory reaction, which is associated with occurrence, development and prognosis of the disease. TXB₂ reflected coagulation function in vivo. Common complication of CVVH is thrombosis in pipeline and in vivo, with morbidity rate of 1.5- 3% and a high mortality rate (14). Compared with CVVH, HVHF better improved procalcitonin, hs-CRP and TXB₂ in vivo.

In conclusion, HVHF appeared to be a safer and more efficient method for treatment of infantile sepsis with stable hemodynamics, shorter filtration time, better APACHE II score and ability to alleviate inflammatory reactions.

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LETTER TO THE EDITOR

TRANSURETHRAL FLUORESCENCE CYSTOSCOPY GUIDANCE FOR TOTAL RESECTION OF BLADDER TUMOR

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Received August 21, 2017 – Accepted March 27, 2018

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The purpose of this work is to investigate the total resection of bladder tumor under transurethral fluorescence cystoscopy. Nineteen patients with bladder tumor, from which we resected a total of 26 tumors, including 16 single tumors with diameters of 0.5~2 cm, were enrolled in the study. All tumors were located in the posterior wall or neck of the bladder. For the surgery, the size and location of tumors in the bladder were observed by fluorescence cystoscopy. Then, plasma electrocision was used to cut the full-thickness of the bladder to the fat outside of the bladder along the near-end of the tumor, then along the left and right side of bladder (to the far-end), and the full-thickness of the tumor was resected. Finally, the far-end tumor was removed and the full-thickness of the bladder at the bottom was completely resected. All operations were completed successfully within 10-40 min. There was little bleeding during surgery and no secondary bleeding after surgery. Tumor staging found 17 patients at T1 stage (20 tumors) and 2 patients at T2 stage (6 tumors). Patients were followed up for 6~12 months without any recurrence. We show here that total resection of bladder tumor can be accomplished under transurethral fluorescence cystoscopy and preventative resection can be conducted on the suspicious bladder wall with precision to eliminate tumor residue that promotes recurrence.

To the Editor,

Transurethral resection of bladder tumor (TURBT) is a commonly used surgical method for treating invasive non-muscle bladder cancer. But this method can easily leave tumor residues that are responsible for a significant number of tumor recurrences (1). Based on the principle of whole tumor removal, complete tumor resection from the normal tissue surrounding it can effectively lower the chances of dissemination and growth of tumor cells (2). We report here the diagnosis

and treatment of 19 bladder tumor patients to total tumor resection under transurethral fluorescence cystoscopy.

MATERIALS AND METHODS

Patients

Nineteen patients with bladder tumor (ages 44~83 years, average: 63.53±12.35 years) were enrolled in the study and signed the informed consent form of diagnostic electrocision before surgery. The patients

Key words: bladder tumor, cystoscopy, recurrence, surgery, follow-up

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0393-974X (2018)

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received urinary system color Doppler ultrasound before surgery. Three hydronephrosis patients underwent intravenous pyelography and computed tomography (CT) examination of the whole abdomen, and upper urinary tract tumors were excluded. Next, sacral canal block anesthesia was administered. A total of twenty-six tumors with diameters 0.5~2 cm were resected. All tumors were located in the posterior wall or neck of the bladder. The size and position of tumors in the bladder were observed by fluorescence cystoscopy. Then, plasma electrocision was used to cut open the full-thickness of the bladder to resect the entire tumor, which was sent for pathological examination.

Methodology

GYRUS plasma kinetic vaporization was used as surgical tool. The surgery was conducted under the supervision of a TV monitoring system. Patients received sacral anesthesia at lithotomy position. Normal saline was used for flushing during surgery.

The size and position of the tumors in the bladder were observed by fluorescence cystoscopy: injection of 40 ml (40 mg) (2''R)-4'-O-tetrahydropyranyl doxorubicin (THP) for 20-30 min. The fluorescence cystoscope was inserted to evacuate the THP from the bladder, which was repeatedly flushed by 500 ml normal saline. The bladder was filled with flushing fluid. When there was no reductus on the bladder wall, we used white and fluorescence lights to observe the bladder wall at short range and observe tumor size, position in the bladder, and whether there was any other suspicious areas.

All minimal lesions positive for fluorescence in the bladder were first treated with electrocision or electrocautery. Then plasma kinetic vaporization was used to cut open the bladder mucosa, submucosa, and muscular layer along the tumor near end until reaching

the fat outside the bladder. The incision was conducted from both the left and right sides towards the far end until the whole layer was cut off. Finally, the tumor far end was cut off, and the tumor and the basal bladder whole layer were both cut off. The bleeding was stopped after completing the surgery. The sample was directly extracted with an Elik flusher from the sheath of plasma kinetic vaporization. The complete tumor and the all the underlying bladder layers were removed and sent for pathological examination. After surgery, a catheter was maintained for 7 days and it was not necessary to continue bladder irrigation.

RESULTS

A fragment of the tissue was sent for testing drug sensitivity after bladder surgery. According to the result of the drug sensitivity test, the most effective chemotherapeutics were selected for bladder irrigation one week after surgery. Of the 19 cases, 13 received epirubicin, 5 THP, and 1 mitomycin for infusion chemotherapy.

The patients were examined by fluorescence cystoscopy three, six, and twelve months after surgery. The wounds were well healed without formation of vesical diverticulum. Two cases observed were at T2 stage and one case at T1 stage with suspicious wound by fluorescence cystoscopy. Five basal tissues were sent for pathological examination. Pathology indicated bladder with chronic inflammation accompanied by proliferation of fibrous tissue. No recurrence was found among this group of patients 12 months after surgery.

In summary, patients received sacral anesthesia and all tolerated the surgery. The operation time was 10~30 min, averaging 23.95 ± 8.39 min. There was rarely bleeding during surgery and no secondary bleeding after surgery. Staging of pathological section of tumor after surgery found 17 patients at T1 stage (20 tumors) and 2 at T2 stage (6 tumors). Patients were followed up for 6~12 months without any instance of recurrence (Table 1).

DISCUSSION

In China, the morbidity of male bladder cancer

Table 1. Pathological staging and number of tumors.

Pathology	Cases	Number of tumors
T1	17	20
T2	2	6

ranks seventh among all malignant tumors, but for females is not among the top ten cancers (3). At present, most hospitals perform transurethral resection as the most common approach for invasive non-muscle bladder cancers. This procedure resects the tumors totally until the normal bladder wall muscular layer is exposed. However, for low risk invasive non-muscle bladder tumors, the recurrence rate after standard TURBT is 35~70%, with 10~50% of tumors having further development. In particular, the recurrence rate of single tumor within 3 months is 0~15% and the recurrence rate of multiple tumors is 7~45% (4). Routine WLC cannot effectively identify the minimal lesions of the mucous membrane, like atypical hyperplasia, flat lesion, and carcinoma *in situ*. This results in incomplete tumor resection by traditional TURBT, which is the major reason for recurrence of bladder tumor (5). Fluorescence cystoscopy (FC) has been increasingly applied in the diagnosis and treatment of bladder tumors, and TURBT guided by FC has proved to be effective for identifying all tumor fragments and reducing the recurrence rate (6). The Chinese Urinary Surgical Diseases Treatment Guideline of 2014 recommended electric resection under FC guidance to improve the discovery of tumors and reduce the recurrence rate of bladder tumors (7). Data suggest that the diagnosis and treatment of bladder cancer with FC has 87-98% sensibility, 50-75% specificity (8, 9), and nearly 95% sensibility of diagnosis and treatment of carcinoma *in situ* (10). G. Heqing et al. found that performing TURBT with 5-ALA FC greatly reduces the recurrence at early postoperative period for non-muscle invasive bladder cancer (11).

Conventional TURBT consists of cutting the tumor into pieces, then the Ellik flusher is used to extract tumors via the resectoscope sheath. This procedure can cause the spread of tumor cells throughout the whole bladder cavity, which is against the non-tumor principle and can easily cause tumor cell seeding. One century ago, Albarran and Imbert proposed that the postoperative recurrence of bladder tumors is related to seeding of floating tumor cells. Based on the non-tumor principle, precise resection of the whole tumor from the normal tissue can effectively reduce the chances of dissemination and seeding of tumor cells. Y.

Hualiang et al. compared the efficacy of transurethral 2 μ m laser partial bladder excision and transurethral bladder tumor electrocision to cure T1G3 bladder cancer. Reexamination via cystoscope four weeks after surgery revealed that pathological analysis of wound surface biopsy showed 6 positive cases (23.1%) in the electrocision group compared to no positive results in the 2 μ m laser group (12). The analysis also showed that after the first electrocision, 23.1% of patients had tumor cell residues, thus causing easy recurrence after surgery. Although a second round of TURBT could be performed, it would aggravate the pain of patients and their financial burden (13).

The bladder wall consists of three layers: the internal mucous layer, the middle smooth muscle layer, and the external fibrous membranes composed of connective tissue. It is relatively easy to peel off the connective tissues between the muscular and outer layers of the bladder. Under the resectoscope, it is easy to find the dissection plane, making it possible to cut the whole layer of tumor by bladder partial excision under endoscope as the open bladder partial excision. Some Chinese hospitals have reported good efficacy by conducting partial bladder excision via 2 μ m laser, which can be applied for many indications. However, the high cost of the laser makes it prohibitive for many hospitals. Instead, plasma electrocision has been popularized in many hospitals in China and this technology can be easily popularized in bladder partial excision for treating bladder tumors. Since the obturator nerve reflex may cause bladder perforation and bladder upper wall, anterior wall perforation into enterocoelia, plasma bladder partial excision is usually applied for patients with tumors at the posterior wall or bladder neck.

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LETTER TO THE EDITOR

EFFECTS OF LOW MOLECULAR WEIGHT HEPARIN IN THE TREATMENT OF VENOUS THROMBOEMBOLISM IN PATIENTS WITH GASTROINTESTINAL CANCER

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This study aimed to observe the safety and effectiveness of different dosing regimens of low molecular weight heparin in the prevention of venous thromboembolism (VTE) and to provide a guideline for the treatment of individualized VTE prophylaxis in patients with postoperative gastrointestinal tumors. Forty patients with high risk for VTE after surgery for gastrointestinal tumors treated in Hongqi Hospital of Mudanjiang Medical University in the period October 2016 to May 2017 were included in the study. The patients were randomly divided into two groups, group A that included 24 patients treated with low molecular weight heparin (LMWH) 5000 IU every 12 hours, and group B comprised of 16 patients treated with LMWH 5000IU every 24 hours. On the seventh day post-surgery anti-Xa activity values reached effective anticoagulation (>0.5 IU/mL) in both groups. In group A, there was one case of anti-Xa activity exceeding 1.0 IU/mL, but no bleeding complications occurred. Using LMWH 5000 IU subcutaneous injection every 12 hours could also prevent postoperative VTE of gastrointestinal tumor, but the risk of bleeding complications is higher compared with 24-hour administration. Monitoring of plasma D-dimer within 7 days after gastrointestinal surgery did not show a great value for VTE monitoring. This study demonstrated that subcutaneous injection of LMWH can prevent VTE after gastrointestinal tumor surgery and provide a new alternative for VTE prevention.

To the Editor,

Venous thromboembolism (VTE) refers to a series of diseases caused by the abnormal coagulation of intravenous blood and obstruction of vena cava, including deep venous thrombosis (DVT) and pulmonary embolism (PE) (1). Abnormal coagulation may occur in chronic liver disease that affects the synthesis of coagulation factors such as non-alcoholic fatty liver, hepatic cirrhosis or vitamin K-dependent coagulopathies (2, 3). It is a disease with high incidence and high mortality in hospitals but also preventable (4). In 2004, the United States proposed in ACCP (American College of Clinical

Pharmacy) venous thromboembolism guidelines for internal medicine or general surgery. According to ACCP, the incidence of hospital-correlated DVT is 10-40% and the incidence of orthopedic surgery postoperative hospital-correlated DVT is 40-60% (5). Thus, the safe and effective prevention of VTE is a serious problem that has to be solved.

As early as the eighteenth century, Virchow described three major causes of thrombosis: venous wall damage, slow blood flow, and hypercoagulability. For patients to accept surgery for gastrointestinal cancer there are some factors that increase the risk of postoperative VTE. Old or

Key words: venous thromboembolism, gastrointestinal tumors surgery, low molecular weight heparin

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0393-974X (2018)

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bedridden patients appear to be more vulnerable. Postoperative hypercoagulable state, tumors, central venous indwelling tube condition and the degree of postoperative systemic inflammatory response are some of the factors that raise the risk of postoperative VTE. In this study, patients who underwent abdominal gastrointestinal tumor surgery were given single subcutaneous injection of LMWH (Low molecular weight heparin) to prevent VTE.

MATERIALS AND METHODS

Forty patients with high risk of postoperative VTE who underwent gastrointestinal tumor surgery from October 2010 to December 2011 at the Hebei People's Hospital Second General Surgery Department were randomly selected. Twenty-four of the patients were males and 16 females with average age 55.9 years.

Inclusion criteria: i) male or female aged 18-80 years; ii) body mass index (BMI) 18.5-24.9 Kg/m²; iii) patients conducted with radical resection of gastrointestinal tumor under general anesthesia; iv) higher postoperative VTE risk; v) agreement to test and sign the informed consent.

Exclusion criteria: i) aged <18 years or >80 years; ii) patients in critical condition, abnormal liver and kidney function [alanine aminotransferase (ALT), aspartate aminotransferase (AST) or creatinine were higher than the normal level]; iii) patients with contraindications to anticoagulation, such as ulcer, bleeding, infective endocarditis, hypertension [systolic blood pressure (SBP) > 200 mmHg, or diastolic blood pressure (DBP) > 120 mmHg] or cerebral hemorrhage within 3 the previous months; iv) patients with abnormal coagulation function [prothrombin time (PT) is 3 seconds higher than the normal value, activated partial thromboplastin time (aPTT) is 10 seconds higher than the normal value, and the original content of fibrin is lower than the normal value]; v) patients who are allergic to heparin or low molecular weight heparin.

The patients were divided into 2 groups; Group A included 24 cases with 16 males and 8 females, group B included 16 cases with 8 males and 8 females. All included cases underwent standard D2 tumor resection. Abdominal cavity was rinsed and soaked for 10 min with warm distilled water after surgery. When the irrigating fluid was totally absorbed, a drainage tube was placed in a pelvic cavity. After the patients were awake (after the surgery),

they were left to rest so as to observe the drainage. The study has the Ethical approval of Affiliated Hongqi Hospital of Mudanjiang Medical University. All patients were informed and signed consent for participation.

Twelve hours after surgery, both groups (excluding patients with contraindications to anticoagulation) were administered LMWH treatment. If drainage was concentrated bloody and greater than 200 mL, then LMWH treatment start time was prolonged to 24 h after surgery. Group A was administered LMWH 5000 IU (US Sigma-Aldrich Company) subcutaneously every 12 h, while group B was administered LMWH 5000 IU subcutaneously once every 24 h. On the first, second, and seventh day after surgery, patients in both groups performed blood sampling 3.5 h after receiving LMWH treatment. The blood samples (2.7 ml) were rapidly injected into a vacuum tube containing 3 ml of 0.109 moles sodium citrate. 3000 r/min centrifugation was conducted at room temperature for 10 min. Then, the plasma was transferred to a tube and was stored at -80°C. From 1st to 7th day after surgery, the patients were examined once a day to check whether or not there was tenderness along the deep vein, edema of the lower extremities, Homans signs, chest tightness, palpitation, chest pain or hemoptysis. If the above symptoms or signs were detected, the plasma D-dimer was tested immediately. If the above symptoms were not present, the plasma D-dimer was tested seven days after surgery. If D-dimer was $\leq 300\mu\text{g/l}$, VTE was excluded; if D-dimer was $> 300\mu\text{g/l}$, double lower extremity deep venous ultrasound was performed and, if necessary, pulmonary artery spiral CT was also checked. The bleeding complications were observed at 1st-7th day after surgery and the number of platelets was monitored.

Termination

In any patient if one of the following symptoms/conditions appeared, the experimental treatment was terminated: i) any bleeding complications, such as hematoma and abdominal bleeding; ii) appearance of heparin-induced thrombocytopenia (HIT), defined as platelet count $<100 \times 10^9/\text{l}$ or lower than 50% of preoperative laboratory measured value; iii) when DVT was confirmed by deep venous ultrasound or PE was confirmed by pulmonary artery CTA (termination of the experiment, but continuation of the appropriate treatment); iv) low molecular weight heparin allergy.

Anti-Xa activity determination

Equipment: Enzyme marker model Denley Dragon Wellsan MK3 (Finland Thermo Corporation); Centrifuge model 80-2B (Jiangsu Zhengji Instrument Co., Ltd.); Digital display water heater type incubator PYX-DHS (Shanghai Yuejin Medical Instrument Factory); Plate washing machine Wellwash 4 MK2 (Finland Thermo Corporation); Electric constant temperature blower drying box DHG-9023A (Shanghai Jinghong Laboratory Equipment Co., Ltd.); anti-Xa activity kit (US Pacific Blood Products Inc.); Heparin standard (US Sigma Corporation); Low molecular weight heparin sodium 5000IU/ branch (Qilu Pharmaceutical Co., Ltd.)

Methods for the determination of anti-Xa factor

activity: Plasma anti-Xa activity was determined by chromogenic substrate method and the appropriate kit (US Pacific Blood Products Inc.). The determination was carried out in strict accordance with the manufacturer's instructions. The definition of effective anticoagulation is plasma anti-Xa activity peak > 0.5 IU/ml. The chromogenic substrate method comprises the following steps: addition of excessive purified antithrombin (ATIII) in the measured plasma, forming AT III -LMWH complex, followed by addition of excessive known overdose purification factor Xa, forming ATIII -LMWH- factor Xa complex and residual factor Xa. The residual factor Xa mixed with chromogenic substances composes p-nitroaniline (PNA). At the wavelength of 405 nm, the plasma LMWH has an

Table I. Demographic characteristics of enrolled patients.

	Group A	Group B	P Value
Gender(Male/Female)	16/8	8/8	0.341 [#]
Age(year)[mean(SD)]	53.28(11.1)	58.01(9.49)	0.176*
BMI (Kg/m ²) [mean(SD)]	23.25(1.89)	22.67(1.79)	0.332*
Lt (min) [mean(SD)]	231.19(26.42)	224.28(30.87)	0.452*

*Two independent-samples *t* test; [#]chi-square of constituent ratio; Lt: lasting time of surgery

Table II. Complications following LMWH prophylaxis for VTE.

	Group A	Group B
72 hour drainage fluid(mL)	96.66±32.03	87.89±25.69
Incision hematoma	N/A	N/A
Other parts of the bleeding	N/A	1 case of upper gastrointestinal bleeding
HIT	N/A	N/A
Anti-Xa activity on day 1 postoperatively	0.69±0.16	0.67±0.15
Anti - Xa activity on day 2 postoperatively	0.70±0.05	0.69±0.16
Anti-Xa activity on day 7 postoperatively	0.74±0.09	0.69±0.12
distribution of anti-FXa (<0.5)	0	0
distribution of anti-FXa (0.5≤anti-Xa<1.0)	23(95.83%)	17(100%)
distribution of anti-FXa (>1.0)	1	0

inverse relationship with the amount of PNA. The heparin standard solution was diluted to make the standard curve, and then the anti-Xa factor activity unit was calculated based on the standard curve.

Plasma D-dimer and routine blood tests were determined by the laboratory of our hospital. For plasma D-dimer determination, double antibody sandwich ELISA method was used.

Statistical analysis

Data were input into SPSS 13 statistical software package for statistical analysis. All data were tested by normality test and represented by mean $\pm$ standard deviation. The *t*-test was used to compare the parameters between groups. Comparison of composition ratio was expressed by χ^2 and $P < 0.05$ indicated that the difference was statistically significant. The differences in analysis of repeated measurement materials were used to study the difference of anti-Xa activity between the two groups and different time points.

RESULTS

There was no statistical difference between the demographic characteristics in the two groups (Table I).

In the morning of the seventh day after surgery,

one patient from group A showed hematemesis (vomiting of dark red, coffee-like residues), melena and palpitation discomfort. This condition was attributed to bleeding stress ulcer causing digestive tract bleeding (later diagnosed as gastric cancer). The experiment was terminated and recorded as adverse events. The corresponding data were excluded from statistical analysis. After the effective suppression of acid, hemostasis, blood transfusion and other treatments, the patient's bleeding stopped and there were neither bloody and coffee-like residues in the gastric tube nor black stools. The anti-Xa activities were monitored on the first and second day after surgery and were found to be 0.75 IU/ml and 0.82 IU/ml, respectively. According to LMWH pharmacokinetics, they were within the safe anti-Xa activity concentration range (0.5-1 IU/ml). When digestive tract hemorrhage occurred, blood testing was performed immediately. Four factors of blood coagulation were measured: platelet count (PLT) = $347 \times 10^9/l$, prothrombin time = 12.5 seconds (normal value of 10-14 seconds), activated partial thromboplastin time = 36.3 seconds (normal value of 20-35 seconds) and fibrinogen content = 4.34 g/l (normal value of 2-4 g/l).

Seventy-two hours after surgery, the drainage

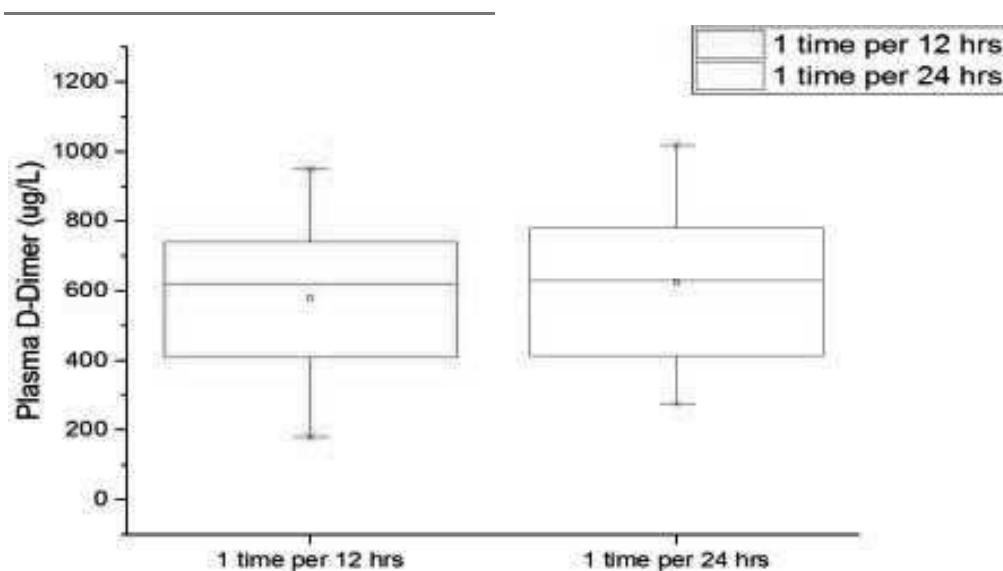


Fig. 1. The comparison of plasma D-dimer of the two groups.

fluid in group A was 96.69 ± 31.99 ml and the corresponding fluid in group B was 87.96 ± 24.74 ml. Both groups showed weak drainage fluid and there was no significant difference between them ($P = 0.419 > 0.05$). After excluding gastrointestinal bleeding cases, there were no other bleeding complications in the two groups, and there was no HIT (Table II).

Two independent-samples *t*-test were conducted for 72 hours' drainage, with no statistically significant difference between the two groups ($P = 0.419 > 0.05$).

After clinical observation, patients of both groups experienced no lower extremity edema, no tenderness along deep vein, no Homans sign, no tenderness, no chest tightness, no chest pain, no palpitation, and no hemoptysis. On the seventh day after surgery, patients were checked for plasma D-dimer. Patients with positive D-dimer were checked with double lower extremity deep venous ultrasound. The concentration of plasma D-dimer in group A was 606.78 ± 276.47 $\mu\text{g/l}$ and in group B was 621.25 ± 226.85 $\mu\text{g/l}$ ($P > 0.05$). In group A 93.7% of the patients showed plasma D-dimer higher than $300 \mu\text{g/L}$, while the corresponding percentage for group B was 82.6% ($P = 0.305 > 0.05$), as shown in Fig.1. The VTE was diagnosed with double lower extremity deep vein ultrasound examination in both

groups.

Anti-Xa activity monitoring after surgery was as follows: on the first day, anti-Xa activity of group A was 0.67 ± 0.15 IU/mL and of group B 0.69 ± 0.16 IU/ml; on the second day, anti-Xa activity of group A was 0.69 ± 0.16 IU/ml and of group B 0.70 ± 0.05 IU/ml; on the seventh day, anti-Xa activity of group A was 0.69 ± 0.12 IU/ml, while anti-Xa activity of group B was 0.74 ± 0.09 IU/ml. All values of anti-Xa activity were in the recommended range of 0.5-1.0 IU/ml. In general, there was no statistical difference between the two groups (ANOVA F-test statistic=0.299, $P = 0.588 > 0.05$) and there was no statistical difference between the patients of each group, separately ($F = 2.368$, $P = 0.112 > 0.05$ (Table II).

In the first day after surgery, for 95.65% of group A patients anti-Xa activity value showed effective anticoagulation (>0.5 IU/ml). In one case of this group anti-Xa activity value was more than 1.0 IU/ml, but without bleeding complications. 87.5% of group B cases reached effective anticoagulation (>0.50 IU/ml) with anti-Xa activity value no more than 1.0 IU/ml. There was no statistical difference between the two groups in the effective rate of anticoagulation ($P > 0.05$). On the second day after surgery, 95.65% of group A patients' anti-Xa activity

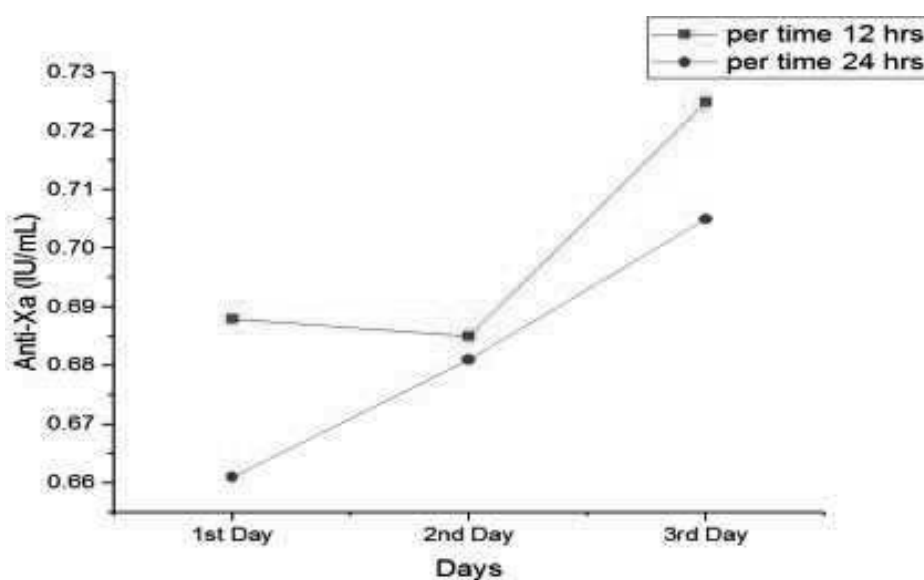


Fig. 2. The changes of anti-Xa following LMWH prophylaxis for VTE.

value reached effective anticoagulation (>0.50 IU/ml), and the corresponding percentage for group B was 93.75%. The anticoagulation effective rate in the two groups showed no significant difference ($P>0.05$). There were no cases with anti-Xa activity value more than 1.0 IU/ml in either group. On the seventh day after surgery, anti-Xa activity values of the two groups reached effective anticoagulation (>0.5 IU/ml). Group A had one case of anti-Xa activity value exceeding 1.0 IU/ml, but no bleeding complications occurred (Table II).

The recommended anti-Xa activity in blood is 0.5-1.0 IU/ml. Blood samples were taken at 3.5 hours after regimens and anti-Xa activity was measured. The results showed that the effective anticoagulant drug concentration in each group of anti-Xa activity can be reached in 3.5 hours after medication. In group A, 95.65% of cases reached effective anticoagulation in the first two days, while on the seventh day, all cases reached effective anticoagulation. In group B, on the first day, 87.5% of cases achieved effective anticoagulation, while on the second day the percentage increased to ($P > 0.05$). On the seventh day, all cases reached effective anticoagulation. The anti-Xa factor activities of the two groups on the first day were 0.69 ± 0.14 IU/ml and 0.66 ± 0.14 IU/ml, respectively. On the second day, the anti-Xa factor activities of the two groups were 0.69 ± 0.07 IU/ml and 0.68 ± 0.14 IU/ml, respectively. Finally, on the seventh day the anti-Xa factor activities of the two groups were 0.73 ± 0.10 IU/ml, and 0.70 ± 0.11 IU/ml, respectively. The number of anti-Xa factor activities monitored for three days seemed to be gradually increased, but there was no significant difference in the activity of anti-Xa ($P<0.05$) (Fig. 2). It can be concluded that patients who undergo surgery due to suffering from gastrointestinal neoplasms, with normal liver and kidney function, can reach effective anticoagulation 3.5 h after using LMWH for the prevention of VTE if they are not affected by postoperative hemodynamic instability, high metabolic state, etc. The continuous application of LMWH continued to be an effective anticoagulant factor until the seventh day post-surgery when no obvious pharmacodynamic cumulative effect was observed. According to the pharmacokinetics

of LMWH, there is a significant dose-dependent anticoagulant effect of LMWH, and the half-life is about 3.5 hours, which was consistent with the results of this study.

DISCUSSION

The incidence of VTE is high and harmful; nevertheless it is a preventable disease (6). In recent years, various countries and medical organizations presented plans related to different groups of population (7). In February 2012, the United States ACCP proposed the latest VTE prevention guidelines (8-10). It was recommended that patients with non-orthopedics surgery, whose postoperative VTE risk is high, and after the exclusion of high-risk bleeding, should be administered LMWH or low-dose unfractionated heparin (LDUH) to prevent VTE. There are three major causes of VTE formation; venous wall damage, slow blood flow, and hypercoagulability. Taking measures against these causes can effectively prevent VTE (11, 12), including general, mechanical, and drug prevention measures, such as early ambulation, postoperative intermittent pressure therapy, pressure gradient compression stockings, low-dose heparin or low molecular weight heparin (12-13).

Based on our results, we can conclude that administration of LMWH 3.5 h after surgery of patients diagnosed with gastrointestinal neoplasms, leads to prevention of VTE and effective anticoagulation. Seven days after the surgery, the anticoagulation intensity becomes stable and there is no significant pharmacodynamics cumulative effect. Using LMWH for subcutaneous injection every 12 or 24 hours can effectively prevent postoperative abdominal tumor VTE, however the 12 hour regimen seems to increase the risk of bleeding.

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LETTER TO THE EDITOR

PREDICTION OF LENGTH OF HOSPITAL STAY AND MORTALITY IN PATIENTS WITH DELIRIUM: A PROSPECTIVE COHORT ANALYSIS OF 200 ICU PATIENTS

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Delirium develops in most critically ill Intensive Care Unit (ICU) patients and is associated with longer hospital stay, increased rate of mortality and increased cost of healthcare. The purpose of this study was to evaluate the incidence of delirium in non-ventilated ICU patients and demonstrate its clinical association with the hospital/ ICU length of stay and in-patient mortality. A total of 200 ICU patients (aged 18 years or over) who were admitted to the ICU with specific primary diagnoses were selected for the study and followed up until either hospital discharge or death. Delirium status of each patient was assessed every morning by trained study personnel using the Confusion Assessment Method for Intensive Care Unit (CAM-ICU). Data collection included baseline demographics, diagnosis (at the time of admission) and severity of illness using the Acute Physiology and Chronic Health Evaluation II (APACHE II) score. Upon evaluation of the effects associated with age, gender, mortality and other variables, it was found that age had a direct effect on the incidence of delirium, with the probability of delirium increasing with the increase in the age of patients. It was also shown that mortality, either in the ICU or during the hospital stay, was associated significantly with the occurrence of delirium. This study shows that duration of delirium in ICU is an independent predictor of long-term hospital stay and is significantly associated with mortality. These findings are crucial for health care professionals and future research trials dealing with critically ill ICU patients.

To the Editor,

Delirium, also known as acute encephalopathy, is an acute disturbance of cognition and consciousness that has the tendency to fluctuate in severity. It can be described as a pathological alteration in cognitive activities associated with an attenuation and an underlying or latent medical/systemic comorbidity or metabolic imbalance (1). It has a tendency to occur in 8 out of 10 ICU patients (2). Although ICU delirium has been considered as an iatrogenic event by most physicians, it is evident from the literature

that it is associated with medical complications, poor treatment outcome, increased length of hospital stay, functional disability, cognitive impairment and dementia after discharge (3, 4). However, there is no clarity in the literature with regard to the association between delirium and mortality: while some studies documented that delirium is an independent predictor of mortality in ICU patients, other studies noticed no significant association between delirium and mortality (1, 4, 5). Even though new technology and advancements in critical care have scaled down

Key words: delirium, critically ill patients, intensive care unit, mortality, length of hospital stay

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0393-974X (2018)

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the mortality rate in critically ill patients (6), the development of delirium in ICU patients has always prolonged their ICU/hospital stay and is generally considered as an independent predictor of increased mortality.

Potential risk factors associated with the development and incidence of delirium can be categorised into two groups. First and foremost, they include the primary reasons for admission, along with co-morbidities that can increase vulnerability during the ICU stay, such as advanced age, dementia, prior cerebrovascular disturbance, depression, epilepsy, alcoholism, and any prior treatments with psychoactive substances. Secondly, additional precipitating factors or events during the ICU stay that are secondary to the disease should be taken into account; these factors and events include disease severity, metabolic disturbances, anaemia, infection, hypoxemia, hypotension and other iatrogenic/environmental factors in the ICU (7). It has recently been emphasized that one of the major priorities of public health departments is to diagnose, prevent and treat delirium in the ICU-admitted or critically ill patients (8). Due to the fact that many problems and triggering factors resulting in delirium can be prevented or treated, a daily delirium monitoring has been advised for ICU patients if adverse outcomes are expected. Keeping these arguments in mind, we performed this study to investigate the delirium occurrence in non-ventilated critically ill ICU patients and evaluate its association with overall hospital stay and in-patient mortality.

MATERIALS AND METHODS

This observational cohort study was approved by the institutional review board. Informed consents were obtained from all the patients. Patients included in the study were 18 years of age or older. They were referred and admitted to the ICU for at least one day or twenty four hours but did not need any invasive mechanical ventilation. Two hundred patients that met these criteria were included in this study during the 6-month study period and were followed up till either discharge from hospital or death.

Every morning, the delirium status of each patient was

assessed by trained study personnel using the Confusion Assessment Method for Intensive Care Unit (CAM-ICU) (5). Patients were categorised into two groups based on the delirium score by CAM-ICU and those with positive CAM-ICU score were categorized into a “delirium” group. The other patients were categorized into a ‘no delirium’ group. Data was collected on the basis of many parameters that include baseline demographics, diagnosis (at the time of admission) and severity of illness by the Acute Physiology and Chronic Health Evaluation II (APACHE II) score (9). The Charlson Comorbidity Index (CCI) that accounts for the number and seriousness of pre-existent comorbidities as the total sum of weighted indices was calculated using ICD-9 codes, as described by Deyo et al. (10). The primary reason for admission to ICU of each patient was recorded by the concerned clinician as a diagnostic category that was the most representative reason for ICU admission, and tabulated accordingly. As this was not an interventional study, delirium was not treated specifically by any method. With regards to the delirium and sedation, it was decided by the medical team on a patient-by-patient basis. The results were statistically analysed using Excel and vassarstat.net to obtain mean averages and distribution, and to run *chi*-squared test and Fisher exact probability test. Correlations were duly noted and recorded to prognosticate the influence of variables.

RESULTS

Two hundred patients aged 18 years or more, who were referred and admitted to the ICU for at least twenty-four hours but did not need any invasive mechanical ventilation, were involved in the study. Sample distribution of the patients by gender was 42% males and 58% females (Table I), mean age of the patients was 57.3 years (no delirium group) and 66.4 years (delirium group). Although the age of the enrolled patients ranged between 18 and 80 years, delirium occurred mainly in the patients aged between 61 and 70 years. Table I shows the differences in mean age and gender among patients who presented with or without delirium. The data allow to conclude that only the association of delirium with the age was significant. Similarly, significant differences were noted in patients with or without delirium in the mean difference calculated

for incidence of mortality.

Fig. 1 shows a comparative evaluation of reasons for ICU admission in 200 patients. The most common diagnosis was Myocardial Infarction/Arrhythmia/ Congestive Heart Failure, which accounted for 41% of patients (82 cases). It was followed by ARDS/ sepsis (21.5% of patients, 43 cases), hepatic/renal failure (11.5%, 21 cases), drug overdose (7%, 14 cases), gastrointestinal bleeding (6.5%, 13 cases), COPD/pneumonia (4.5%, 9 cases) and malignancies

(3.5%, 7 cases). Upon careful assessment by the examination team, the delirium was diagnosed in 142 patients. Fifty-eight patients did not present with delirium.

Table II shows the average values of studied parameters in patients with and without delirium. For the APACHE II ($P=0.0001$) and Charlson Comorbidity Index ($P=0.003$) scores, the differences were statistically significant between the two patient groups. The SOFA score ($P=0.68$) did not show a

Table I. Incidence of delirium and mortality in different groups of patients .

	No delirium	Delirium	P-value
Age	57.3	66.4	0.003
Gender			
Male	28	56	0.5
Female	30	86	
Mortality			
in ICU	4	14	0.68
in hospital	12	38	

Table II. Evaluation of variables in patients monitored for the presence of delirium during ICU stay.

Variable	No delirium	Delirium	P-value
APACHE II	22.9	24.9	0.0001
Charlson Comorbidity Index	3.2	3.9	0.003
SOFA Score	9.3	9.5	0.54

Table III. Number of patients monitored in ICU vs days after admission.

No of days (after admission)	No delirium	Delirium
0	58	142
1	54	141
5	44	131
10	36	119
15	5	99
20	1	39
25	0	18
30	0	2
35	0	0

significant difference between the two categories of patients.

All 200 patients were monitored for the development and prognosis of delirium for 50 days after admission to ICU. Fifty-eight patients who never developed delirium (no delirium group) stayed in ICU for 14.4 days on average. In this group, most patients were moved out of ICU 10-15 days after the initial admission, and the maximum duration of their stay was 25 days (Tables III). By contrast, 142 patients from the delirium group spent an average of 19.8 days in the ICU. The maximum ICU stay of patients in this group was 35 days, and most patients left within 20 days (Tables III). This comparison shows that on average patients with delirium stayed in the Intensive Care Unit for around 5 days longer. At any point of time during the hospital stay, the patients who developed delirium were provided with personalised nursing care and careful monitoring.

DISCUSSION

In this study, the overall incidence of delirium among the ICU patients was 71%, which correlates well with most of the published international data that report 60-80% incidence rate (11). However, the study conducted at the Reina Sofía Clinic in Bogota,

Colombia, reported 21% incidence of delirium in sedated ICU patients (12).

The development of delirium in ICU patients was associated with increased risk of mortality of these patients. Clinical experience and literature evidence also suggest that there is a statistically-proven association between development of delirium and mortality in ICU patients. Using a validated protocol for evaluation of delirium, Klouwenberg et al. (13) estimated the mortality attributed to delirium in 1,112 critically ill patients. They concluded that although delirium prolongs the ICU stay, it is not an independent predictor of mortality in critically ill patients. The authors advised that further studies should be focused on the episodes and/or events related to persistent delirium and their long-term sequelae as a part of follow-up. Following this study, a systemic review was carried out by Salluh et al. (14) in 2015 with the objective to determine the relationship between delirium in critically ill patients and their health outcomes in the short term (in the ICU and in hospital) and after discharge from hospital. This study concluded that approximately one-third of ICU patients developed delirium during their stay in hospital. Mortality rate was higher among these patients, their hospital stays were longer, and significant cognitive disturbances were

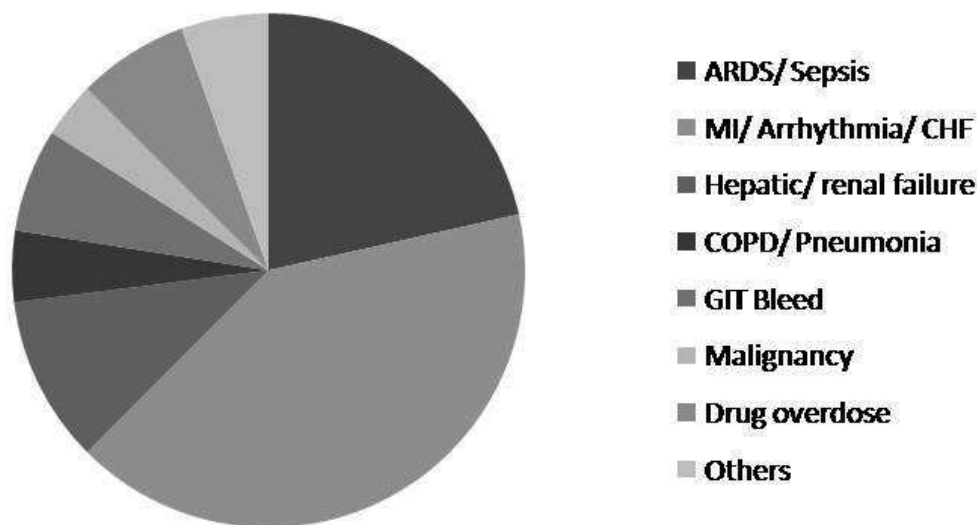


Fig. 1. Comparative evaluation of reasons for ICU admission.

observed after discharge from hospital. The results of our study show similar findings. Also, the delirium development was significantly associated with the prolonged Intensive Care Unit stay and the overall stay in hospital.

In our study, cardiovascular diseases, such as myocardial infarction, arrhythmia and congestive heart failure, were the most common documented reasons for ICU admission, followed by acute respiratory distress syndrome and sepsis. Other major reasons behind ICU admission included hepatic/renal failure, drug overdose and gastrointestinal bleeding. These reasons for ICU admission were consistent with the findings of other studies (15).

Upon evaluation of the effect associated with age, gender, mortality prediction and other variables, it was found that age had a direct effect on the incidence of delirium, with the probability of delirium increasing with the increase in the age of patients. It was also shown that mortality, either in the ICU or during the hospital stay, was associated significantly with the occurrence of delirium. This study shows that duration of delirium in ICU is an independent predictor of long-term hospital stay and is significantly associated with mortality. These findings are crucial for health care professionals and future research trials dealing with critically ill ICU patients.

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LETTER TO THE EDITOR

PERIPHERAL T LYMPHOCYTE IMMUNITY AND L-DOPAMINE IN PATIENTS WITH PARKINSON'S DISEASE

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This study aimed to evaluate the efficacy of T-cell immune function and L-dopamine (L-DOPA) in patients with Parkinson's disease (PD). Sixty subjects (included in the study group) with PD who were patients of the Neurology Department of The Affiliated Hospital of Hangzhou Normal University from July 2015 to March 2017 were selected. The study group was then categorized into groups according to the age of the patients, severity of disease, level of cognition, and treatment of L-DOPA. The control group (30 cases) was from the healthy population of the check-up center at The Affiliated Hospital of Hangzhou Normal University. The peripheral blood T-lymphocyte subsets of the study group were measured by direct immunofluorescence flow cytometry staining and compared with the control group. At the same time, correlation analysis was carried out on patients with different degrees of disease severity according to staging, different accompanying symptoms, and whether L-DOPA was administered. The results of the study show that the levels of CD4+, CD8+, CD3+, and CD4+/CD8+ peripheral blood in PD patients were significantly lower than those in the control group ($P<0.05$). It was found that the levels of CD4+, CD8+, CD3+, and CD4+/CD8+ decreased with age. The CD4+, CD8+, CD3+, and CD4+/CD8+ in patients with advanced stage PD were more significant than those with low PD stages ($P<0.05$). The levels of CD4+, CD8+, CD3+, and CD4+/CD8+ in the dementia group were significantly lower than those in the non-dementia group ($P<0.05$). The levels of CD4+, CD8+, CD3+, and CD4+/CD8+ in PD patients treated with L-DOPA were higher than those of PD patients without L-DOPA treatment ($P<0.05$). In conclusion, the immune function of T cells in patients suffering from PD is low, and the immune function of T cells in patients with severe disease is lower. Therefore, it is of certain significance to further study the pathophysiological mechanism of PD.

To the Editor,

Parkinson's disease (PD) is a common type of neurodegenerative disease affecting people in middle age and older (1). With the development of an aging population, the incidence of the disease is increasing, which has caused a negative impact (2). The primary pathological features of PD are the degeneration and necrosis of dopaminergic

neurons in the nigrostriatal system and the presence of Lewy corpuscles in the brain. The mechanism of the disease is not completely clear. It may be related to the mechanisms of genetic and environmental factors, oxidative stress, excitatory neurotoxicity, apoptosis, and other mechanisms (3).

Studies have shown that the incidence of PD is associated with abnormal cell and humoral immunity

Key words: Parkinson's disease, t-lymphocyte subset, L-dopamine, immune function.

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0393-974X (2018)

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in peripheral blood and antibody of dopaminergic neurons (4). The T-lymphocyte subgroup is the main cellular immune system in the human body, and the changes of lymphocyte subsets in patients with PD are mainly manifested in T-lymphocyte subgroup disorder (5). At present, a number of studies have suggested that T cells are involved in the pathogenesis of PD and may be associated with the activation of microglia (6). Microglia, as a macrophage in the brain, can secrete a variety of cytokines, such as TNF- α , IL-10, IL-6, and epidermal growth factor. The PD immune mechanism was discussed from clinical aspects and the effect of T-cell subgroup disorder on PD patients was studied.

MATERIALS AND METHODS

Sixty subjects (study group) with PD who were patients of the Neurology Department of The Affiliated Hospital of Hangzhou Normal University between July 2015 and March 2017 were selected. Among them, there were 33 males and 27 females with an average age of 63.52 ± 9.98 years. The control group was from the physical examination center of The Affiliated Hospital of Hangzhou Normal University. Thirty cases were without hereditary diseases, history of nervous system disease, acute and chronic inflammation, connective tissue diseases, hematological diseases, malignant tumors, or liver and renal failure. Among them, there were 17 males and 13 females with an average age of 64.15 ± 8.41 years.

A detailed questionnaire survey, physical examination, and laboratory testing were used to collect clinical data of all subjects. The age, sex, physical examination, Hoehn-Yahr staging, history of drug use, and other diseases of all subjects were recorded in both the study group and the control group. The collection and classification of clinical data were the responsibility of the specialist in the neurology department. All patients in the group signed informed consent to participate in the study which was approved by the ethics committee of The Affiliated Hospital of Hangzhou Normal University.

Admission criteria: After detailed inquiries about medical history, physical examination, and MRI head examination result, these cases were consistent with the clinical diagnostic criteria for Parkinson's disease formulated by the UK Parkinson's Disease Society Brain Bank (7).

The Hoehn and Yahr Scale (H-Y) was used to evaluate the disease stages (8).

Exclusion criteria: Other extrapyramidal diseases such as secondary PD and multiple system atrophy were excluded. Some diseases such as recurrent head trauma, encephalitis, tumor, aorto-arteritis, glial disease, drugs, blood diseases, severe liver and kidney diseases, thyroid diseases, and other diseases that may affect the peripheral T-cell immune system were excluded.

Study group characteristics

According to the age of the patient, the severity of the disease, the level of cognition, and the treatment of L-DOPA, the study group was sub-grouped as follows:

PD patients were divided into five groups according to age. The first group (PD-1) included 5 patients younger than or equal to 50 years. The second group (PD-2) included 16 patients aged 50-59 years. The third group (PD-3) included 19 patients aged 60-69 years. The fourth group (PD-4) included 17 patients aged 70-79 years. The fifth group (PD-5) included 3 patients aged 80 years old or older.

These groups were further divided into 3 subgroups according to the H-Y Scale with 5 standard stages: 22 patients in stage I-II were included in the PD-I group; 25 patients in stage II-III were included in the PD-II group; and 13 patients in stage IV-V were included in the PD-III group.

The PD patients were also classified into two groups according to their cognitive level: 15 patients were included in the dementia group (PD-D), and 45 patients were included in non-dementia group (PD-ND).

The PD patients were also classified into two groups according to the use of L-DOPA therapy: 22 patients (PD-NL) were not treated with L-DOPA, 38 patients (PD-L) were treated with L-DOPA for more than 2 weeks.

Method

The peripheral blood T-cell subgroup was detected by direct immunofluorescence staining. All the subjects underwent 2-ml peripheral venous blood analysis in the early morning on an empty stomach. Then, 100 uL heparin anticoagulant was extracted, and the anti-human CD3FITC/CD4FITC/CD8PE monoclonal antibody labeled with 20 uL fluorescein was added, respectively (provided by the US Beckman-Coulter Company). After

mixing, it was incubated at room temperature (18 -20°C) for 15 min, and 1 mL erythrocyte lysis was added. After the red cells were dissolved, it was centrifuged and washed two times. The Epics-XL type flow cytometry (provided by the US Coulter Company) was used for testing. The results were calculated with the percentage of positive rates.

Statistical analysis

The data are expressed in mean $\pm$ standard deviation. Variance analysis and Q-test were used in each group, and the paired *t*-test was used for comparison. The difference was statistically significant when $P < 0.05$. The main indexes were analyzed by linear correlation.

RESULTS

General clinical data

As shown in Table I, there was no statistical difference in age and sex between the two groups ($P > 0.05$).

Detection of T cell subsets

Compared with the control group, the levels of CD4+, CD8+, CD3+, and CD4+/CD8+ peripheral blood in the study group were significantly lower ($P < 0.05$).

In the study group, the level of CD4+, CD8+, CD3+, and CD4+/CD8+ peripheral blood showed

Table I. Basic information.

Type	Study group	Control Group	P-Value
Age (year)	63.52 $\pm$ 9.98	64.15 $\pm$ 8.41	0.069
Cases (male/female)	33/27	17/13	0.173

Table II. Determination of T-lymphocyte subsets in patients.

Groups	Cases	CD3+ (%)	CD4+ (%)	CD8+ (%)	CD4+/CD8+
Control group	30	64.63 $\pm$ 5.54	58.02 $\pm$ 8.23	44.82 $\pm$ 9.23	1.83 $\pm$ 0.89
PD group	60	54.65 $\pm$ 7.13*	48.71 $\pm$ 9.65*	36.16 $\pm$ 9.44*	1.25 $\pm$ 0.64*
PD-1	5	56.91 $\pm$ 9.36	45.72 $\pm$ 5.35	37.36 $\pm$ 6.54	1.22 $\pm$ 0.32
PD-2	16	53.52 $\pm$ 7.75	42.28 $\pm$ 5.66	36.12 $\pm$ 5.78	1.18 $\pm$ 0.31
PD-3	19	50.48 $\pm$ 7.87	40.59 $\pm$ 6.36	35.73 $\pm$ 5.56	1.17 $\pm$ 0.20
PD-4	17	48.17 $\pm$ 5.76	39.81 $\pm$ 5.65	34.21 $\pm$ 6.82	1.16 $\pm$ 0.18
PD-5	3	46.57 $\pm$ 4.88*	38.82 $\pm$ 6.33*	33.08 $\pm$ 4.05*	1.15 $\pm$ 0.30*
PD-I	22	61.25 $\pm$ 4.82	43.91 $\pm$ 5.24	38.18 $\pm$ 5.54	1.25 $\pm$ 0.53
PD-II	25	55.08 $\pm$ 6.76	42.38 $\pm$ 6.15	34.20 $\pm$ 3.37	1.20 $\pm$ 0.39
PD-III	13	49.77 $\pm$ 7.36*	39.53 $\pm$ 5.43*	32.08 $\pm$ 5.27*	1.17 $\pm$ 0.20*
PD-ND	45	59.65 $\pm$ 6.86*	38.37 $\pm$ 4.61*	39.21 $\pm$ 7.72*	1.24 $\pm$ 0.19*
PD-D	15	48.43 $\pm$ 6.15	35.17 $\pm$ 5.24	35.16 $\pm$ 5.26	1.13 $\pm$ 0.23
PD-NL	22	50.71 $\pm$ 7.08*	24.52 $\pm$ 6.41*	33.83 $\pm$ 5.56*	1.18 $\pm$ 0.17*
PD-L	38	58.32 $\pm$ 5.54	31.83 $\pm$ 7.54	37.16 $\pm$ 6.27	1.24 $\pm$ 0.19

* $P < 0.05$. According to age, the PD patients are divided into 5 groups, PD-1: less than 50 years for the first group; PD-2: 50-59 years old; PD-3: 60-69 years old; PD-4: 70-79 years old; PD-5: more than 80 years old. According to the Hoehn-Yahr5 grading standards, the PD patients are divided into 3 groups. PD-I is I-II stage patients and PD-II is II half- III stage patients. PD-III is IV-V stage patients. Based on the cognitive level, the PD patients are divided into 2 groups: dementia group (PD-D) and non-dementia group (PD-ND). In addition, the PD patients are divided into two groups according to whether or not L-Dopa drug treatment is applied. They are PD-NL (without L- dopamine drugs) and PD-L (with L- dopamine therapy for more than 2 weeks).

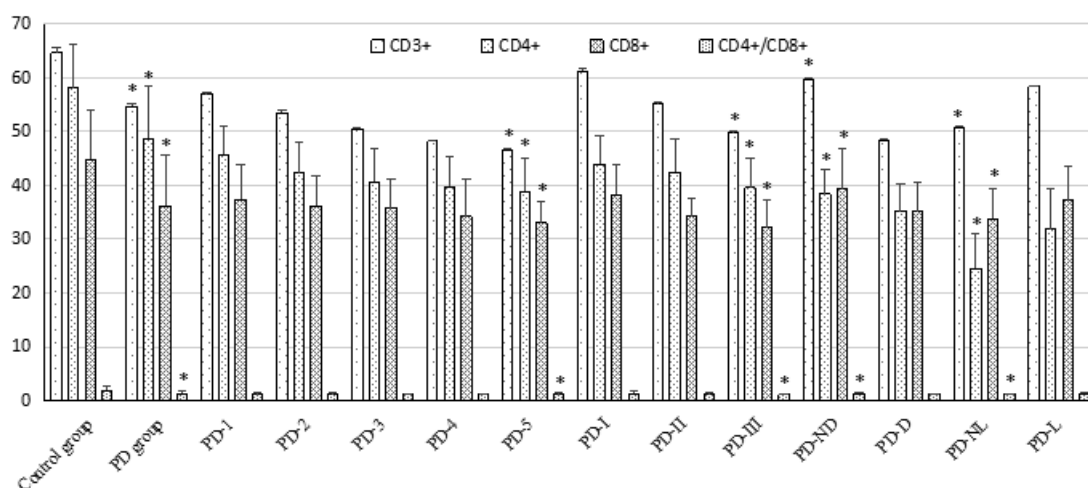


Fig. 1. A column graph for the determination of T-lymphocyte subsets in patients. * $P < 0.05$

a decreasing trend with age in all five groups of PD-1~PD-5 ($P < 0.05$). According to the H-Y Scale, the level of CD4+, CD8+, CD3+, and CD4+/CD8+ decreased significantly in high-stage patients ($P < 0.05$). Compared with the PD-ND group, the level of CD4+, CD8+, CD3+ and CD4+/CD8+ in the PD-D group was significantly lower than that in PD-ND group ($P < 0.05$). With the L-DOPA treatment, the level of CD4+, CD8+, CD3, and CD4+/CD8+ in the untreated group was significantly lower than that in the L-DOPA group ($P < 0.05$). The specific data are shown in Table II and Fig. 1.

DISCUSSION

Recent studies have shown that PD, central neurodegeneration, substantia nigra, and striatum injury are closely related to the inflammatory response mediated by cellular immunity (9). However, the correlation between autoimmunity and PD is still not clear. CD4+ cells can help B cells secrete antibodies and modulate the immune response of other T cells. CD8+ cells often exhibit cytotoxic activity and are the major cytotoxic effector cells (10-11). If the CD4+/CD8+ balance is disturbed, the immune response will be disturbed. The data showed that the levels of CD4+, CD8+, CD3+, and CD4+/CD8+ peripheral blood were lower than those in the control group

which supports the basis that PD has a low T-cell immune function. Furthermore, it was found that the decrease of the ratio of CD4+ to CD4+/CD8+ was particularly obvious. As a neurodegenerative disease, PD is commensurate with autoimmune disease. The decrease of CD4+/CD8+ ratio indicates that the immune status of the body is out of balance. Furthermore, the results showed that the ratio of CD4+, CD8+, CD3+, and CD4+/CD8+ in PD with dementia was lower than that in the non-dementia group. The possibility of progressive decrease in cellular immune function was associated with dementia.

Barbanti (12) observed that the expression of dopamine receptors in the lymphocytes of the peripheral blood of PD was increased. It is presumed that the abnormal cellular immune function of PD may be related to the regulatory mechanism of dopamine on immunity. In this study, the level of CD4+ and CD3+ in patients with L-DOPA therapy was higher than that of the patients who did not use L-DOPA. Given that the patients in this study were not observed for a long period, there is no clear conclusion.

ACKNOWLEDGEMENTS

This work was supported by the Medical and

Health Science and Technology Foundation of Zhejiang Province, China (2017KY126).

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LETTER TO THE EDITOR

APATINIB TREATMENT IN EXTENSIVE METASTATIC ADVANCED THYMIC CARCINOMA

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Apatinib is a novel oral, anti-tumor, angiogenic-targeting drug that can selectively target vascular endothelial growth factor receptor-2 (VEGFR-2). In clinical trials, this new tyrosine kinase inhibitor (TKI) has been shown to be an effective and safe treatment for a variety of malignancies. Currently, there is a lack of studies of patients with thymic carcinoma; therefore, we present a case of advanced thymic carcinoma treated with apatinib after chemotherapy failure with multiple lung metastases. This patient has been taking a dose of 500 mg of apatinib per day, and his efficacy has achieved partial response (PR), according to the RECIST 1.1 standard, and progression-free survival (PFS) is 6.3 months at this point. Apatinib will continue as his maintenance treatment. During the treatment, drug-related toxicity and side effects were tolerable. Thus, apatinib may be a meaningful option for the treatment of advanced metastatic thymic carcinoma after chemotherapy failure.

To the Editor,

Thymoma is the most common primary tumor of the upper mediastinum, and the World Health Organization (WHO) uses the Muller-Hermelink classification to classify thymoma into Types A, AB, B1, B2, B3, and C. Type C thymoma is thymic carcinoma, the incidence of which is low, however, the rates of recurrence, metastasis, and mortality are high after treatment. The treatment of metastatic thymic carcinoma after surgery and radiochemotherapy failure has been a bottleneck (1).

Apatinib, a new oral angiogenic-targeting drug, is a type of small molecule angiogenesis that acts as an antitumor mechanism. In previous studies, apatinib had been proved effective and safe in the treatment of chemotherapy-refractory advanced gastric cancer (2, 3), and it also has been effective in the treatment

of advanced hepatocellular carcinoma, metastatic breast cancer, advanced non-squamous non-small-cell lung cancer, and other malignant tumors (4-6).

At present, there is a lack of information on the role of apatinib after the failure of radiochemotherapy in advanced metastatic thymic carcinoma, which is the focus of the current study.

Case report

A 35-year-old male patient underwent excision of an anterior mediastinal mass and intraoperative radiotherapy at Chinese People's Liberation Army (PLA) General Hospital in January 2016. The mass was seen in the anterior superior mediastinum, with a maximum volume of ~5cm×5cm×8cm during the operation, and was invading the upper lobe of the left lung. The postoperative pathology diagnosis

Key words: advanced thymic carcinoma, apatinib, targeting drug, multiple lung metastases

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0393-974X (2018)

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confirmed the thymic as a poorly differentiated squamous cell carcinoma (SCC; see Fig. 1). After the operation, the patient was administered three cycles of chemotherapy with gemcitabine gemcitabine and cisplatin (GP regimen).

In June 2016, the patient visited our hospital for review. The results of the chest computed tomography (CT) scan showed that local irregular density images could not exclude the recurrence of thymic cancer; the right shoulder blade and the left femoral head were destroyed, and bone metastases were considered. The nature of the small nodules in both lungs remained to be determined. No abnormalities were found in the pelvic CT examination. Thereafter, the patient underwent docetaxel and carboplatin chemotherapy for two cycles, and the procedure was uneventful. Subsequently in August 2016, the patient was scheduled for radiation therapy, and the thymus lesions were almost completely relieved under a CT simulation positioning scan. Radiotherapy was carried out for the postoperative tumor bed with a dose of 50Gy/25 fractions. In late August 2016, the patient underwent magnetic resonance imaging (MRI) examination of the lumbar spine, which indicated abnormal signals of the fifth lumbar spine and the first

sacral vertebra and were recommended for review. In October 2016, the patient repeated the CT, and it was found that both lung nodules were increased in size and identified as double lung metastases. The patient also repeated the MRI of the lumbar spine, which revealed the abnormalities in the signal range increased in the fifth lumbar spine and in the first sacral vertebra. Subsequently, he was treated with docetaxel plus cisplatin for three cycles of chemotherapy. In mid-November 2016, this patient received radiation therapy for the left adrenal gland, the fifth lumbar, and the first sacral vertebra metastatic tumors. At the end of the treatment, in late December 2016, the patient was reexamined via CT, which showed that no thymic lesion was found. The left adrenal gland was evaluated as partial response (PR), whereas the efficacy of the double lung metastatic tumors was stable disease (SD), according to the WHO's Response Evaluation Criteria in Solid Tumors (RECIST 1.1). The MRI of the lumbar spine indicated abnormal signals of the left pubic bone, acetabulum, and upper femur, which were considered as new bone metastatic tumors. The patient had no obvious bone pain at this time. In early January 2017, this patient began to take apatinib with a dose of 500mg/d. After one month, the chest CT

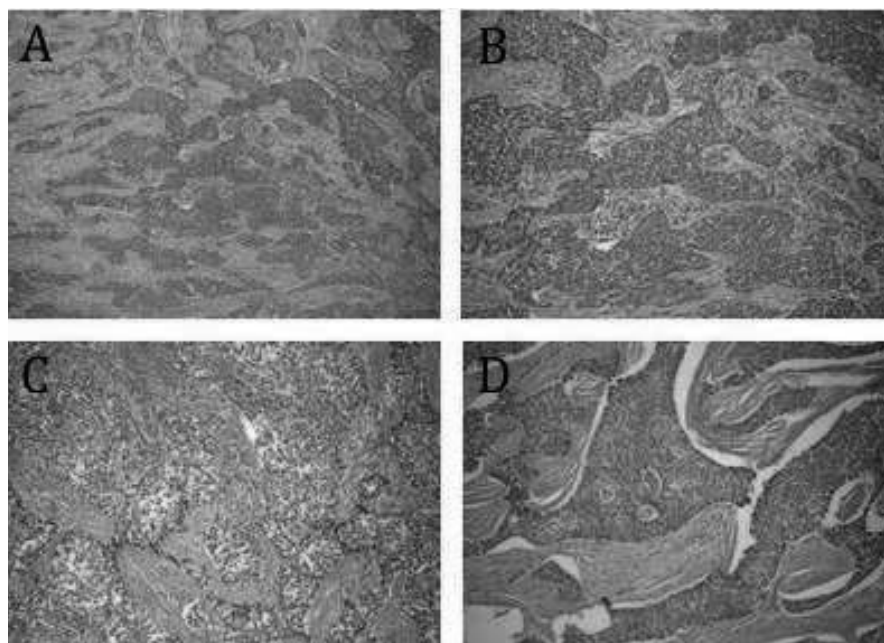


Fig. 1. Hematoxylin and eosin staining of a tumor section ($A \times 400$, $B \times 1100$, $C \times 2100$, $D \times 3100$). The pathological diagnosis is the thymic is a poorly differentiated SCC.

revealed that the lung metastases were completely responsive and the other lesions were stable. In May 2017, the patient suffered from increased pelvic pain, and was treated with radiation therapy (46Gy/23 fractions) for palliation and relief of symptoms of the pelvic metastases. The bone pain of this patient has been completely relieved. In late August 2017, the

patient repeated the CT, which indicated a complete response of both the lung and left adrenal metastatic tumors (see Figs. 2 and 3 and Table I). To date, we have not found disease progression in this patient. Since he began taking apatinib, the progression free survival of other lesions (PFS), except bone metastases, has reached 6.3 months.

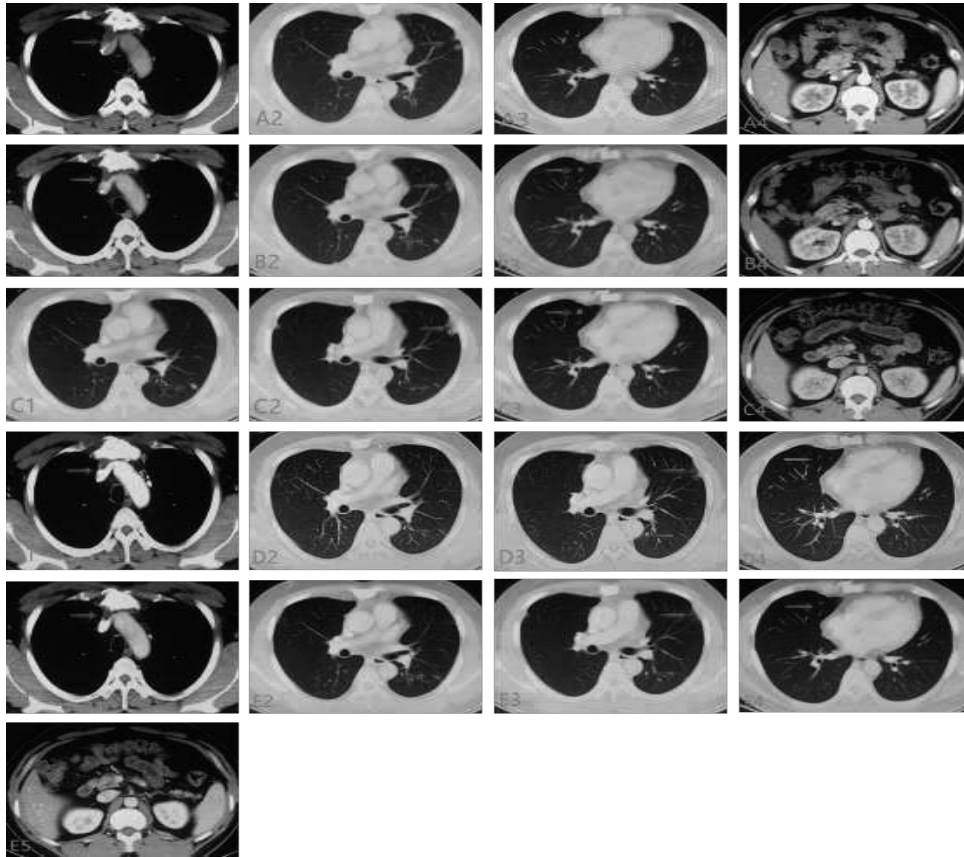


Fig. 2. CT images during treatment (A1-4:2016-06; B1-4:2016-10; C1-4:2016-12 late; D1-4:2017-02; E1-5:2017-08). The arrows mark lesions.

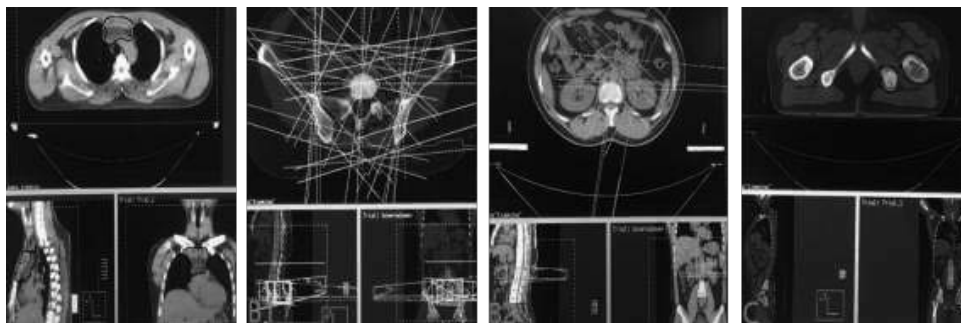


Fig. 3. Radiotherapy and target volume. A) thymus lesion, B) 1-2: the fifth lumbar spine and the first sacral vertebra lesions, left adrenal lesion, C) pelvic and the left upper femur lesions).

Table I. *Timeline of patient management.*

Time	Examination	Diagnosis or Progression	Treatment	Efficacy
Early Jan. 2016	CT and Pathology	Thymic poorly differentiated SCC	Operation and postoperative chemotherapy (gemcitabine and cisplatin)	--
Jun. 2016	CT	Suspected thymic cancer recurrence Right shoulder blade and the left femoral head bone metastases Double lung nodules	Chemotherapy (docetaxel and carboplatin)	--
Early Aug. 2016			Radiotherapy for postoperative tumor bed	--
Late Aug. 2016	MR	The fifth lumbar spine and the first sacral vertebra bone metastases		--
Oct. 2016	CT	Double lung metastases Left adrenal metastasis	Chemotherapy (docetaxel plus cisplatin)	--
Mid Nov. 2016			Radiotherapy for left adrenal, the fifth lumbar spine and the first sacral vertebra bone metastases	Osteodynia relief
Late Dec. 2016	CT and MR	The left pubic bone, acetabulum and upper femur bone metastases		Thymic lesion complete response(CR) Left adrenal metastasis --PR Double lung metastases--SD
Early Jan. 2017			Oral apatinib	
Feb. 2017	CT			Double lung metastases--CR
May 2017			Radiotherapy for pelvic and the left upper femur bone metastases	Osteodynia relief
Aug. 2017	CT			Thymic lesion --CR

During the administration of apatinib, the patient experienced some toxicities. In the initial stage of taking this medicine, he experienced a grade of stomach upset and loss of appetite; after reducing the dose for one week and administering oral omeprazole, the symptoms were relieved and the initial dose was resumed later. In addition, he

also presented with hand-foot syndrome (grade 1), characterized by tingling at the end of fingers and toes, and transient hypertension (grade 1), but these adverse events were tolerable.

Currently, the patient has been taking apatinib as a maintenance treatment; we will continue to follow-up regularly with this patient. The patient

agreed to the treatment plan and with this case report, and has signed the therapeutic communication and informed consent.

DISCUSSION

We chose the apatinib treatment for several reasons. First, the National Comprehensive Cancer Network (NCCN) Guidelines recommend chemotherapy primarily for the treatment of extensive metastatic advanced thymic carcinoma with platinum-based combination chemotherapy regimens, but unfortunately, thymic carcinoma is not sensitive to chemotherapy. Secondly, similar to apatinib, sorafenib and sunitinib are also tyrosine kinase inhibitors that target vascular endothelial growth factor receptors and have been demonstrated to effectively and safely treat thymic carcinoma in several cases in the literature (7, 8). Thirdly, to the best of our knowledge there is not a case report in clinical studies about apatinib in thymic carcinoma treatment, but it has been shown to be effective and well tolerated in the treatment of a variety of solid tumors, including a number of rare malignancies (9). Fourthly, the thymic carcinoma patient could not afford the expensive cost of sorafenib and sunitinib. Fifthly, after repeated chemotherapy, the patient had experienced a marked toxic response, whereas apatinib's toxicity is relatively less and therefore more tolerable (10, 11).

Additional studies and cases are necessary to confirm that apatinib is effective for advanced metastatic thymic carcinoma. We can probably change the dilemma and pattern if this can be confirmed at the molecular level and from more clinical data. From this case, we considered whether we could make case for a combination of radiotherapy and apatinib in future research (12), not limited to the treatment of thymic carcinoma.

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LETTER TO THE EDITOR

PROTECTIVE EFFECTS OF *HERPETOSPERMUM CAUDIGERUM* EXTRACTS AGAINST LIVER INJURY INDUCED BY CARBON TETRACHLORIDE IN MOUSE

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Received December 3, 2017 – Accepted January 9, 2018

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Herpetospermum caudigerum (*H. caudigerum*; HC), popularly known as “Sejimeiduo” in Tibet, it is widely used in Tibetan traditional medicine for the treatment of dyspepsia, liver and colic diseases. This study was designed to evaluate the effect of *H. caudigerum* extract (HCE) on suppressing liver injury induced by carbon tetra chloride (CCl₄). For this purpose, we used CCl₄ to induce acute liver injury in mouse model. The protective effects of HCE against liver injury were evaluated by biochemical parameters, histopathological and immunohistochemical staining. The results showed that the superoxide dismutase (SOD) activity was significantly increased with the increasing dose of HCE as compared to the CCl₄-treated group ($p < 0.01$); while AST and ALT levels in serum, MDA and MPO in liver were reduced in a dose-dependent manner. The histopathology showed that HCE treatment promoted the recovery of histopathological changes in liver in a dose-dependent way. Meanwhile, there was a higher expression of caspase-3 and NF- κ B in the nucleus of several liver cells in the CCl₄-induced group, and a low expression of caspase-3 and NF- κ B were found with the increasing dose of HCE. Therefore, the present study suggests that HCE is a potent hepatoprotective agent that can treat acute liver injury and this ability may be attributed towards its anti-inflammatory and antioxidant potential.

To the Editor,

Herpetospermum caudigerum (*H. caudigerum*) belongs to the cucurbitaceae family, which is widely distributed in the Himalayan region of the southwest of China with an average height of 2300–5000m (1). It is known as “Sejimeiduo” among the Tibetan traditional healers and is used for the treatment of liver diseases, colic and dyspepsia (2). In previous

investigations, an ethanol extract of *H. caudigerum* seeds showed a positive effect to inhibit hepatitis B virus (3, 4). Recently, many chemicals, drugs and viruses have induced liver injury and have been recognized as a toxicological problem (5). CCl₄ is a potent chemical hepatotoxin that can cause acute hepatocellular injury. Despite recent studies and improved knowledge of our understanding of

Key words: liver injury, carbon tetrachloride, Tibetan medicine, *Herpetospermum caudigerum* extract (HCE), hepatoprotective

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0393-974X (2018)

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pathogenesis and novel drug discovery, there are still limited effective therapeutic interventions for liver diseases.

In the present study, we investigated the *in vivo* activity of an extract solution of *H. caudigerum* (ESHC) seeds against experimentally-induced liver injury by carbon tetrachloride (CCl_4). Therefore, the purpose of the present study was to investigate the effect and the possible mechanism of ESHC to protect CCl_4 -induced acute liver injury in mice via the biochemical parameters, antioxidant activity, and histopathological and immunohistochemical staining.

MATERIALS AND METHODS

All the experiments were conducted after the approval of the Ethics Committee guidelines of Huazhong Agricultural University, Wuhan, China (Approval number: 31272518), and guidelines of Proclamation of the Standing Committee of Hubei People's Congress (PSCH No. 5), China.

Preparation of HCE

The seeds of *H. caudigerum* were purchased from the Tibetan medicine market in Linzhi, Tibet Autonomous Region, China, and oven-dried at 60°C for 24 h. The seeds (100 g) were then crushed into powder and extracted with ligarine using sonication for 30 min, 3 times (the volume of ligarine was 200 ml, 150 ml, 150 ml, respectively). After filtration, the extract of ligarine was concentrated by rotary vacuum evaporator and then vacuum-dried lower than 60°C . Then the yield obtained was extracted with chloroform using sonication for 30 min, 3 times (the volume of chloroform was 100 ml, 50 ml, and 50 ml, respectively). After filtration, the chloroform solution was concentrated and vacuum-dried, and then added with 100 ml distilled water, tween-80 as solubilizer. The chloroform solution was extracted from seeds of HC and then the aqueous extract was prepared in water solution for the seeds of HC.

Animal treatment and experimental groups

Fifty mice were randomly divided into five groups, (10 mice in each group): group I (control); group II (acute liver injury); groups III, IV and V used HCE prophylactically

for 7 days with 3 different doses (10, 30 and 60 g/kg/day through intragastric administration, respectively). Then, the mice in groups II-V received a peritoneal injection of CCl_4 (5ml/kg 1% CCl_4 solution in bean oil). The control group was injected with the same amount of physiological saline alone. The mice were sacrificed after 16 h of the CCl_4 treatment. Blood samples were collected from each group for biochemical analysis and liver tissues were fixed in 4% paraformaldehyde for hematoxylin & eosin (H&E) staining.

Determination of MDA, SOD, ALT and AST

The levels of MDA, SOD, ALT and AST activity in liver were measured by using the commercial assay kit as suggested by previous studies (6, 7).

Histopathological and immunohistochemistry studies

The liver samples were stained for H&E according to previous study (8). The caspase-3 and NF- κB in immunohistochemistry analysis was measured according to previous studies as described by Zhang et al. (9).

Statistical analysis

The results were analyzed with a *chi*-squared test using SPSS 18.0. The differences were considered statistically significant if $P < 0.05$.

RESULTS

AST and ALT levels in serum

The level of ALT and AST enzymes in serum were increased significantly after the single injection of CCl_4 in group II as compared to the control group. Whereas, pre-treated animals with different doses of HCE (10, 30 and 60 g/kg/day) for 7 days showed a significant reduction in activities of serum AST and ALT as compared to group II (Table I).

MDA and SOD levels in liver tissue

MDA levels were significantly increased in the liver of mice subjected to CCl_4 as compared to the normal control mice. Administration of different doses of HCE (10, 30 and 60 g/kg/day) in mice prior to CCl_4 depicted a marked reduction in the mean MDA level (Table II).

The mice exposed to CCl_4 showed a significant

Table I. Effect of HCE on serum biochemical parameters in mice intoxicated with CCl_4

Groups	Pretreatment (g/kg)	ALT ($\text{U} \cdot \text{L}^{-1}$)	AST ($\text{U} \cdot \text{L}^{-1}$)
Group I (Normal group)	Vehicle	26.07±3.23	66.09±8.99
Group II (CCl_4 model group)	Vehicle	121.04±9.81 ^b	231.84±18.32 ^b
Group III (CCl_4 + HCE)	10	53.99±3.21 ^d	139.67±13.98 ^d
Group IV (CCl_4 + HCE)	30	37.25±9.80 ^d	126.63±8.53 ^d
Group V (CCl_4 + HCE)	60	30.40±2.44 ^d	99.63±36.89 ^d

^bCompared with normal group: $p < 0.01$; ^dCompared with CCl_4 model group: $p < 0.01$.

Table II. Effect of HCE on MDA level in mice intoxicated with CCl_4

Groups	Pretreatment (g/kg)	MDA ($\text{nmol} \cdot \text{mg}^{-1}$)	SOD ($\text{U} \cdot \text{mg}^{-1}$)
Group I (Normal control)	Vehicle	6.35±1.49	45.2±6
Group II (CCl_4 model control)	Vehicle	11.74±1.07 ^b	22.8±4.36 ^b
Group III (CCl_4 + HCE)	10	8.16±1.15 ^d	33.9±1.72 ^c
Group IV (CCl_4 + HCE)	30	7.90±0.38 ^d	36.61±6.84 ^c
Group V (CCl_4 + HCE)	60	7.85±0.90 ^d	37.47±5.29 ^c

^bCompared with normal group: $p < 0.01$; ^dCompared with CCl_4 model group: $p < 0.05$; ^cCompared with CCl_4 model group: $p < 0.05$.

decrease in SOD activity in liver as compared to the normal group. Administration of different doses of HCE (10, 30 and 60 g/kg/day) in mice prior to CCl_4 showed a significant increase in the mean SOD activity (Table II). These results indicate that HCE-treated animals effectively scavenged the free radicals released in the liver.

MPO activity in liver tissues

The mice exposed to CCl_4 showed a significant rise in MPO levels in liver as compared to the control mice. Administration of different doses of HCE (10, 30 and 60 g/kg/day) in the mice before CCl_4 resulted in significant decrease in MPO level (Table III).

Histopathological observations

The results obtained from serum enzyme assays were also supported by the histological observations. Liver histological sections from group I showed normal hepatic cells, normal lobular architecture, a well-defined nucleus and nucleoli with a well-preserved cytoplasm (Fig. 1A). Livers challenged with CCl_4 , upon histopathological examination showed centrilobular necrosis, inflammatory infiltration of lymphocytes, nucleus fragmentation and central veins having plenty of blood (Fig. 1B). The histology of liver of mice pre-treated with HCE before CCl_4 challenge showed that HCE was effective by increasing in a concentration manner to

Table III. Effect of HCE on MPO activity in mice intoxicated with CCl_4 .

Groups	Pretreatment (g/kg)	MPO ($\text{U} \cdot \text{mg}^{-1}$)
Group I (Normal control)	Vehicle	56.7 ± 5.09
Group II (CCl_4 model control)	Vehicle	162.91 ± 9.86^b
Group III (CCl_4 + HCE)	10	83.56 ± 8.73^c
Group IV (CCl_4 + HCE)	30	74.07 ± 9.02^c
Group V (CCl_4 + HCE)	60	67.95 ± 7.57^c

^bCompared with normal group: $p < 0.01$; ^cCompared with CCl_4 model group: $p < 0.05$.

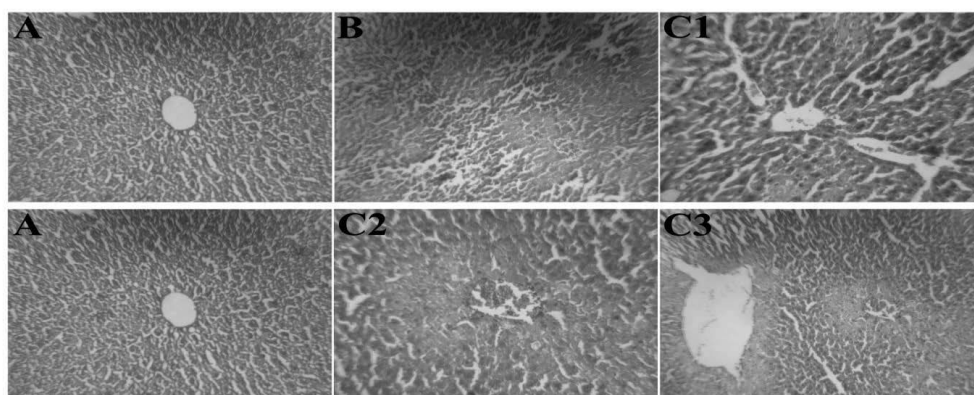


Fig. 1. Effect of HCE on the histopathological changes in liver of CCl_4 -intoxicated mice. **A)** Group I showing normal structure. **B)** Group II, showing inflammatory infiltration of lymphocytes, nucleus fragmentation and central veins with plenty of blood as well as the disappearance of nuclei. **C)** Pre-treated mice groups with HCE at 10, 30 and 60g/kg respectively. All liver samples were stained with hematoxylin and eosin ($200\times$).

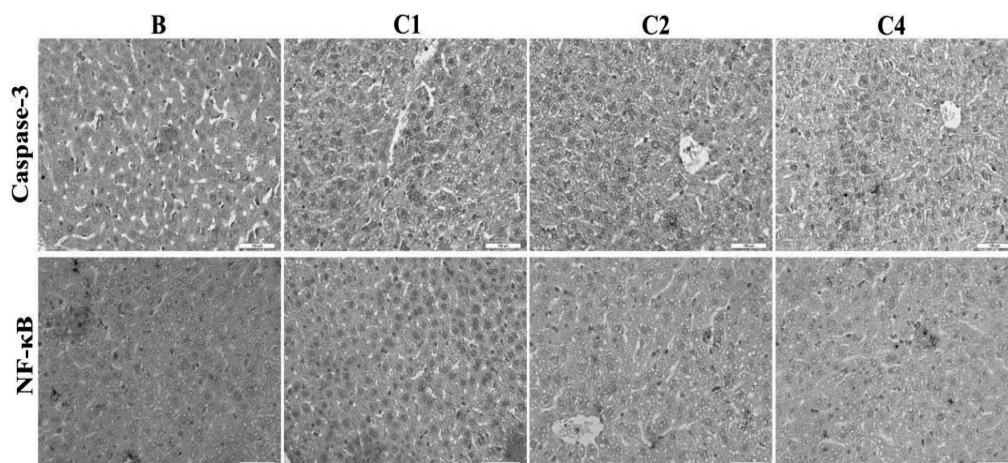


Fig. 2. Immunohistochemistry staining of caspase-3 and NF- κ B.

stop the histopathological variations (Fig. 1C). The liver of mice which were pre-treated at 60g/kg/day (highest dose) showed no change in structure.

Immunohistochemistry of cleaved caspase-3 and NF- κ B

In group II, most of the liver cells in the cytoplasm showed positive staining. Caspase-3 was more expressed in the CCl₄-treated mice (group II), compared to the HCE. In other groups (group III-V) which were pretreated with HCE, there was less localization of caspase-3, because some of liver cells showed positive staining in the cytoplasm. The CCl₄-treated mice (group II) showed that hepatic cells were bordered by positive-stained cytoplasm for NF- κ B. In contrast, in other groups pretreated with HCE, there was less expression of NF- κ B.

DISCUSSION

In this study, we checked the anti-inflammatory and free radical scavenging effects of HCE via different experimental test models. During the last decade, most of studies were conducted on the liver of animals along with animal model normal liver. Due to the damaging effect of allopathic medicine on liver, many medicinal plants have been recommended to treat liver disorders (10, 11). However, the application of medicinal plants needs a detailed pharmacological study before their recommendation for clinical use. Recently, it has been recognized that many chemicals and drugs have induced liver injury (5). CCl₄ is a potent chemical hepatotoxin that can cause acute hepatocellular injury (12). Serum AST and ALT are important biomarkers for the diagnosis of liver damage (5). The increased level of liver enzymes indicates halofantrine-induced hepatocellular damage. Results showed that CCl₄ caused hepatocellular damage via change in serum enzyme (AST and ALT) activities in comparison with non-toxicity group (group I) in mice. This study suggested *in vivo* potential of HCE to prevent chemical-induced liver injury, as the histopathology analysis showed the absence of liver injury in pre-treated HCE groups. One previous study (13) also mentioned the hepatoprotective effect of herbal

drugs. The treatment of animals with CCl₄ caused a significant increase in lipid peroxidation and decrease in the activities of antioxidant enzymes. These results indicate that HCE can attenuate the lethal oxidative stress triggered by CCl₄. This antioxidant capacity of HCE may be due to the presence of lignans, saponins, unsaturated fatty acids and other phenolic compounds.

Caspase-3 is involved in the final execution phase of apoptosis (14). Our results indicated that caspase-3 was more expressed in the CCl₄-treated mice (group II), and less expressed in animals pretreated with ESHC. Taken together, the results of this study suggest that ESHC attenuates CCl₄-induced apoptosis in group II. NF- κ B plays an important role in the gene expression of pro-inflammatory cytokines (15). Our results also support that liver specimens were positively stained for NF- κ B in group II, and negatively in the ESHC pretreated groups III-V.

This study demonstrates that ESHC has an *in vivo* anti-hepatotoxic activity, and therefore it could be used as a protective agent against acute liver injury. The findings are important for human/ animal health care to implement seeds of *H. caudigerum* as a remedy.

ACKNOWLEDGEMENTS

The study was supported by National Key Research and Development Program of China (Project No. 2017YFD0502200) and the Chinese Agricultural Research Systems (CARS-37).

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LETTER TO THE EDITOR

PYRAZINAMIDE-RESISTANT MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM KHYBER PAKHTUNKHWA AND *rpsA* MUTATIONS

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Received March 6, 2018 – Accepted April 11, 2018

Pyrazinamide (PZA) is a key first-line antibiotic used for the short-course treatment of drug-sensitive and multidrug-resistant (MDR) isolates of tuberculosis. PZA exhibits potent action against semi-dormant bacilli in acidic environments. However, mutations that occur in target genes may cause technical difficulties in the diagnosis of PZA resistance during drug susceptibility testing. The objective of the current study is to identify mutations in *pncA*^{WT} *rpsA* and *rpsA*^{WT} *panD* genes among PZA-resistant isolates of *Mycobacterium tuberculosis* (MTB) circulating in the Pashtun dominant region, Khyber Pakhtunkhwa, Pakistan. We selected 18 PZA-resistant *pncA*^{WT} strains from the Provincial Tuberculosis Reference Laboratory (PTRL) Khyber Pakhtunkhwa to investigate mutations in the coding region of *rpsA* and *panD* genes. The experiments were repeated for drug susceptibility testing using MGIT 960 automated system. In addition, eighteen PZA-resistant *rpsA* genes along with 5 susceptible strains and one H37Rv strain were sequenced. All 18 isolates were PZA-resistant. The majority of these isolates exhibited multidrug resistance (MDR) (13/18). We identified 14 non-synonymous and one synonymous mutation in the coding region of *rpsA* in 11 strains. All mutations were scattered throughout the gene and not reported previously. Further, we did not identify any mutation in 7 *rpsA*^{WT} *panD* genes. Mutations in *rpsA* but not in *panD* occur in PZA-resistant *pncA*^{WT} MTB isolates circulating in Khyber Pakhtunkhwa, Pakistan.

To the Editor,

Tuberculosis (TB) is one of the major public health problems caused by *Mycobacterium tuberculosis* (MTB). The WHO recommended six-month therapy of four first-line drugs, rifampin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB). Pakistan has been ranked among the top five countries with TB prevalence of 56%. The total incidence of drug resistance (MDR/

RR-TB) is twenty-six thousand (16–36) and 14 (8.5–19) per hundred thousand population (1).

Pyrazinamide (PZA) is an important component of first-line antituberculosis drugs that targets latent stage MTB at low pH. The MGIT 960 automated system is the most reliable (2) and the only available phenotypic testing method. However, due to some technical and buffering issues of PZA DST, the results of phenotypic resistance are not always

Key words: resistance, pncA, DST

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0393-974X (2018)

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reliable (3). Molecular methods to detect mutations in the targets gene are better choice for susceptibility testing. Mutations in the *pncA* gene are most commonly associated with pyrazinamide (PZA) resistance (3). Molecular detection of PZA resistance is more complicated given that resistance-conferring mutations are scattered throughout the entire *pncA* gene (3). In some studies, PZA-resistant isolates lack mutations in the *pncA* gene, whereas *rpsA* (ribosomal protein S1 involved in trans-translation) and *panD* (encoding aspartate decarboxylase involved in the coenzyme A biosynthesis) gene mutations were reported to correlate with PZA resistance (4). Here, we aimed to identify *rpsA* gene mutations among PZA-resistant isolates and gene mutations among

wild-type *pncA* (*pncA*^{WT}) and *panD* with wild-type *rpsA* (*rpsA*^{WT}) isolates circulating in Khyber Pakhtunkhwa, Pakistan.

MATERIALS AND METHODS

Ethical considerations

Ethical clearance was obtained from Provincial Tuberculosis Reference Laboratory (PTRL) Khyber Pakhtunkhwa (KPK) and the Institutional Ethics Committee of Capital University of Science and Technology (CUST) Islamabad. However, the results were not linked with individual patient data.

Study samples

We collected 18 samples from the Provincial Tuberculosis Reference Laboratory (PTRL) which had been previously identified as PZA-resistant but *pncA*^{WT}. PTRL is the only central reference laboratory of KPK which receives patient samples for drug susceptibility testing.

Drug susceptibility testing

Drug susceptibility testing (DST) was performed using the automated BACTEC MGIT 960 system. *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* were used as negative and positive controls, respectively. Growth at a critical concentration 100 µg/ml PZA was considered to indicate PZA resistance.

DNA extraction, PCR amplification and sequencing

PZA-resistant samples were subjected for Genomic DNA extraction using the sonication method (5, 6). The

Table I. Demographic data of PZA-resistant *pncA*^{WT}.

Resistant Type	Resistant	Total
MDR	13	13
Poly_Resistant	4	4
XDR	1	1
Gender		
Female	12	12
Male	6	6
History		
Diagnostics	14	14
Follow Up	4	4

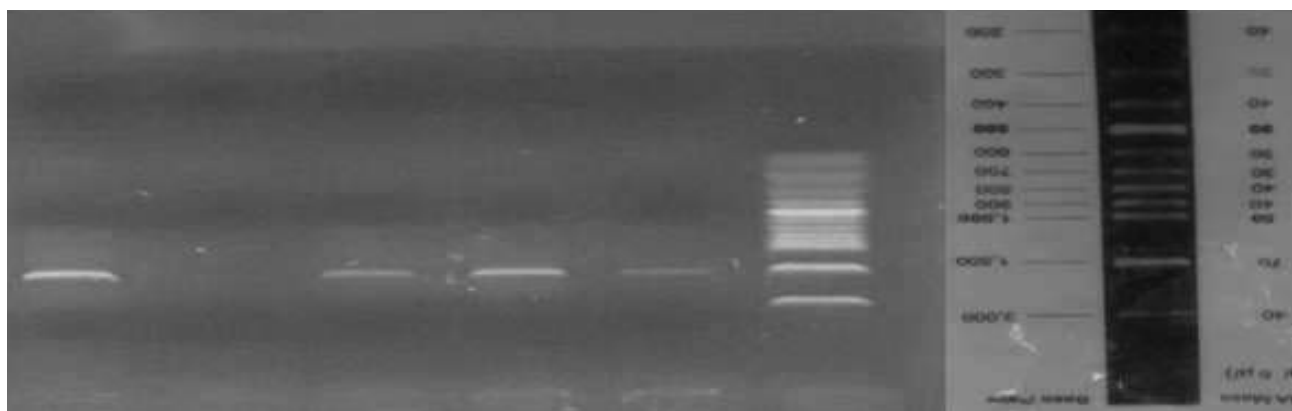


Fig. 1. *rpsA* gene PCR product.

fragments containing *rpsA* and *panD* were amplified using the following previously reported primers: *rpsA*-F (5'-CGGAGCAACCCAACAATA-3'), *rpsA*-R (5'-GTGGA CAGCAACGACTTC-3') (7), *panD* (F:5-TCAACGGTTCCGGTCGGCTGCT-3) and *panD*_R (5-TATCCGCCA CTGCT GCA CGACCTT-3) (8). Each 50- μ l PCR reaction contained 0.1 μ l of each DNTs, 3 μ l $MgCl_2$, 5 μ l PCR buffer, 0.8 μ l Taq (New England Biolabs, UK), 1 μ l each forward and reverse primers, 34.8 μ l molecular grade water and 4 μ l of genomic DNA. The PCR conditions were 5 min at 94°C for the denaturation step; 30 cycles of 30 s, 30 s at 56°C, and 72°C for 1 min; an extension step at 72°C for 5 m as previously described. The PCR product was analyzed using 6 Applied Biosystems 3730xl (Macrogen, Korea).

Statistical analysis

The sequence data were loaded into Mutation Surveyor V5.0.1 and compared with *rpsA* (Rv1630)

and *panD* (Rv2043c) gene sequences from the RefSeq (NC_000962.3) database of NCBI.

RESULTS

Drug susceptibility pattern

All 18 samples were determined to be PZA-resistant using the BACTEC MGIT 960 automated system. The majority of these isolates were MDR (13/18). The resistant isolates along with five sensitive isolates and one H37Rv isolate were sequenced to analyze mutations in 1,544-bp fragment of *rpsA*.

Mutation in PZA-resistant and PZA-susceptible isolates

We investigated mutations in the *rpsA*-coding region (1,544-bp fragment) in both resistant and susceptible isolates (Tables I). Of 18 PZA-resistant isolates, 11 (61%) harbored fifteen different mutations, whereas seven PZA-resistant isolates

Table II. Mutations in *rpsA* gene in PZA-resistant *pncA*^{WT} isolates.

SNO	Nucleotide Position	Codon No.	Codon Change	Amino Acid Change	Frequency (No. of strains)
	76delA	26	ATA	Ile26FRAME	1
	220G>A	74	GTC>ATC	Val74Ile	1
	278A>G	93	AAG>AGG	Lys93Arg	1
	618G>A	206	TTG>TTA	Leu206Leu	2
	636A>C	212	CGA>CGC	Arg212Arg	2
	830A>G	277	AAG>AGG	Lys277Arg	1
	971C>T	324	TCC>TTC	Ser324Phe	1
	973G>A	325	GAG>AAG	Glu325Lys	3
	1021G>C	341	GGC>CGC	Gly341Arg	1
	1024G>A	342	GAC>AAC	Asp342Asn	4
	1027G>A	343	GAC>AAC	Asp343Asn	6
	1030G>C	344	GCG>CCG	Ala344Pro	6
	1051A>T	351	ATC>TTC	Ile351Phe	3
	1108A>C	370	ACC>CCC	Thr370Pro	1
	1207T>G	403	TGG>GGG	Trp403Gly	1

Table III. Mutations in each PZA-resistant strain of *pncA*^{WT} *rpsA* gene.

Sample No.	Mutation 1	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9	Mutation 10
1439										
3264										
3806	973G>A	1021G>C	1024G>A	1027G>A	1030G>C	1051A>T				
1164										
5328	636A>C	1207T>G								
5769	220G>A	278A>G								
1162										
303/17										
644/17										
169/17	76delA									
6408	1030G>C									
2748	1027G>A									
2192	618G>A									
3124	1030G>C	636A>C	1024G>A	1027G>A						
3807	973G>A	1027G>A	1030G>C							
2164										
2550	618G>A	1051A>T	830A>G	971C>T	973G>A	1024G>A	1027G>A	1030G>C	1051A>T	1108A>C
1855/17	1024G>A	1027G>A	1030G>C							

were *rpsA*^{WT} (Table II). We sequenced a 420-bp fragment of the *panD* gene in seven wild-type *rpsA* isolates to identify gene mutations. However, we did not find any *panD* mutations in PZA-resistant isolated with *rpsA*^{WT} or sensitive isolates.

The majority of *rpsA* mutations were found in the 973-1051 coding region. The 1024-1030 region was a hot spot region for mutations. The mutation (Asp342Asn) was identified in four isolates, whereas the latter two mutations (Asp343Asn and Ala344Pro) were detected in six PZA-resistant isolates (Table II). All the variants were substitutions except a single deletion, 76delA.

DISCUSSION

The lack of mutations in *pncA* in eighteen PZA-resistant MTB isolates could be clarified by the identification of mutations in other target genes, including *rpsA*, which encodes the 30S ribosomal protein S1 (*rpsA*) and plays a role in trans-translation, and *panD*, which encodes aspartate decarboxylase (7). We report fifteen mutations in 11 (61%) isolates (Table II), and seven PZA-resistant isolates were *pncA*^{WT} and *rpsA*^{WT}. However, no *panD* gene mutations were detected among the 7 PZA-resistant *pncA*^{WT} and *rpsA* isolates. Gu et al. also identified

6 *pncA* and *rpsA* wild-type PZA-resistant strains that lacked any *panD* mutations (9). These findings suggest a new mechanism of PZA resistance in MTB. Tan et al. identified *rpsA* gene mutations in 3 PZA-resistant *pncA*^{WT} isolates and one in susceptible strain (10). All the above reports revealed a weak correlation between *rpsA* gene mutations and PZA resistance. However, Gu et al. identified 26 mutations dispersed along the entire coding region of *rpsA* (9). We also report 14 novel nonsynonymous and one synonymous mutation. We did not identify any *panD* gene mutations in 7 PZA-resistant *rpsA*^{WT} isolates. A possible new target of PZA, the *clpC1* (unfoldase) gene that encodes a family of ATPases, was identified in PZA-resistance isolates (11). However, the role of these genes (*rpsA*, *panD* and *clpC1*) in PZA resistance is minimal compared with *pncA*. A more recent study identified four new efflux proteins, including Rv0191, Rv1667c, Rv3756c and Rv3008, involved in PZA/POA resistance (12). Future studies are needed to assess their roles in PZA resistance in clinical strains (12). These findings suggest the role of reported efflux proteins in PZA resistance in addition to *pncA*, *rpsA* and *panD*. Further investigations are needed to identify the quantitative role of all targets of PZA resistance for better management of drug-resistant TB. The role of *rpsA* gene mutations in PZA resistance should be evaluated from geographically diverse environments, especially in countries with a high TB burden.

ACKNOWLEDGEMENTS

This study was ethically and technically supported by the Project Director Provincial Tuberculosis Reference Laboratory Khyber Pakhtunkhwa, Pakistan.

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LETTER TO THE EDITOR

COMPARATIVE EFFICACY OF ORAL CONTRACEPTIVE *VERSUS* LOCAL TREATMENT *VERSUS* INTENSE PULSED LIGHT COMBINED WITH VACUUM IN ENDOCRINE ACNE IN WOMEN

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Received February 27, 2018 – Accepted March 30, 2108

All Authors contributed equally to this study

Acne is the most common affection of adolescents, although it can be also found in adult women. Our study was aimed at the comparative assessment of three different therapies over a three-month period, applied to women with moderate comedogenic and papulo-pustular endocrine acne. In the study 116 female patients with endocrine localized face acne were included and divided into three groups: group I with 42 patients was treated with a combination of contraceptive pill + local treatment + pulsed-vacuum light; group II with 38 patients was treated with contraceptives and pulsed-vacuum light and group III with 36 patients was treated only with local treatment. The acne evaluation was made using the Global Acne Grading System (GAGS). Statistical data processing was carried out using the STATA software. For the comedogenic form of acne, the good and very good results were superior in group I vs group II and III (83.33% vs 31.58% vs 5.56%) at the end of the three months of treatment. For the papulo-pustulous form of acne, good and very good results were similar in groups I and II (92.86% vs 73.68%) both after the first month of treatment and at the end of the study, well above the local treatment group (13.99%). Our study highlighted the superiority of laser treatment combined with hormonal treatment, compared to hormonal and local treatment in the comedogenic form of acne, and the superiority of hormonal treatment combined (or not) with laser treatment in the papulo-pustular form compared to local treatment.

To the Editor,

Acne is the most common affection of adolescents, over 80% of whom suffer from one of the clinical

forms of the disease (1). Liver diseases, such as non-alcoholic fatty liver, may influence the normal synthesis of estrogens and promote the occurrence

Key words: acne, contraceptive pill, local treatment

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0393-974X (2018)

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of acne in adult women (2). Various endocrine disruptors play a role in the development of acne by influencing the synthesis of ovarian estrogen hormones (3, 4).

However, the disease may begin at birth or perinatally and may extend to adulthood, especially in females (1). Depending on the clinical form of the disease, the age of the patients, the associated pathology, etc., the treatment may range from a simple local treatment to a complex treatment, generally based on intense pulsed light, general antibiotic, isotretinoin or hormonal treatment (1, 5). In the case of female patients, it is essential to distinguish a physiological acne from endocrine acne in pathological conditions. Our study aimed at the comparative evaluation of the results obtained from a combined treatment of contraceptives, high pulsed light + vacuum and local treatment on a group of patients with moderate endocrine acne form located on the face.

MATERIALS AND METHODS

We conducted a randomized, comparative, blind study between January 2016 and January 2017 on a group of 116 Caucasian patients with endocrine acne, moderate comedogenic form and papulo-pustular localized on the face. Each patient signed an informed consent to participate in the study. The study group comprised of three parallel groups. The age of the patients ranged between 18-35 years.

Each study group was evaluated monthly over a three-month period. The patients were recruited on the first-come, first-served basis. For the allocation of patients, a computer-generated list of random numbers was used. Before each enrollment, the patient allocation to one group or another was transmitted by telephone to the principal investigator by a computer specialist not involved in this study.

Patients were classified as having endocrine acne if they met at least one of the following criteria:

- Increased serum testosterone (between 10-200 ng / dl) and dehydroepiandrosterone (DHEA) (normal levels ≤ 1 mg/24 h) (ELISA method, ELx808IU device, BioTek, Winooski, VT, USA)

- Acne resistant to conventional treatment performed

according to international guides (local, general antibiotic or isotretinoin)

- Acne accompanied by signs of hyperandrogenism (hypertrichosis, intense seborrhea of the face and scalp, menstrual cycle disorders - oligo/amenorrhea defined as less than 8 menstrual cycles/year, androgenic alopecia of the scalp, voice thickening, clitoromalgia).

- Patients with refractory acne to conventional treatment accompanied by polycystic ovaries highlighted by transvaginal ultrasound

- Refractory acne refractory to a woman with a body mass index of over 29 kg / m² (BMI ≥ 29 kg / m²)

The severity of the disease was assessed using the Global Acne Grading System (GAGS) scale: no injuries - Grade 0; comedones present - Grade 1; papules present - Grade 2; pustules present - Grade 3; nodules present - Grade 4.

The local score is calculated as factor x grade (0-4). The factor had the following values: forehead - 2, right cheek 2, left cheek - 2, chin -1.

The overall score was made up of the sum of the local scores. Accordingly, the severity of the disease was appreciated as follows: mild-acne - score 1-18; moderate acne - score 19-30; severe acne - score 31-38; very severe acne - score ≥ 39 .

The inclusion as comedogenic acne was made for patients with predominantly comedonal lesions, while the papulo-pustular form was diagnosed when predominantly papulo-pustular lesions were present.

The inclusion criteria were: women with endocrine acne, moderate, localized, face-shaped, assessed according to the above criteria; and women with Fitzpatrick I-III.

The exclusion criteria were: patients with autoimmune diseases or other endocrine diseases; patients treated with isotretinoin in the previous 6 months; pregnant/nursing women; patients with superinfected injuries, very dry skin; patients unable to comply with the study protocol; and recently tanned patients

Prior to enrollment, each patient signed an informed consent agreeing to participate in the study. The study protocol followed the guidelines of the Helsinki Declaration (1975) and was approved by the Ethics Committee of DR Ianosi Medical Center.

To assess the evolution of the disease, each patient was photographed at the beginning of the treatment and subsequently monthly for three consecutive months using

the Canfield Visia II Complexion Analysis System. The evaluation of the results was made blindly, monthly, with the help of two sub-investigators who did not participate in the recruitment of patients, using the Global Acne Grading System (GAGS) (6) as follows:

- insignificant results - reduction of papulo-pustular comedones and lesions between 0-25%
- modest results - reduction of comedones and papulo-pustular lesions between 26-50%
- good results - reduction of comedones and papulo-pustular lesions between 51-75%
- very good results - reduction of comedones and papulo-pustular lesions between 76-100%.

The following treatments were performed:

- contraceptive pills consisting of 2 mg cyproterone acetate +0.035 mg ethinylestradiol
- intense pulsed light (IPL) combined with vacuum (Acleara Acne Grading System manufactured by Theravant INC for Palomar Medical Technologies, Burlington VT, USA), a continuously-cooled system FDA-cleared for mild and moderate comedonal and papular-pustulous inflammatory acne lesions.
- local treatment consisting of tretinoin combinations 0,02% + clindamycin 0,8%

The patients were divided into three groups: group

I included 42 patients treated with a combination of contraceptive pill + intense pulsed light + vacuum + local treatment; group II included 38 patients treated with a combination of contraceptive pill and local treatment; and group III with 36 patients treated only with local treatment.

The contraceptive pill was given from day 1 to day 21 of the menstrual cycle for three consecutive months.

For patients treated with pulsed light combined with vacuum, the manufacturer's recommended working protocol was observed: steam exposure for 10 min before therapy; 2 passings were performed for each patient with energy level 6, V3 vacuum for chin and S3 for forehead, double pulse, 3 ms pulse width and pulse delay of 750 ms for skin type I-III. Each patient received 2 treatments per month for 3 consecutive months. Local treatment was applied every night for all patients for three consecutive months after a pre-cleaning. In the morning, each patient applied a moisturizing cream with SPF 30 specific to acne skin.

All patients were advised to avoid exposure to the sun throughout the study.

Statistical analysis

All data were introduced into Microsoft Excel (Microsoft corporation, USA) and after several

Table I. Results of treatments applied in the two forms of acne after three months of treatment.

Acne Form	Treatment	Good /Very Good	Modest	Insignificant	Chi ² test (vs L)		Multivariate regression		Treatment effects analysis		P	Survival analysis	
					Risk Ratio	P	Odds Ratio	P	ATET	PO mean		Hazard Ratio	P
Global	LAL	35 (83.33%)	4 (9.52%)	3 (7.14%)	7.22	<0.001	13.32	<0.001*	0.033	0.894	<0.001*	1.26	0.346
	AL	26 (68.42%)	8 (21.05%)	4 (10.53%)	5.92	<0.001	4.70	<0.001*	0.284	0.611	<0.001*	8.26	<0.001
	L	3 (8.33%)	19 (52.78%)	14 (38.89%)	-	-	-	-	-	-	-	1	-
COMED	LAL	35 (83.33%)	4 (9.52%)	3 (7.14%)	10.00	<0.001	13.04	<0.001*	0.517	0.316	<0.001*	4.26	0.004
	AL	12 (31.58%)	16 (42.11%)	10 (26.32%)	4.11	0.007	1.98	0.007*	0.232	0.083	0.070*	5.08	0.138
	L	3 (8.33%)	16 (44.44%)	17 (42.22%)	-	-	-	-	-	-	-	1	-
PAP	LAL	39 (92.86%)	2 (4.76%)	1 (2.38%)	6.68	<0.001	10.94	<0.001*	0.192	0.737	<0.001*	1.26	0.462
	AL	28 (73.68%)	6 (15.79%)	4 (10.53%)	5.30	<0.001	8.67	<0.001*	0.598	0.139	0.016*	9.60	0.03
	L	5 (13.99%)	18 (50.00%)	13 (36.11%)	-	-	-	-	-	-	-	-	-

COMED: comedonal form; PAP: papulo pustular form; LAL: contraceptive pill + intense pulsed light + vacuum + local treatment; AL: ontraceptive pill + local treatment; L: local treatment only; ATET: average treatment effect among the treated; PO: potential outcome mean

arrangements and variable generation, data was introduced in two separate files for transversal and longitudinal data analysis into the software STATA (Stata Corp, USA).

Numerical variables were expressed as mean $\pm$ standard deviation. Proportions were always expressed as percentages. The required sample size required for the study was calculated a priori based upon previous studies (5) using the statistical power computation procedure of STATA, which give 14 patients per group. We enrolled almost triple the number of patients.

Treatment effects statistical analysis is a procedure that helps to prove causality hypothesis and this specially developed for comparing the effect of multiple treatments in homogeneous lot of patients with a given disease. We used the estimator inverse-probability-weighted regression adjustment (IPW-RA) to calculate average treatment effect among the treated (ATET) and potential outcome mean as measures of treatment effects.

The longitudinal data analysis included calculation of incidence rates, statistical modelling using longitudinal regression models and time-to-event analysis.

Statistical modeling used longitudinal regression models, namely random-effects ordered logistic regression. The actual values taken on by the outcome were irrelevant, although larger values are assumed to correspond to higher outcomes. The conditional distribution of the outcome variable given the random effects is assumed to be multinomial with success probability determined by the logistic cumulative distribution function. These type of models are used to assess the influence of the time-independent variables (as is the type of treatment applied) when the outcome have a high individual variation, as is well-known that treatment response have a high variability among the individuals due to a large variety of factors. By accounting for randomness of the treatment outcome that is inherent to biological systems, the model can better predict the chance for success of every treatment type, compared with the usual transversely applied regression models which ignore the time factor.

The time-to-event analysis (survival analysis) was performed using the good/very good result as failure variable and time to event expressed in whole months from the beginning of the treatment. This produced hazard ratios which can be assimilated to risk ratios in the transverse analysis and which can be used to compare treatment success rates per unit of time, so in the future

the patients can make informed decisions on the type of treatment outcomes and time required.

RESULTS

At the end of the three months of treatment, of the 116 patients enrolled, 31 had a modest overall result, 64 had good and very good overall results, and 21 were refractory to treatment, the results being insignificant. Good and very good results were encountered in 35 (83.33%) of the 42 the patients treated with laser + contraceptive pills + local (LAL), 26 (68.42%) of the 38 patients treated with contraceptives + local (AL) and 3 (8.33%) of the 36 patients treated only locally (L) (Table I).

The results of the treatment effect analysis showed that in the comedonal form, LAL treatment had a higher potential outcome (PO) mean than AL treatment (0.316 vs 0.083), whereas in the papulopustular form, LAL treatment had also a higher PO mean than the AL treatment (0.737 vs 0.139) (Table I).

Comparing the good and very good results of LAL vs AL, it was observed that comedonal lesions occurred in 83.33% of patients treated with LAL, vs 8.33% of patients treated with local treatment (Risk Ratio (RR) = 10.00, $p < 0.0001$). In the case of papulopustular lesions, good and very good results occurred in 92.86% of patients treated with LAL, vs 13.99% of patients treated locally (RR = 6.68, $p < 0.001$).

A similar comparison of the AL and L treatments showed an RR of 4.11 in the cases of comedonal lesions ($p = 0.007$) and 5.30 for papulopustular lesions ($p < 0.001$) (Table I).

Multivariate analysis compared the effect of applying the three types of treatments to the chance of obtaining a good result (in comedogenic and papulopustular form). In the comedonal form, LAL treatment had an OR of 13.04 ($p < 0.001$) and AL treatment of 1.98 ($p = 0.007$) vs L treatment. In the papulopustular form, LAL treatment had an OR of 10.94 ($p < 0.001$) and the AL treatment of 8.67 ($p < 0.001$) compared to L treatment. There was a much better effect by combining the intense pulsed light with anti-contraceptives in the comedonal form compared with using contraceptive treatment alone ($p < 0.001$). In the papulopustular form, the effects of the two treatments

were comparable ($p = 0.351$) (Table I).

The longitudinal data analysis of the results showed that in the case of comedonal form the good and very good results of LAL treatment occurred from the first month in 20 patients (47.62%), in the second month in 11 patients (26.19%) and in the third month in 4 patients (9.52%), while for the AL treatment, good and very good results occurred from the first month in 6 patients (15.79%), in the second month in 4 patients (10.53%) and in the third month in 2 patients (5.26%). Local treatment achieved good results in 1 patient in the first month and 1 in the second month (Table II).

In the case of the papulopustulus form, LAL treatment had good and very good results of LAL treatment in the first month in 23 patients (54.76%), in the second month in 6 patients (14.29%) and in the third month in 10 patients (23.81%), while for the AL treatment, good results occurred from 2

months (55.26%), in the second month in 5 patients (13.16%) and in the third month in 2 patients (5.26%). Local treatment achieved good results in 1 patient in the first month and in 4 patients in the third month (Table II).

Survival analysis

The overall incidence rate for achieving a good result in comedogenic form was 8.52 per 100 patients x months and 20.39 per 100 patients x months for LAL treatment, 4.71 per 100 patients x months for AL treatment and 0.93 per 100 patients x months for local treatment. The Cox proportional hazard regression model showed a hazard ratio of 4.26 ($p = 0.004$) for laser treatment and 5.08 ($p = 0.138$) for AL treatment. For this form of acne LAL treatment is clearly superior to AL treatment, the latter having an insignificant coefficient. In the comedonian form, the chance to achieve a very good result through LAL

Table II. Monthly treatment results in comedonal acne.

MONTH	COMEDONAL ACNE					PAPULO-PUSTULAR ACNE				
	Very good	Good	Moderate	Failure	Total	Very good	Good	Moderate	Failure	Total
Treatment Laser+Pills+Local (LAL)										
1	10 (23.81%)	10 (23.81%)	14 (33.33%)	8 (19.05%)	42 (100%)	13 (30.95%)	10 (23.81%)	9 (21.43%)	10 (23.81%)	42 (100%)
2	13 (30.95%)	18 (42.86%)	7 (16.67%)	4 (9.52%)		17 (40.48%)	12 (28.57%)	5 (11.90%)	8 (19.05%)	
3	21 (50.00%)	14 (33.33%)	4 (9.52%)	3 (7.14%)		23 (54.76%)	16 (38.10%)	2 (4.76%)	1 (2.38%)	
Treatment Pills+Local (AL)										
1	3 (7.89%)	3 (7.89%)	10 (26.32%)	22 (57.89%)	38 (100%)	8 (21.05%)	13 (34.21%)	10 (26.32%)	7 (18.42%)	38 (100%)
2	5 (13.16%)	5 (13.16%)	13 (34.21%)	15 (39.47%)		16 (42.11%)	10 (26.32%)	8 (21.05%)	4 (10.53%)	
3	5 (13.16%)	7 (18.42%)	16 (42.11%)	10 (26.32%)		17 (44.74%)	11 (28.95%)	6 (5.79%)	4 (10.53%)	
Treatment Local (L)										
1	0 (0.00%)	1 (2.86%)	10 (28.57%)	24 (68.57%)	35 (100%)	1 (2.78%)	1 (2.78%)	14 (38.879%)	20 (55.56%)	35 (100%)
2	0 (0.00%)	2 (5.71%)	19 (54.29%)	14 (40.00%)		1 (2.78%)	1 (2.78%)	17 (47.22%)	17 (47.22%)	
3	1 (2.78%)	2 (5.56%)	16 (44.44%)	16 (44.44%)		2 (5.56%)	3 (8.33%)	17 (48.57%)	13 (36.11%)	

treatment was 23.81% in the first month, increasing to about 30.95% in the second month and 50% in the third month (Table I).

The overall incidence rate for obtaining a good papulo-pustular result was 14.38 per 100 patients x months and 23.95 per 100 patients x months for LAL treatment, 18.89 per 100 patients x months for AL and 1.89 per 100 patients x months for local treatment. The fact that the rate of incidence in LAL treatment is higher than AL treatment shows that although the success rate is similar, LAL treatment results appear faster than AL treatment, which was to be expected because treatment with intense pulsed light + vacuum light contributes to the disappearance of papulo-pustular lesions by its anti-inflammatory effect.

The Cox proportional hazard regression model showed a hazard ratio of 1.26 ($p = 0.462$) for the LAL treatment and 9.60 ($p = 0.003$) for AL treatment, *vs* L treatment. We can conclude that for the papulo-pustular form a superiority of LAL *vs* AL in terms of achieving good results treatment could not be demonstrated. Both types of treatments were well superior to local treatment in this form of acne

(Table I). For both forms of acne, it was not possible to estimate the effect of local treatment due to lack of a control group (for ethical reasons all patients were treated).

It is noted that in the papulo-pustular form the chance to achieve a very good result by LAL treatment was 30.95% in the first month, increasing to about 40.48% in the second month and to about 54.76% in the third month (Fig. 1). For local treatment, the results appeared in a single patient (2.78%) after the first month of treatment and in 2 patients (5.56%) at the end of the three months.

DISCUSSION

Acne affects women of all ages. The age at which women are affected vary a lot from individual to individual, beginning at adrenarche, between the ages of 8 and 10 years, and peaks around age 17. In recent years adult acne started to be fairly common, occurring in 5% to 12% of adult women (6) and affecting also women in the postmenopausal period (7, 8). The treatment plan for women with acne consists of combined oral contraceptives and other

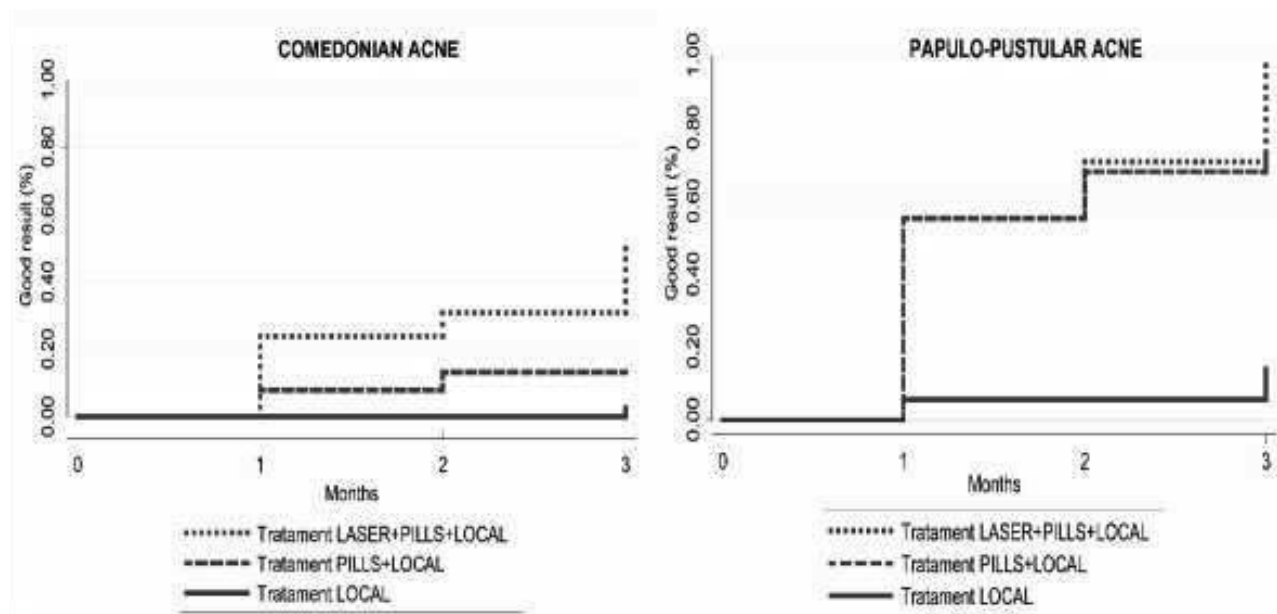


Fig. 1. The hazard function for the good effect of the 3 treatments in the comedonian and papulo-pustular form.

hormonal treatments that can be effective in this group of patients (9).

The key mechanism of combined oral contraceptives pills (cOCPs) action is folliculogenesis inhibition through either suppression of pituitary gonadotrophin secretion or direct influence on ovarian function when the cOCPs agent has weaker anti-gonadotropin activity. cOCPs can also lead to a decrease in dehydroepiandrosterone sulphate and androgen production (10). Estrogens are known to decrease sebum production.

Current therapeutic guidelines recommend that differential therapy be carried out according to the clinical form of the disease. In endocrine acne in women, the role of using hormonal therapy is particularly essential for inflammatory lesions (1). Within 3–6 months of PCOS treatment, inflammatory acne lesion counts are reduced by 30–60%, with improvement in 50–90% of patients.

Intense pulsed light combined with vacuum is a modern therapy to reduce cutaneous porphyrins responsible for the production of inflammatory lesions (11). The Acleara™ Acne Treatment System delivers a combination of vacuum with up to 3 psi pressure (which lifts the dermal structures towards epidermis and expels follicular contents onto the skin's surface) with a broadband pulsed light (500–1200 nm). Due to absorption in the 500–600 nm range, Acleara has an important effect on activates endogenous porphyrines and decreases peri-lesional erythema. The light output between 800–1000 nm has a role on heating the material which clogs the pilosebaceous duct (5, 12).

In our study, for comedonal forms of the disease, the combined LAL treatment determines a healing rate of 13.32 times higher than the local treatment ($p < 0.001$), while the AL treatment produces a healing rate of 4.7 times higher than the local treatment ($p < 0.001$). The effectiveness of combined IPL + vacuum + contraceptive treatment is explained by the fact that the comedone effect of contraception and of local treatment is low.

In the papulo-pustular form, good and very good results are similar for groups I and II of patients compared to group III treated with local treatment only. In conclusion, all the statistical procedures

used demonstrated the superiority of laser treatment combined with hormonal treatment to hormonal and local treatment in the comedogenic form of acne, and the superiority of hormonal therapy combined with or without laser treatment in the papulo-pustular form compared to local treatment.

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LETTER TO THE EDITOR

COMPARISON OF AUTOMATIC AND MANUAL REPOSITION TREATMENT FOR HORIZONTAL SEMICIRCULAR CANAL BENIGN PAROXYSMAL POSITIONAL VERTIGO

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The purpose of this investigation was to compare the efficacy of automatic to manual reposition treatment for patients with horizontal semicircular canal paroxysmal positional vertigo (BPPV). Sixty patients diagnosed with BPPV were equally and randomly divided into either a manual reposition treatment group or an automatic reposition treatment group. The groups were compared regarding difference in pain [visual analog scale (VAS)], extent of vertigo disorder [dizziness handicap inventory (DHI)], quality of life (SF-36), and therapeutic effect. Improved VAS, DHI and SF-36 were observed in both groups, however the efficacious rate of the automatic reposition group (96.7%) was 13.4% higher than that of the manual reposition group, reaching a statistical significance ($p < 0.05$). In conclusion, in the treatment of BPPV patients, automatic reposition is more effective than manual reposition and can improve the patient's quality of life.

To the Editor,

Benign paroxysmal positional vertigo (BPPV), a peripheral vertigo disease, is caused by various etiologies and may develop into a more severe debilitating condition (1, 2). In patients with BPPV, the otolith migrates from the utricle and enters the semicircular canal. When the head changes position the otolith floats in the semicircular canal, causing the endolymph to move, resulting in changes in both the position and discharge frequency of hair cells in the semicircular canal ampulla, resulting in BPPV. The major symptoms of BPPV are paroxysmal vertigo during position change, which usually lasts 2-3 seconds. In some patients, the vertigo of BPPV is associated with vomiting, sweating, nausea and even neurological disorders (3, 4). These symptoms can affect not only the patient's normal physiological function but also their mental acuity. Therefore,

timely treatment for symptoms is critical to promote early recovery and improve the patient's prognosis. For patients with persistent symptoms, a first line of treatment is repositioning maneuvers; these maneuvers attempt to reposition the free-floating canalith particles from the semicircular canals to the utricle using gravity. The purpose of this study is to compare the efficacy of automatic or manual reposition treatment for patients with BPPV.

Sixty patients diagnosed with horizontal canal BPPV from December, 2015 to December, 2016 were randomly and equally divided into either a manual or an automatic reposition group. The criteria for subjects included in the study were: i) age range from 30 to 80 years; ii) a positive clinical rotation test (vertigo caused by a specific position of head lasting no more than one minute); and iii) willing to sign an informed consent. Patients with other aural diseases were excluded.

Key words: benign paroxysmal positional vertigo, automatic reposition treatment, manual reposition treatment, quality of life

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0393-974X (2018)

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The study focused on horizontal semicircular canal cupulolithiasis patients. To eliminate the effect of posterior canal reposition, all patients with posterior canal cupulolithiasis were first treated with horizontal semicircular canal cupulolithiasis followed by a treatment of manual reposition after a negative rotation test. Patients in the manual group were treated with traditional manual repositioning (Barbecue rotation Manoeuvre) as follows (5): they were placed in the supine position and with their heads turned 90 degrees toward the healthy side. Patients were then placed in the prone position with their heads turned 90 degrees toward the healthy side. The patients were placed in the lateral position with the diseased side below, with their heads turned

90 degrees to the healthy side. Lastly, the patients were placed back supine position, with their heads turned 90 degrees to the diseased side. Four rotations comprised one cycle, and every rotation lasted approximately one minute until the nystagmus was relieved. Patients in the automatic group underwent a treatment using an automatic reposition with Thomas Richard-Vitton chair (TRV) (SYNAPSYS co; Marseille, France). Patients were strapped into the swivel chair and wore eye goggles. During the treatment, the patients had to open and close their eyes in order to record the nystagmography. The rotation time was same for both manual and automatic groups.

The degree of pain, the extent of vertigo disorder,

Table I. Comparison of VAS score between groups.

Group	Case	VAS score	
		Before treatment	After treatment
Control	30	8.14±2.13	6.11±1.82
Study	30	8.17±2.22	4.18±1.17
t	-	-0.05340	4.8857
P	-	0.6721	0.001

($\bar{x} \pm SD$)

Table II. Three dimensions of DHI table in different groups.

Groups	Time relative to treatment	DHI Overall score	Body	Function	Emotion
Control n=30	Before	70.9±8.1	21.2±3.2	30.9±2.1	18.2±5.1
	After	45.2±6.1	13.7±3.9	16.1±2.9	16.1±1.9
Study n=30	Before	72.3±7.9	21.3±3.4	32.3±2.9	19.8±4.9
	After	31.9±6.8	9.2±4.1	12.3±3.9	10.9±2.7
t	-	13.8820	8.1428	22.6400	2.1134
P	-	0.001	0.001	0.001	0.001

($\bar{x} \pm s$)

and the quality of life of every patient before and after treatment were accessed. The degree of pain was measured using a visual analog scale (VAS) score, which ranged from 0 to 10 points (the higher VAS score corresponded to more severe pain). The extent of vertigo disorder was quantified according to the dizziness handicap inventory (DHI) table, the dimensions of which included body, function and emotion (6). There were 25 sub-dimensions with a total possible score of 100 points (the higher points represented more severe dizziness); for each sub-dimension, there were 3 scores: none (0 points), sometimes having (2 points) and often (4 points). The quality of life was assessed via SF-36 table with 4 dimensions (7, 8) and a total score of 100 points

(the higher score indicated a better quality of life). There are 3 levels (significantly effective, partially effective and non-effective) of treatment efficacy. Significantly effective was defined as a positive rotation test and a complete relief of vertigo and positional nystagmus. Partially effective was defined as an incomplete relief of vertigo and positional nystagmus. No treatment benefit was defined as non-effective. Overall, the effective rate was determined as the sum of significantly effective rate and the partially effective rate.

All analyses were performed with SPSS software (IBM, USA). Age, VAS, DHI, SF-36 scores were expressed as a mean±standard deviation ($\bar{x} \pm SD$). Gender, type of BBPV and effective rate of treatment

Table III. *SF-36 in different groups.*

Groups	Time relative to treatment	Dimensions of DHI				
		Overall	Psychological	Physiological	Social status	Emotional status
Control n=30	Before	59.2±12.3	59.7±18.5	59.9±20.1	45.9±12.3	40.1±17.6
	After	62.5±18.8	66.8±11.7	64.9±19.8	61.9±11.8	66.3±19.7
Study n=30	Before	58.3±11.9	59.3±17.1	60.3±21.9	44.2±11.9	39.1±18.4
	After	77.2±20.7	73.9±18.2	76.1±26.7	75.3±13.8	76.9±23.4
t	-	4.3355	3.2021	2.5060	9.3480	6.9551
P	-	0.001	0.001	0.001	0.001	0.001

($\bar{x} \pm s$)

Table IV. *Effective rate in different groups.*

Groups	Case (n)	Significantly effective (n)	Partially effective (n)	Non-effective(n)	Overall effective rate (%)
Control	30	16	9	5	83.3
Study	30	22	7	1	96.7
X ²	-	6.7734	4.1575	21.3457	18.4151
P	-	0.001	0.001	0.001	0.001

were expressed as the number of cases or percentage (n%). The differences of continuous and categorical data were assessed by student *t*-test and *chi*-squared test, respectively (statistical significance was defined as $p < 0.05$).

In the automatic group, there were 12 male patients and 18 female patients with an average age of 56.6 ± 1.6 years. Eighteen patients were diagnosed with horizontal geotropic nystagmus, 4 patients with horizontal apogeotropic nystagmus and 8 patients with posterior canal BPPV. In the manual group, there were 11 male patients and 19 female patients, with an average age of 57.1 ± 1.2 years. Seventeen patients were diagnosed with horizontal geotropic nystagmus, 4 patients with horizontal apogeotropic nystagmus, and 9 patients with posterior canal BPPV. There was no significant difference in the demographics data between the two groups.

Markedly improved VAS score was observed in both groups after treatment. However, improvement of VAS was significantly higher in the automatic group ($p < 0.05$) (Table I). Significant improvement of the DHI score was shown in both groups. Additionally, DHI of the automatic group was significantly lower than that of the manual group (Table II). A significantly higher improvement of quality of life (SF-36 score) was displayed in the automatic group compared to manual group ($p < 0.05$) (Table III). The effective rates for the manual group and the automatic group were 83.3% and 96.7%, respectively. The effective rate of the automatic group was significantly higher than that of the manual group ($p < 0.05$) (Table IV).

Evidence has shown that the cause of BPPV is the canal cupulolithiasis affecting the upper, posterior and horizontal semicircular canal; the cause could be multifactorial including overtiredness, poor sleep quality or dental drilling (1, 9, 10). BPPV was the most common canal disease in clinical practice with complicated symptoms of nystagmus. The benefit of repositioning treatment could be affected by misdiagnosis. Manual repositioning maneuver, which physically relieved the symptoms, was a first line treatment of BPPV (11). The manual repositioning maneuver needed a highly skilled physician to observe and make correct judgment on the nystagmus

of patients during treatment; therefore, successful treatment was variable (6). In this investigation, TRV (a 360 degree rotation treatment machine to treat BPPV patients) was utilized. TRV could provide rotation in both the horizontal and vertical direction and adjust the speed of rotation. The different electro-magnetic locked position in the horizontal direction of TRV could be used to treat different types of BPPV. For instance, when the horizontal axes were locked in the standard position (above head) or supine position (0/180 degrees), TRV could be used to treat BPPV. Another advantage of TRV is that it can record the status of nystagmus, which facilitates the ability of the physician to recognize the presence and direction of the nystagmus via a monitor. This allows for more accuracy and ease of future treatment. Since the patient's body and head are fixed during the treatment, it directly eliminates the dizziness caused by rotation. All the rotation is performed by the TRV, and there is no need for patients to rotate their heads. Results from this study of 60 BPPV patients demonstrated the effective rate of TRV treatment of 96.7%, which was significantly higher than that of the manual reposition treatment group (83.3%) ($p < 0.05$); the improvement of VAS, DHI, and SF-36, were also statically significantly higher in the automatic reposition group ($p < 0.05$). Our results indicate that automatic repositioning conducted using TRV is superior to manual repositioning treatment, specifically as it relates to pain, the extent of vertigo, and the quality of life. In conclusion, the treatment with TRV appears to be a safe and effective treatment for BPPV.

ACKNOWLEDGEMENTS

This study was supported by Xuzhou Science and Technology Project (No. KC16SL143).

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LETTER TO THE EDITOR

DIAGNOSIS WITH SPIRAL CT IMAGING BEFORE GASTRIC CARCINOMA SURGERY

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Received December 21, 2017 – Accepted February 9, 2018

This study was carried out to study multi-slice spiral CT imaging for patients with gastric carcinoma and explore the values of multi-slice spiral CT imaging in staging prior to gastric carcinoma (GC) surgery. Forty-eight patients with GC underwent multi-slice spiral CT, and the scanning results were compared with the pathological results. The similarity of the results was observed, and the accuracy was calculated. Of 48 patients, 8 did not undergo surgery because of metastasis. In the diagnosis of the remaining 40 patients, the sensitivity of multi-slice spiral CT in the diagnosis of staging of invasive depth of GC was 77.5%; $\kappa = 0.642$ in the analysis of consistency; there was no significant difference with the pathological results ($p > 0.05$). The overall accuracy of diagnosis for stage N was 80%. The accuracy of multi-slice CT in detecting distant metastasis of GC was 87.5%. Multi-slice spiral CT can determine and evaluate various metastases of GC. The diagnostic results obtained using multi-slice spiral CT was probably consistent with the pathological results.

To the Editor,

Gastric carcinoma is one of the most common tumor diseases worldwide (1). The number of patients in China is 4 to 8 times that of the European and American developed countries and has distinct difference between rural and urban areas, which is in a close correlation with the living environment and dietary habits of patients (2). GC can be classified into early-stage and progressive (3), the latter including middle-stage and late-stage. For early-stage, many factors influence the clinical detection rate. Computed tomography (CT) is important for detection, as GC can easily be missed by the

physician (4). The detection rate of protruded GC is the highest (5).

Currently, surgery is still an irreplaceable treatment of GC. Detecting the stage of the disease is the most important factor related to the treatment. In recent years, the application of multi-slice spiral CT can effectively solve the problem of clinical staging of GC (6).

Ni et al. (7) pointed out that the sensitivity and specificity of multi-slice spiral CT was superior to other methods, and has played a crucial role in the development of clinical medicine. Zhong et al. (8) found that multi-slice spiral CT scanning could more

Key words: GC, multi-slice spiral CT, preoperative staging

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0393-974X (2018)

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directly provide information, such as the actual size and shape of lesions and the anatomical relationship of surrounding structure. This study investigated the application of multi-slice spiral CT in staging diagnosis of GC before surgery through comparing the scanning with the pathological results. The results suggested that spiral CT could help to better understand the specific conditions of lesions and also provide evidence for the preoperative evaluation of GC.

MATERIALS AND METHODS

Study subjects

Eighty-eight patients who were admitted to the Tumor Hospital of Shandong University, Shandong, China between June 2016 and August 2017, diagnosed with GC by gastroscopy or biopsy, and had not previously undergone chemotherapy or radiotherapy were selected. There were 56 males and 32 females, aged 30–71 years (average 49 ± 6.1 years). There were 18 cases of gastric fundus cardiac cancer, 22 cases of gastric body cancer, 40 cases of gastric antrum cancer and 8 cases of other types. The patients were informed of the study and signed consent. This study was approved by the ethics committee of the Tumor Hospital of Shandong University, Shandong, China.

The patients were forbidden to eat 12 h before scanning and drink 6 h before scanning. Each patient was given 500–1000 mL of warm water to drink 10 min before scanning. Those who had no contraindications to glaucoma or prostate were injected with a muscle hypotonic agent 15–20 min prior to scanning to slow down gastric peristalsis and prevent spasm or other conditions during scanning which might affect the results.

All the patients were scanned in a supine position by Siemens Definition AS 128-slice spiral CT from the diaphragmatic dome to the pelvic floor. Firstly, upper abdominal CT plain scanning was performed. The thickness and space between layers were both set as 0.625 mm, the screw pitch was 0.984:1, and the scanning speed was 0.8 s. Perfusion surface was selected according to the scanning results. Four layers on both sides of the largest surface of the GC lesion were selected and processed by perfusion imaging. The voltage used was 120 kV, the current was 80 mA, and the total exposure time 50 s. One hundred ml of ioversol non-ionic contrast agent at

a concentration of 320 mg/mL (SFDA approval number: H4230127) was injected intravenously at an injection rate of 5 mL/s using a double-tube high-pressure injector, and 30 s later scanning was performed; the results obtained were at arterial phase. The second time of scanning was performed 60 s after the injection, and the results obtained were at portal venous phase. The contrast agent was injected again, at a dose of 20–40 ml. The interval between the two times of injection was 150 s. Scanning was performed 50 s after the second injection (delayed phase). The rotation time of the gantry for one cycle was 0.35 s, and the scanning scope was between 15 and 25 cm.

Image analysis

The data obtained through CT imaging was sent to ADW4.3 workstation. Coronal view, sagittal view and curved surface were reconstructed to clearly display the lesions and the surrounding of the stomach. The thickness of the focal zones and the particle size were measured. The enhancement values of the lesions were measured at different real phases. The highest data was regarded as the maximum enhancement value of the scanning. The metastasis of GC in different sites was tested, and the results were recorded. The CT diagnostic criteria for GC extraserosal invasion included black shadows in the space between GC extraserosal fat or presence of protruded nodules; the criterion for lymphatic metastasis was 8 mm larger diameter of lymph glands in or around the abdominal cavity; criteria for peritoneal metastasis included multiple cord-like shadows in the space between peritoneal fat, abnormal peritoneal activities and peritoneal effusion.

Three experienced radiologists, blinded to the pathological results, evaluated and recorded the results, taking the three CT diagnostic criteria into account. Discussion was needed when the opinions were not unified.

Staging criteria

The criteria for the invasion depth (9), distant metastasis (10) and regional lymph nodes are shown in Table I. TNM classification referred to the Union for International Cancer Control (2009) (the 7th version) (16); T stage included diameter of primary tumor, local invasion and tumor nodules, N stage involved whether or not there was regional lymph node metastasis, and M stage involved whether there was distant visceral metastasis or not.

Statistical analysis

Statistical analysis was performed using SPSS ver. 22.0. The results were processed by *t*-test and expressed as mean $\pm$ standard deviation (SD). Enumeration data were expressed as percentage and processed by *Chi*-squared test. The difference was statistically significant if $p < 0.05$. Analysis of consistency was performed using kappa test; the consistency was considered as favorable if $\kappa \geq 0.75$, as general if $0.4 < \kappa < 0.75$, and as poor if $\kappa \leq 0.4$ (11).

RESULTS

Of the 88 patients with GC, eight patients were confirmed having distant metastasis but did not undergo surgery, and the remaining 80 patients underwent surgery. The CT images of the 80 patients

were evaluated and compared with the postoperative pathological results as detailed hereafter.

Comparison between the CT results and postoperative pathological results of GC extraserosal invasion

There were 8 cases of stage T1, 26 cases of stage T2, 30 cases of stage T3 and 16 cases of stage T4 in the pathological experiment; there were 6 cases of stage T1 (75.0%), 20 cases of stage T2 (76.9%), 24 cases of stage T3 (80.0%) and 12 cases of stage T4 (75.0%) according to the evaluation results of spiral CT scanning. The overall diagnostic accuracy was 77.5% (62/80) (Table II).

The consistency of the diagnostic results was analyzed, and the results were $t=0.846$, $P > 0.05$ and $\kappa = 0.642$. The results suggested that the diagnostic

Table I. Staging criteria for the invasion depth, distant metastasis and regional lymph nodes of GC.

Stages		Manifestations
Invasion depth of primary carcinoma (T)	T1	Infiltrating to mucous membrane or submucosa and muscularis mucosa
	T2	Infiltrating to muscular layer or subserosa
	T3	Infiltrating and passing through serosa
	T4	Infiltrating and passing through surrounding organization structure or passing through intracavity and transferring to duodenum and esophagus
Distant visceral metastasis (M)	M0	No distant metastasis
	M1	With distant metastasis
Regional lymph gland	N0	No regional lymph gland metastasis
	N1	1 to 6 regional lymph gland metastasis
	N2	7 to 15 regional lymph gland metastasis
	N3	More than 15 regional lymph gland metastasis

Table II. Comparison between the pathological experiment results and spiral CT scanning results.

Examination mode	No. of cases	Stage T1	Stage T2	Stage T3	Stage T4	Total
Pathological experiment	80	8	26	30	16	80
Spiral CT	80	6	20	24	12	62
Accuracy (%)	-	75.0	76.9	80.0	75.0	77.5

Table III. Comparison between the determination results of GC at stage M using multi-slice CT and the postoperative pathological results.

Examination mode	Number of cases	M0	M1	Total
Pathological experiment	80	47	33	80
Spiral CT	80	44	28	72
Spiral CT	-	93.6	84.8	90

Table IV. Comparison between the determination results of GC at stage N using multi-slice CT and the postoperative pathological results.

Examination mode	Number of cases	N0	N1	N2	Total
Pathological experiment	80	36	30	14	80
Spiral CT	80	30	24	10	64
Spiral CT	-	83.3	80.0	71.4	80.0

results obtained through spiral CT and pathological experiment had no obvious difference, indicating the high accuracy and reference value of spiral CT.

Comparison between multi-slice spiral CT and postoperative pathological results of GC with lymphatic metastasis (stage M)

Forty-seven cases were found at stage M0 by pathological examination and 33 cases at stage M1. However, the results of evaluation based on multi-slice spiral CT suggested 44 cases of M0 (93.6%) and 28 cases of M1 (84.8%). The accuracy rate of the diagnosis was 90% (72/80). A favorable consistency was found between the diagnostic results and the postoperative pathological results ($\kappa = 0.808$), and was not statistically significant ($p > 0.05$) (Table III).

Comparison of CT and postoperative pathological results of GC with lymphatic metastasis (stage N)

Fig. 1 shows a CT image of GC with lymphatic metastasis of a patient. Lymphatic node metastasis is one of the metastatic approaches of malignant carcinoma, and there are many complex lymphatic systems around the stomach. Hence lymphatic metastasis is also an important factor influencing the prognosis of patients with GC. Lymphatic metastasis and metastasis tendency were determined based on the diameter of lymph nodes, but inflammation

and tuberculosis may result in lymphadenectasis, reducing the accuracy of staging determination. When the enlarged lymph nodes were nibble-like, cystiform and granophytic and had high surrounding density, low middle density, relative high density, bead-like arrangement and vascular compression, there was presence of metastasis.

As shown in Table IV, there were 36 cases of stage N0, 30 cases of stage N1 and 14 cases of stage N2 according to the results of the pathological experiment; there were 30 cases of stage N0 (83.3%), 24 cases of stage N1 (80.0%) and 10 cases of stage N2 (71.4%). The accuracy of multi-slice CT in determining the stages of GC with lymphatic metastasis was 80.0% (64/80); its consistency with the postoperative pathological results was favorable ($\kappa=0.77$), and the difference with the postoperative pathological results had no statistical significance ($p > 0.05$).

Comparison between CT and postoperative pathological results of GC with distant metastasis

Fig. 2 shows a CT image of a patient with GC with peritoneal metastasis. The CT image of GC with peritoneal metastasis presented omental cakes, irregular thickening of peritoneum, enhanced nodules, mass ascites, thickening of mesentery and multiple cord-like shadows in the space between fat.



Fig. 1. CT image of GC with lymphatic metastasis.



Fig. 2. CT image of GC with peritoneal metastasis.

In this experiment, 10 cases of GC with liver metastasis and 4 cases of GC with peritoneal metastasis were diagnosed accurately and underwent no surgery. One patient who was diagnosed as peritoneal metastasis by the pathological experiment was not diagnosed by CT examination. Therefore the success rate of detecting GC with distant metastasis

with multi-slice CT was 87.5% (14/16), and the results were highly consistent with the postoperative pathological results ($\kappa=0.55$).

DISCUSSION

GC is the most common digestive tract malignancy in China, and GC surgery-induced complications may affect the life of patients and even cause death (12). CT with relatively high density and spatial resolution can precisely detect the invasion and distant metastasis of GC, hence it has been the first choice in the preoperative examination (13). Multi-slice spiral CT scanning greatly improves the success rate of determining metastasis of GC.

If GC appears in glands, the glands and blood capillaries are difficult to observe, and blood flow slows, which may extend the retention of ioversol in glands. Multi-slice CT as a cutting-edge technology in the new century can more efficiently and effectively examine, scan and image (14) and deepen the display of three layers of the stomach wall, which is beneficial to determine T staging of GC. T staging based on multi-slice spiral CT scanning can help determine the invasion depth of tumor (15).

In this study, 80 out of 88 patients with GC underwent multi-slice spiral CT. The comparison between the diagnostic results and pathological results suggested that the diagnostic accuracy was 77.5% for stage T (62/80), 90% (72/80) for stage M, and 80.0% for stage N (64/80), indicating a high consistency.

In conclusion, multi-slice spiral CT can make accurate diagnosis on the disease conditions of patients, and the diagnostic results are basically the same as the postoperative pathological results. Based on this, the physician can formulate the most suitable individual treatment for patients.

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LETTER TO THE EDITOR

THE IMPORTANCE OF EXPRESSING PDCD4 AND PDCD5 ANTI-ONCOGENES IN GLIOMA

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Received November 6, 2017 – Accepted December 21, 2017

Glioma is the most common malignant tumor of the brain, which is difficult to be completely resected. The recurrence and mortality rates are high and the prognosis is poor. The aim of this study was to investigate the expression of anti-oncogene programmed cell death 4 (PDCD4) and programmed cell death 5 (PDCD5) in glioma and their influence on the progression of the disease in order to provide new therapeutic approaches. Reverse transcription polymerase chain reaction (RT-PCR) analysis was used to investigate PDCD4 mRNA and PDCD5 mRNA expression in 66 glioma patients who served as the study group and 22 patients who suffered from craniocerebral injuries or hematencephalon who were used as controls. The experimental group was divided into a low malignant group (tumors grade I - II) and a high malignant group (tumor grades III-IV). The PDCD4 mRNA and PDCD5 mRNA expression in the experimental group was 0.545 ± 0.202 and 0.687 ± 0.174 and in the control group was 0.942 ± 0.131 and 0.868 ± 0.190 , respectively ($P < 0.05$). The PDCD4 mRNA and PDCD5 mRNA expressions in the low malignant group were 0.628 ± 0.240 and 0.750 ± 0.198 , respectively, and in the high malignant group were 0.464 ± 0.185 and 0.553 ± 0.170 , respectively ($P < 0.05$). The results showed a downregulation of PDCD4 mRNA and PDCD5 mRNA expression in the experimental group compared with the control group. This downregulation was correlated with the pathological grade of glioma. In the high malignant group the PDCD4 mRNA and PDCD5 mRNA expressions were significantly decreased compared with the low malignant group and the control group. PDCD4 mRNA and PDCD5 mRNA expressions are promising targets for the diagnosis and treatment of glioma.

To the Editor,

Glioma, originating from nerve epithelium, is the most common malignant tumor in the human brain. Glioma can affect the brain function and can be life-threatening (1). The type of glioma determines the form of treatment and the prognosis of the disease according to the location and the rhythm of growth. Symptoms are diverse and depend on the type of tumor, its size, localization and growth rate. The symptoms may include migraines, mental confusion, nervousness, psychosis-like personality changes

(2). Classic treatment for glioma includes several approaches. Surgical treatment consists of total or partial resection of the tumor but with high bleeding risk in vitamin K-dependent coagulopathies (3). Radiotherapy is combined with chemotherapy, but its side effects are severe with neutropenia and severe immunosuppression, favoring the development of bacterial infections which are very difficult to eradicate. Targeted therapy includes drugs that attack the tumor cells by focusing on abnormalities to destroy them. It is important to find a target gene

Key words: glioma, PDCD4, PDCD5, RT-PCR, anti-oncogene

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0393-974X (2018)

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that can influence the prognosis of this disease.

Programmed cell death 4 (PDCD4) is a new anti-oncogene found in recent years. It was once considered as a target gene for cancer treatment. For example, Asangani et al. (4) investigated the effect of PDCD4 on colon cancer cells. Data show that functions of PDCD4 may depend on the type of stem cells or genetic backgrounds (5). Some studies confirm that programmed cell death 5 (PDCD5) gene is positively correlated with apoptosis and its expression decreases in various pathological conditions. For instance, Shi et al. (6) found that its expression is decreased in leukemia and osteosarcoma. Furthermore, one study indicates that PDCD5 can promote apoptosis (7). In fact, tumor is the result of combined actions of several genes. Few studies focus on the expression of PDCD4 and PDCD5 in glioma and their relevance. In the present study, we used RT-PCR to explore mRNA expression of PDCD4 and PDCD5 in glioma in order to show whether any correlation exists with the occurrence and development of the tumor.

MATERIALS AND METHODS

Patients and samples

Two groups were included in the study: an experimental group consisting of 66 patients (39 males and 27 females aged 5-74 years: average 33.14 ± 19.52 years) diagnosed with glioma and operated in the Department of Surgery of Affiliated Hong Qi Hospital of MuDanJiang Medical University, Mudanjiang City, China between February 2016 and February 2017; and a control group of 22 patients who suffered from craniocerebral injuries or hematencephalon. All patients suffered from a tumor for the first time and had not undergone radiotherapy, chemotherapy or other treatment before surgery. According to the World Health Organization guidelines from 2007 for central nervous system tumors classification (8), the study group had 12 cases classified as grade I, 24 cases as grade II, 16 cases as grade III and 14 cases as grade IV. According to the degree of disease severity, patients in the experimental group were classified into a low malignant group (grade I-II) with 36 cases and a malignant group (grade III-IV)

with 30 cases. Twenty-two patients who suffered from craniocerebral injuries or hematencephalon were chosen to form the control group. Tissue specimens were collected during surgery from all the patients included in the study. After the collection, all specimens were snap frozen and kept at -80°C until determination. The study was approved by the Ethics Committee of the Affiliated Hong Qi Hospital of Mu DanJiang Medical University and all patients signed informed consent to participate.

Experimental methods

The full length mRNA of PDCD4 and PDCD5 was obtained from GenBank, the primer and Primer 5.0 software were adopted for primer design. The designed primer was also compared with Blast primer base in PUBMED. After analysis, the specificity of the primer sequence was evaluated. All primers and internal control β -actin were manufactured by Beijing Dingguo Biotechnology Co., Ltd, China.

The RT-PCR procedure comprised RNA extraction, measurement of RNA concentration, purity and integrity, RT polymerase chain reaction (RT-PCR) and amplicon detection by gel electrophoresis.

RNA extraction

Approximately 50-100 g of tissue specimen was taken from storage, placed in a mortar and ground with liquid nitrogen and then transferred to an Eppendorf tube. Next, after keeping the sample for 5 min at room temperature and adding Trizol reagent, it was centrifuged at 12,000 rpm for 5 min. The sediment was discarded and chloroform was added to the supernatant, the tube was vigorously shaken and mixed. After 15 min the tube was centrifuged for 15 mins at 12,000 rpm and the supernatant was transferred to another tube with isopropanol, placed for 30 min at -20°C and then centrifuged at 12000 rpm for 10 min. Next, the supernatant was discarded and ethanol was added to the sediment, mixed and centrifuged again at 8,000 rpm for 5 min. The sediment was dried at room temperature and finally cryopreserved at -80°C .

Measurement of RNA concentration and purity

In a test tube, 5 μl RNA sample was added to

60 ml of 0.1% EDPC. Another tube containing only 0.1% EDPC was used as the control. An ultraviolet spectrophotometer was used to quantify RNA absorbance/optical density (OD) at 260 nm (A_{260}) and 280 nm (A_{280}). RNA concentration (mg/ml) was calculated as $A_{260} \times \text{Nucleic acid dilution times} \times 40/1000$ and RNA purity as the ratio A_{260}/A_{280} . The rates for all specimens were limited to $1.7 < A_{260}/A_{280} < 2.0$.

Assessment of RNA integrity

RNA integrity assay was carried out by electrophoresis in 1% agarose gel and observing the migration pattern.

RT-PCR reaction

A reverse transcription system and a semiquantitative PCR system were used. The reverse transcription system had a volume of 20 μ l and contained RNA 3 μ l, 5 Buffer 4 μ l, dNTP 1 μ l, Oligo (dt) 1 μ l, reverse transcriptase 0.5 μ l, DEPC H₂O 10.5 μ l. The Semiquantitative PCR system had a volume of 25 ml and contained cDNA 2 ml, 10 $\times$ PCR Buffer 2.5 ml, DNTPs(10 mM) 0.5 ml, forward primer (20pmol/ml) 0.25ml, reverse primer (20pmol/ml) 0.25ml, Taq enzyme (2u/ml) 0.5ml, ddH₂O 19ml.

Amplicon detection

After RT-PCR reaction, the PCR products were detected by gel electrophoresis. Both sides of the gelator were taped and put on a horizontal surface. Agarose powder was heated until it was completely dissolved. After that, fluorescent dye gold was added. When the gel cooled to 50°C, it was put into the taped gelator and the tape was carefully removed. Then, the gel and the gelator were put into an electrophoresis bath together with the electrophoretic buffer. For cataphoresis a voltage of 90V was used for 10 min and 70V for 20 min. After the electrophoresis process was complete, the gel was taken out and placed in an ultraviolet imaging system for imaging and confirming OD.

The experiment was repeated three times. The average OD value of the three experiments was used to calculate the content of mRNA. We used deionized water as negative control and β -actin as internal control.

In the case of presence of the gene of interest,

a fluorescent band of 157bp (PDCD4) and 129bp band (PDCD50) was present in the image under the ultraviolet light, which can be used as the standard for the positive sample. The internal control (β -actin) produced a fluorescent band at 318bp.

Automatic gel image analysis system BandScan5.0 was used to analyze the image of RT-PCR product and establish the band intensity (BI). BI = average absorbance $\times$ area. The ratio of target BI and internal control BI describes the relative quantity of target gene, namely, BI ratio.

Statistical analysis

The results are expressed as the mean $\pm$ standard deviation. SPSS17.0 package (IBM Company) was used to process data. The comparison between all means was performed using Student's *t*-test. The difference in proportions was assessed using *Chi*-squared test. Correlation analysis was performed by Pearson product-moment correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Expression of anti-oncogene PDCD4 in experimental and control groups

RT-PCR was used to test mRNA levels of PDCD4 in both the experimental and control groups. Agarose gel electrophoresis indicates there was clear objective strip of 157bp and β -actin internal control strip of 318bp, and that the luminance of strip in the control group was apparently higher than that in the experimental group and also in the low malignant group was obviously lower than that of the high malignant group. All details are shown in Fig. 1A. Compared with the control group, PDCD4 mRNA expression in the experimental group decreased significantly ($P < 0.05$); mRNA expression of PDCD4 in the low malignant group was significantly higher than in high malignant group ($P < 0.05$) (Table I).

Expression of anti-oncogene PDCD5 in the experimental and control groups

RT-PCR was used to determine mRNA levels of PDCD5 in both the experimental group and the

control group. Agarose gel electrophoresis indicates a clear objective clear strip of PDCD5 of 129bp and β -actin internal control strip of 318bp. The luminance of strip in the control group was higher than in the experimental group, and the luminance lowered with the grade rising of the tumor. Details are shown in Fig. 1B. Compared with the control group, PDCD5 mRNA expression in the experimental group decreased significantly ($P<0.05$); PDCD5 mRNA expression in the low malignant group was obviously higher than in the high malignant group ($P<0.05$) (Table I).

Relevance of anti-oncogene PDCD4 and PDCD5

Pearson product moment correlation coefficient analysis showed that mRNA of PDCD4 and that of PDCD5 in the experimental group were

positively related. The prognosis of patients with high expression of PDCD5 and high expression of PDCD4 was significantly better than that with low expression of PDCD5, low expression of PDCD4 and high expression of PDCD4 or PDCD5 alone. This result indicates that agents acting on both PDCD4 and PDCD5 may play an active role in the treatment of glioma. The expression of the two genes tends to be the same, and the increase of PDCD4 gene must be accompanied by the increase of the PDCD5 gene. Pearson product momentum correlation coefficient $r=0.581$ and $P < 0.05$, indicated that the difference was statistically significant (Fig. 2 and Table II).

DISCUSSION

Other studies show that in the process of cell

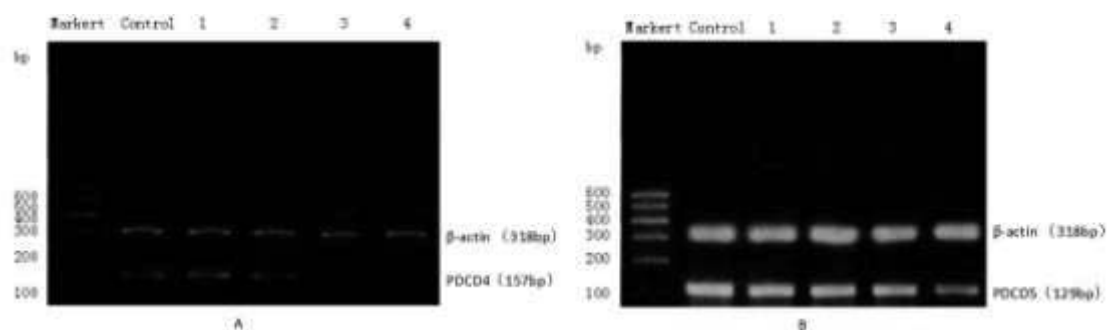


Fig. 1. Agarose gel electrophoresis of PDCD4 gene and PDCD5 gene (1: glioma grade I; 2: glioma grade II; 3: glioma grade III; 4: glioma grade IV). **A)** PDCD4 gene; **B)** PDCD5 gene.

Table I. mRNA Expression of PDCD4 and mRNA expression of PDCD5 in experimental and control groups ($\bar{x} \pm s$).

Group	Number of cases	mRNA expression of PDCD4			mRNA expression of PDCD5		
		IA	t	p	IA	t	P
Control group	22	0.942 $\pm$ 0.131			0.868 $\pm$ 0.190		
Experimental group	66	0.545 $\pm$ 0.202	6.259	0.001	0.687 $\pm$ 0.174	3.161	0.004
Low malignant group	36	0.628 $\pm$ 0.240			0.750 $\pm$ 0.198		
High malignant group	30	0.464 $\pm$ 0.185	3.651	0.000	0.553 $\pm$ 0.170	4.801	0.001

IA: the average absorbance $\times$ the area. The ratio of objective part IA and internal control IA describes the relative quantity of objective part, namely, IA ratio.

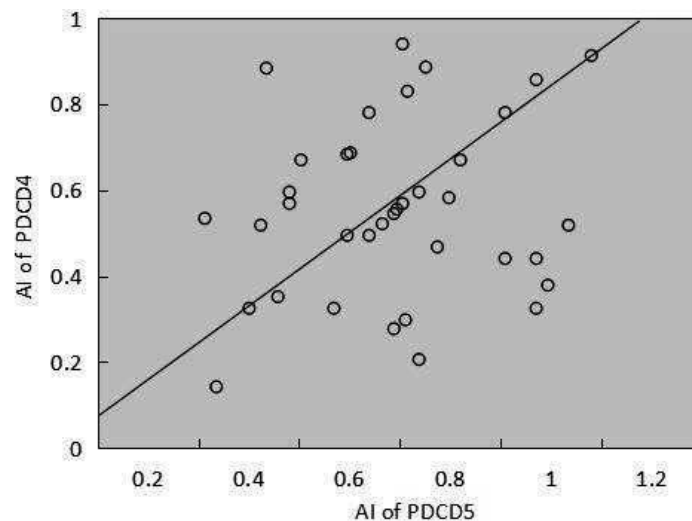


Fig. 2. Correlation between mRNA expression of PDCD4 and PDCD5.

Table II. Correlation of mRNA expression of PDCD4 and PDCD5 in experimental group ($\bar{x} \pm s$).

Test index	Case number	IA	Pearson correlation coefficient	P
PDCD4	66	0.545 ± 0.201	0.581	< 0.0
PDCD5	66	0.686 ± 0.179		

IA: the average absorbance $\times$ the area. The ratio of objective part IA and internal control IA describes the relative quantity of objective part, namely, IA ratio.

apoptosis, PDCD4 can inhibit protein transcription and translation (9, 10). In our study, RT-PCR detection indicated mRNA expression of PDCD4 in the experimental group was lower than in the control group ($P < 0.05$). Further study showed that there were significant differences of mRNA expression of PDCD4 in different grades of glioma ($P < 0.05$). The expression level declined with the increasing severity of the tumor, showing that PDCD4 may aid the prognosis of glioma patients, which is consistent with the study Jansen (11) et al.

Li (12) et al. tested the expression of PDCD5 on glioma cell lines and found a possible effect of PDCD5 on the occurrence and development of glioma. Results of that study show that mRNA expression of PDCD5 in the experimental group was lower than in the control group ($P < 0.05$). Studies

have shown PDCD5 itself may not have an obvious effect on cells, but it can significantly promote apoptosis (13). It has been proved that PDCD5 serves as a promoter rather than an inducer for apoptosis. Analyses of the relatedness of PDCD5 and PDCD4 showed that the two genes were positively related. These results indicate that PDCD4 and PDCD5 play an active role in the treatment of glioma, indicating the feasibility of combining agents acting both on PDCD4 and PDCD5 for the treatment of glioma.

In conclusion, this study showed that mRNA expression of PDCD4 and PDCD5 in glioma was significantly weaker than in the tissues without tumors and declines with the rising grade of the tumor. Expressions of PDCD4 and PDCD5 in glioma are positively related. Both genes are good candidates for targeting diagnosis and treatment of glioma.

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LETTER TO THE EDITOR

TREATMENT OF SYMPTOMATIC ORAL MUCOSITIS WITH SODIUM HYALURONATE AND SYNTHETIC AMINO ACID PRECURSORS OF COLLAGEN IN PATIENTS UNDERGOING HAEMATOPOIETIC STEM CELL TRANSPLANTATION

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Received September 26, 2017 – Accepted March 1, 2018

The purpose of the study is to evaluate the clinical effects of Mucosamin® (a spray preparation containing sodium hyaluronate combined with a pool of amino acids of precursor collagen, including L-Proline, L-Leucine, L-Lysine and glycine) on wound healing and pain management of oral mucositis after hematopoietic stem cell transplantation. The importance of professional dental hygiene by dental hygienist in reducing the severity of oral mucositis as unique therapy or in addition to therapy with Mucosamin® was also evaluated. One hundred thirty-seven patients undergoing hematopoietic stem cell transplantation were recruited in a case-control study and divided into 4 groups: Group A: professional oral hygiene + Mucosamin®; Group B: professional oral hygiene + standard treatment with chlorhexidine 0.20%; Group C: only Mucosamin®; Group D: only standard treatment with chlorhexidine 0.20%. The following evaluations were made: WHO mucositis scale, OMAS mucositis scale, VAS, periodontal recording, days of mucositis. Comparing the groups at the onset of OM on WHO scale, it was observed that Group A grade 1 occurrence was more statistically significant than Group B ($p=0.03^*$); comparison between Group A and D showed a statistically significant difference in favour of Group A ($p=0.0002^*$). Also OMAS scale showed a statistically significant difference between groups who assumed Mucosamin, who developed lower OM grade ($p=0.001^*$). There was a statistically significant difference between group A compared with group B over the overall duration of OM ($p=0.02^*$), as well as between group A and group D ($p=0.03^*$). According to the present study the combination of a careful debridement, correct oral hygiene during hospitalization and the use of Mucosamin® exponentially reduces the severity and duration of mucositis and consequently the discomfort of the patient. Moreover, it can be stated that the use of Mucosamin® also results in a reduction in the extent of chemotherapy lesions. Hyaluronic acid and amino acid-based sprays can be a valuable therapeutic aid in the treatment of mucositis.

To the Editor,

Oral mucositis (OM) can affect up to 80% of patients undergoing hematopoietic stem cell transplantation (HSCT) and it has a direct and

significant impact on quality of life and health care costs, other than effects on patient survival, such as for the risk of infections on oral lesions (1-2): analgesic management is necessary when pain

Key words: oral mucositis, mucosamin, bone marrow, HSCT, sodium hyaluronate, dental care

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0393-974X (2018)

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prevents the patient's intake of food and fluids, requiring parenteral nutrition, and increasing days of hospitalization (3).

There is no standardized therapy for OM, the first-line treatment consists in the elimination of oral infectious foci associated with a scrupulous oral hygiene, before HSC with the aim to reduce intraoral bacterial colonization and prevent infection of ulcers, with mouthwashes and antibiotics (4-5). However, both treatments have been found ineffective in the prevention of OM. Antibiotics and mouthwashes are not able to penetrate the bacterial plaque if it is not previously disintegrated mechanically, as reported in a recent Cochrane review (6).

A new treatment used on patients suffering from oral mucositis is Mucosamin®: a spray compound with sodium hyaluronate (SH) and a pool of collagen precursor amino acids (AAs), which acts on wound healing and pain management. SH acts as a muco-adherent polymer and as an agent barrier, relieving pain thanks to the prolonged contact of the product on the mucosa; the amino acids are collagen precursors and stimulate fibroblast to produce collagen and glycosaminoglycans, for tissue reconstitution during the healing process (7). Thanks to the properties of its components, this spray acts on the stage of ulcerative mucositis, creating a protective layer that reduces pain and accelerates clinical recovery. Our group has recently published a study where 68 patients scheduled for HSCT were treated with Mucosamin®: patients who assumed Mucosamin® on initial lesions developed a less severe OM, and the maximum OM pain, measured with the VAS (Visual Analogue Scale) for pain, was higher in patients who did not assume Mucosamin® (9).

We decided to extend our study to evaluate the clinical efficacy of Mucosamin® in wound healing and pain management in OM after HSCT. We also evaluated the importance of professional oral hygiene by a dental hygienist to reduce the severity of OM in combination with the Mucosamin®.

MATERIALS AND METHODS

A randomized clinical trial study with four groups was drawn up: a total of 137 adult patients (average age

51, range 21-70) prepared for HSCT for allogenic and autologous transplant were recruited at the Stem Cell Transplant Unit, and in the Oral Surgery Department, Dental School, University of Turin from 2012 to 2016.

Inclusion criteria were: i) age > 18 years; ii) candidate to HSCT; iii) informed consent and ability to complete the trial. Exclusion criteria were: i) known allergy to any of the constituents of the compound; ii) inability to check results; iii) patients with systemic disease, other than haematological malignancies, which impair wound healing (i.e. diabetes); iv) muco-cutaneous diseases; v) presence of graft versus host disease; vi) severe deterioration of general health conditions; vii) unable/unwilling to give informed consent.

The study was approved by the Internal Review Board of our Centre and was conducted in accordance with the Helsinki Declaration, and all patients provided informed consent to the proposed study. Each patient was randomly assigned to one of the four groups and the allocation was concealed.

Group A patients (37) underwent a full session of professional oral scaling and root planing by dedicated staff; at the end of the session they were informed about the correlation between oral health and mucositis, instructed on correct oral home hygiene and motivated to continue these instructions even during the hospitalization period. Patients with periodontal issues were subjected to a second session of professional oral scaling and root planing of pathological sites. Patients who received further dental treatment, such as conservative, endodontic or extractive treatment, were reminded to maintain high oral hygiene standards before and after HSCT. In addition, these patients were instructed by the same doctor to recognize the symptoms of oral mucositis and apply Mucosamin spray on these lesions 3-4 times a day after meals and oral hygiene, keeping it *in situ* for about 2 min, and to avoid drinking, eating, and rinsing the mouth for at least an hour. The same doctor controlled the patients during hospitalization to recognize initial and advanced signs of OM and to remind patients how to apply the compound.

Group B patients (32) underwent a full session of professional oral scaling and root planing by dedicated staff; at the end of the session they were informed about the correlation between oral health and mucositis, instructed on the correct oral home hygiene manoeuvres and motivated to continue these manoeuvres even during

the hospitalization period. Patients with periodontal issues were subjected to a second session of professional oral scaling and root planing of pathological sites. However, these patients did not receive Mucosamin but the usual treatment with Chlorhexidine 0.20%.

Group C patients (34) did not undergo a full session of professional oral scaling and root planing by dedicated staff; these patients were instructed by the same doctor to recognize the symptoms of oral mucositis and apply Mucosamin spray on these lesions 3-4 times a day after meals and oral hygiene, keeping it in situ for about 2 min, and to avoid drinking, eating and rinsing the mouth for at least an hour. The same doctor controlled the patients during hospitalization to recognize initial and advanced signs of OM and to remind patients how to apply the compound.

Group D patients (34) did not undergo a full session of professional oral scaling and root planing by dedicated staff and did not receive Mucosamin but the usual treatment with Chlorhexidine 0.20%. In addition, patients were asked whether they had undergone professional hygiene sessions in the previous 6 months.

At the first visit each patient was asked to complete an oral hygiene questionnaire and a mucositis information questionnaire. The staff filed the Periodontal Folder where Plaque Control Record, Full Mouth Bleeding Score and Periodontal Screening and Recording (PSR) were recorded (10).

Starting the day after HSCT, the same observer, blinded to the treatment, an expert trained for the clinical trial, evaluated patients of all groups every day during hospitalization.

The evaluation of patients of the four groups included three scores for OM:

i) The classification of OM according to the World Health Organization of 1979 provides a general assessment of the degree of severity of mucositis by combining some objective characteristics of oral mucosa (erythema and ulcerations) with the ability to drink or eat. The WHO mucositis scale ranges from 0= no symptoms; 1=soreness, erythema; 2=erythema, ulcers but able to eat solids, 3=ulcers but required liquid diet; 4= oral feeding not possible (11);

ii) Visual analogue scale (VAS) ranging from 0=no pain to 10=worst pain in the world (12);

iii) OMAS Scale: Introduced in 1996 by a team of

oral medicine specialists from oncologists and nurses in the United States, Europe and Canada, evaluates the anatomical extension and the severity of OM. The Oral Mucositis Assessment Scale (OMAS) evaluates the presence and size of ulcerations or pseudo membranes for each site of the oral cavity (upper lip, lower lip, right cheek, left cheek, vertical tongue and right side, vertical tongue and left side, oral floor, soft palate and hard palate). The overall evaluation of the mucosal according to the OMAS scale is expressed by adding the scores for the degree of erythema, ulcers and pseudo membranes in certain regions of the oral cavity. OMAS scale: Oral cavity for Ulceration 0=No lesion; 1= Lesion <1 cm²; 2=Lesion 1 to 3 cm²; 3 =Lesion >3 cm²; Oral cavity for Erythema: 0= None; 1=Not severe; 2=Severe (13).

According to WHO and OMAS scales, we divided the lesions into two groups: light mucositis (WHO grade 1 and 2, OMAS grade 1) and severe mucositis (WHO grade 3 and 4, OMAS grade 2 and 3). Primary indicators of mucositis were the degrees of ulceration and redness measured in specific sites in the mouth. Secondary indicators included oral pain, difficulty swallowing and the ability to eat as assessed by the patient. A single score is not produced from this scale, rather a score for ulceration and redness based on different locations in the mouth are used.

Statistical analysis

The results included continuous and categorical variables. The former are reported as mean and standard deviation. Nonparametric tests were used: the Wilcoxon signed-rank test for comparisons of two correlated samples involving matched pairs and the Mann-Whitney test for comparisons of two independent distributions. Categorical variables, reported as count and percentage, were arranged in cross-correlation tables and compared using the χ^2 test with the Yates correction when all expected values were higher than 5 or the Fisher test. Statistical significance corresponded to a probability less than 0.05.

RESULTS

Table I shows demographic detail of patients and other baseline information. All patients were candidate to HSTC for haematological malignancies. The four groups

were homogeneous for age, sex, type of pathology, HSCT type (autologous or allogeneic), conditioning regimen (total body conditioning myeloblastic or reduced), Plaque Index, Bleeding Index and PSR. All these variables did not show statistically significant differences among the various groups.

As for the onset of mucositis, 87% of the whole sample developed a mucositis and the distribution among groups was homogeneous: 30 patients of group A (76.6%), 29 patients of group B (90.6 %), 29 patients of group C (85.3%) and 31 of group D (91.2%). The position of

lesions is described in Table II and it is according to those reported in literature (5-10). Patients who did not develop OM, were excluded from further analysis.

Treatment administration

There was no indication of any intolerance to the product. Based on the interview, all patients, except two, liked the consistency of the product in the mouth and they were able to use it during the day based on the instructions of the dentist.

Table III shows WHO scale occurrence in the 4

Table I. *Patients' baseline characteristics.*

		Group A	Group B	Group C	Group D	P value
	No.	37	32	34	34	
Sex	Male	24	19	13	18	0.13
	Female	13	13	21	16	
Age	< 30 y.	3	2	3	1	0.74
	31-40 y.	6	1	5	3	
	41-50 y.	10	9	5	10	
	51-60 y.	12	10	12	13	
	> 60 y.	6	10	9	7	
Transplant	Autologous	5	7	6	10	0.39
	Allogenic	32	25	28	24	
TBI	Yes	7	4	5	6	0.88
	No	30	28	29	28	
Pathology	Acute Myeloid Leukaemia	11	9	7	11	0.052
	Chronic Myeloid leukaemia	4	1	2	0	
	Hodgkin's Lymphoma	4	2	0	1	
	Non Hodgkin's Lymphoma	4	3	5	8	
	Myelofibrosis	0	1	2	0	
	Lymphoblastic leukemia	7	5	2	1	
	Multiple Myeloma	2	6	3	8	
	Myelodysplastic Syndrome	1	1	5	3	
	Others	4	4	8	2	
FMPS	0-100%	61.0	28.5	58.5	43.5	0.98
FMBS	0-100%	54.5	22.5	32.0	33.5	0.42
PSR	0-4	3	2	3	3	0.09

no.: number of patients; *TBI:* total body conditioning regimen; *FMPS:* full mouth plaque score; *FMBS:* full mouth bleeding score; *PSR:* periodontal score recording.

groups. All comparisons between Group A and group D were statistically significant ($P < 0.05$), and also Group C vs group D showed a statistically significant presence of lower gravity of mucositis in patients treated with Mucosamin®.

With regard to the OMAS scale, results are shown in Table IV: patients in group A and C developed lighter

lesions. In group B there was higher prevalence of the worst lesions, while Group D had a higher number of lesions of all grades.

A statistically significant difference was observed for the OMAS scale mucositis grade 1 between groups A and B for the former group ($p = 0.009^*$) and for grade 3 ($p = 0.04^*$) in favour of the latter group, while between group

Table II. Position of lesions of oral mucositis.

	Group A (% lesions)	Group B (% lesions)	Group C (% lesions)	Group D (% lesions)
Upper/ Lower Lip	5.4 (2)	3.1 (1)	26.5 (9)	5.9 (2)
Tongue	40.5 (15)	21.9 (7)	26.5 (9)	38.2 (13)
Soft Palate	29.7 (11)	6.25 (2)	17.6 (6)	29.4 (10)
Hard Palate	18.9 (7)	9.4 (3)	29.4 (10)	38.2 (13)
Cheek	35.1 (13)	15.6 (5)	26.5 (9)	32.3 (11)
Gums	8.1 (3)	9.4 (3)	35.3 (12)	26.5 (9)
Oral Floor	13.5 (5)	3.1 (1)	20.6 (7)	26.5 (9)

Table III. Evaluation of gravity of oral mucositis.

	WHO 1	WHO 2	WHO 3	WHO 4
	% (n. pts)	% (n. pts)	% (n. pts)	% (n. pts)
Group A	60 (18)	23.3 (7)	6.7 (2)	10 (3)
Group B	17.2 (5)	24.1 (7)	38 (11)	20.7 (6)
Group C	31 (9)	48.3 (14)	13.8 (4)	6.9 (2)
Group D	9.7 (3)	3.2 (1)	51.6 (16)	35.5 (11)
P- value	WHO 1	WHO 2	WHO 3	WHO 4
Group A vs B	0.03*	0.76	0.02*	0.19
Group A vs D	0.0002*	0.03*	0.00005*	0.01*
Group C vs D	0.007*	0.0002*	0.0007*	0.01*

WHO scale $P < 0.05$ (*).

Table IV. Evaluation of gravity of oral mucositis.

	OMAS 1	OMAS 2	OMAS 3
	% (n. lesions)	% (n. lesions)	% (n. lesions)
Group A	73.3 (22)	30 (9)	20 (6)
Group B	31 (9)	44.8 (13)	41.4 (11)
Group C	41.4 (12)	37.9 (11)	24.1 (7)
Group D	48.4 (15)	48.4 (15)	51.6 (16)
P- value	OMAS 1	OMAS 2	OMAS 3
Group A vs B	0.009*	0.20	0.04*
Group C vs D	0.08	0.07	0.04*

OMAS scale $P < 0.05$ (*).

C and D for Grade 3 ($p = 0.04^*$) it was higher in the latter.

For statistical analysis it was decided to divide lesions into two groups: light mucositis (WHO grade 1 and 2, OMAS grade 1) and severe mucositis (WHO grade 3 and 4, OMAS grade 2 and 3). Comparing subjects who contracted a light mucositis in group A vs group B, there is a statistically significant difference ($p = 0.001^*$), as well as when comparing the same groups with severe mucositis ($p = 1.8 \times 10^{-6}^*$)

Finally, the duration of the lesions was analysed: it was observed that more than 70% of Group A injuries disappeared within 7 days (70.3%) and only 37.6% for Group B, 50% for Group C and only 32.4% for Group D. In addition, lesions lasted up to 3 weeks in 25% of Group B and only in 10.8% of Group A, 17.6% in Group C and 14.7% in Group D. Comparing group A with group B, for the overall duration of OM, a statistically significant difference between the two groups ($p = 0.02^*$) was found, as well as between group A and group D ($p = 0.03^*$).

DISCUSSION

Data show that subjects in Group A have predominantly developed lighter mucositis in respect to group B. Since both groups were subjected to a professional oral hygiene session and had the same initial conditions, we can assume that use of Mucosamin® in Group A played a decisive role on the appearance lower OM degree. Comparing groups C and D, similar results were obtained: even the two groups who did not receive a professional oral health care (POHC) by a dedicated operator, showed a statistically significant difference for higher grades of mucositis in patients who had not received Mucosamin®. The OMAS Scale confirms that Mucosamin® has played a protective role against the severity of mucositis. The study also shows a duration reduction in patients using Mucosamin® and undergoing POHC: data confirm our previous study on OM treatment (9), emphasize the importance association of POHC and Mucosamin® and reinforce the need to create a team haematologist-dentist-hygienist to manage oral cavity complications in patients undergoing HSCT, (14-15). Moreover, the risk of developing high levels of OM decreases drastically if Mucosamin is associated with POHC

by a dedicated operator.

The use of Mucosamin® may influence OM type and severity, allowing patients to improve their quality of life. Mucosamin's® effectiveness in reducing pain and accelerating wound healing is due to its ability to repair tissues, activate and modulate inflammatory response, promote proliferation and cell migration, angiogenesis, increase re-epithelisation, deposition of new basal keratinocytes and collagen layers, allowing to form a protective layer on the ulcerative layers (8); it seems more effective than other drugs tested (16).

According to the present study the combination of a careful debridement, correct oral hygiene during hospitalization and the use of Mucosamin® exponentially reduce the severity and duration of mucositis and consequently the discomfort of the patient. Finally, it can be stated that the use of Mucosamin® also results in a reduction in the extent of chemotherapy lesions. Hyaluronic acid and amino acid-based sprays can be a valuable therapeutic aid in the treatment of OM.

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LETTER TO THE EDITOR

SDR® vs TRADITIONAL COMPOSITE IN CLASS II RESTORATIONS IN PRIMARY MOLARS

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Received March 14, 2018 – Accepted April 12, 2018

This study aims to compare the clinical characteristics of a new flowable resin-based composite with those of a traditional composite when applied to deciduous molars for class II restorations. Twenty-eight children between 6-12 years of age, with paired minimal Class II cavities present on their primary molars, were chosen. The paired cavities were restored with either a microhybrid composite or a new flowable resin-based composite. The composites were assigned to cavities in either the right or left side of the mouth, using random allocation tables. Each pair of restorations were clinically assessed at six-month intervals following the United States Public Health Service criteria. The prevalence of carious lesions was higher in maxillary molars (53.6%) compared to the mandibular molars (46.4%). At 24 months, Charlie or Delta scores in the paired groups were not seen in any patient, thus demonstrating a clinical success. The statistical analysis using Pearson's *chi*-squared test did not reveal significant p-values for any parameter except the proximal contact showing significant p-values for this parameter. SDR appears to be the material of choice for the restoration of deciduous teeth due to its clinical features shown at 24 months as well as its ease and speed of application.

To the Editor,

Since the early 1990s, the idea of a global approach to the patient which allows the prevention of disease by implementing the Medical Home has been growing in the US (1). Although the scientific community recommends this approach as the gold standard for pediatric dentistry, the day-to-day reality is quite different.

Caries remain a problem of social order (2), and the dentist should be able to address this problem with appropriate tools and techniques. The American Academy of Pediatric Dentistry (AAPD) states that “the objectives of restorative treatment are to repair or limit damage from caries, protect and preserve

tooth structure, re-establish adequate function, restore aesthetics (where applicable), and facilitate good oral hygiene” (3).

The restorative treatment of deciduous dentition is complex and more difficult to manage in terms of prognosis. There are several options available on the market regarding both treatment and restorative materials; however, the choice often depends on the individual case. A recent Cochrane review (4) states that “Well designed, randomised controlled trials, comparing the different types of filling materials with similar outcomes are urgently needed in dentistry. There was insufficient evidence from the three trials included, to make any recommendations

Key words: caries, deciduous teeth, dental materials, SDR, restorative treatment

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0393-974X (2018)

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about which filling material to use”.

In reply to this need in literature, this study aims to compare the clinical characteristics of a new flowable resin-based composite (Smart Dentin Replacement®, Dentsply) with a traditional composite (Filtek Z250®, 3M ESPE) when applied to deciduous molars for class II restorations.

MATERIALS AND METHODS

Children between 6 and 12 years of age with paired minimal Class II cavities on their primary molars were selected for inclusion in this prospective study. Informed written consent was given by the family or guardian of all individual participants included in the study.

A split-mouth design was used to assign minimal Class II cavities to matching primary molars located on opposite sides of the same dental arch; the caries diagnosis was made through bitewing radiographs (Image n°1a-1b). Patients with clinical or radiographic signs of pulpal or periradicular pathology and pathological wear were excluded. Twenty-eight healthy children were selected, and 56 restorations were performed. These procedures were carried out at the Paediatric Dental Institute of the University of Modena and Reggio Emilia, Italy.

The paired cavities were restored with either a microhybrid composite (Filtek Z250®, 3M ESPE) or a new flowable resin-based composite (SDR®, Dentsply). The composites were assigned to cavities in either the right or left side of the mouth using random allocation tables. Bitewing radiographs of each child were taken at baseline. Further radiographs were taken only if there was a clinical indication and not for the purpose of the study. Each pair of restorations were clinically assessed at six-month intervals, following the United States Public Health Service (USPHS) criteria (5-6).

The investigators attended calibration sessions carried out at regular intervals to standardize both the diagnosis of carious lesions and the placement of restorations. Standardisation of bitewing radiographs was attained by using Rinn holders and the extended cone parallel technique. The technique for the placement of restorations was standardized throughout the practice in plastic and extracted primary molar teeth. All restorations were performed under standardized conditions using topical and local anesthesia and rubber dam isolation.

Dental cavity preparation was performed using a 330 bur in a high-speed handpiece with water coolant and a round carbide bur at slow speed for dentin. All cavity walls had supragingival margins. Metal matrix bands and wooden wedges were used for restoration placement. Prepared cavities were treated with different types of enamel-dentine adhesives following the manufacturers' guidelines. For teeth restored with a microhybrid composite, the proximal wall was filled first, and then increments no larger than 2 mm were added with an oblique incremental technique. For teeth restored with SDR, the cavity was filled in a single step with a 4-mm layer. The occlusion was checked, and the restorations were finished and polished with ultrafine granulation burs and polishing paste.

At baseline and 6, 12, 18 and 24 month intervals (Images n°1c-1d), direct clinical evaluation of each restoration was performed by two calibrated double-blinded evaluators using the USPHS criteria for anatomical form, marginal adaptation, cavosurface marginal discoloration, axial contour, proximal contact and secondary caries. Furthermore, the Visible Plaque Index was used to record the presence or absence of dental biofilm, with scores Alpha and Bravo denoting clinical success and Charlie and Delta representing clinical unacceptability. For the secondary caries criteria, the restoration was considered a failure if it had a score of 1 (Bravo).

Statistical analysis

Stata 12.0 (StataCorp, College Station, TX 2011) was used to compare the prevalences of the different outcomes in the two treatment groups through Pearson's *chi*-squared test and the associated p-value. The odds ratio of achieving a satisfactory outcome for each of the parameters under investigation were also computed by using conditional logistic regression for the matched sets, i.e., comparing the paired teeth which had been treated with different material within each study subject. This matched analysis was repeated within a multivariate model, adjusting for potential confounders (age, sex, ethnic group, income) and stratifying the study population according to gender.

RESULTS

The patients in this study were between the ages of 6 and 12 years, with an average of 8.5 years. The gender distribution was 53.6% male and 46.4%

female. Most of the patients included in this trial were Italians (67.87%), followed by Moroccans (14.28%), Romanians (7.14%), Ukrainians (7.14%) and Argentinians (3.7%). Most patients were of high socio-economic status (50%) followed by middle (21.42%), medium-low (7.16%) and low (21.42%) socio-economic status. The prevalence of carious lesions was slightly higher in maxillary molars (53.6%) compared to the mandibular molars (46.4%).

Only one patient interrupted follow-up, as the studied teeth were exfoliated. This patient's data were analyzed; however, the patient was not available for further discussion. At 24 months, Charlie or Delta scores in the paired groups were not seen in any patient, thus demonstrating a clinical success. Three teeth from both groups had secondary caries at the last follow-up and, therefore, were considered clinical failures.

Statistical analysis

Pearson's *chi*-squared test did not reveal any significant *p*-values for marginal adaptation, anatomical form, cavosurface margin discoloration and axial contour, therefore, it was not possible to

emphasize any difference between the two materials for these parameters.

However, for marginal adaptation, the OR values calculated through logistic regression revealed a progressive decrease in risk favoring SDR in the first 6 months (OR=0.333), which reversed at 24 months (OR=2). Furthermore, for anatomical form it was possible to estimate the risk up to 6 months while the OR values at 12 and 24 months favored the traditional composite (OR=4). Moreover, the OR values for cavosurface margin discoloration revealed a decrease in risk favoring SDR at a constant rate from 6 to 24 months (OR=0.8). For axial contour, the OR values revealed a decrease in risk favoring the traditional composite at baseline (OR=3), which was reversed at 12 and 24 months.

With regard to proximal contact, Pearson's *chi*-squared test showed significant *p*-values for this parameter at 6 months ($p=0.007$), which decreased at 12 months ($p=0.005$) and, finally, slightly increased at 24 months ($p=0.012$). The OR values revealed a decrease in risk favoring the traditional composite at baseline (OR=2.5), which progressively increased up to 6 months (OR=10) and finally decreased at 12 and 24 months (OR=4).

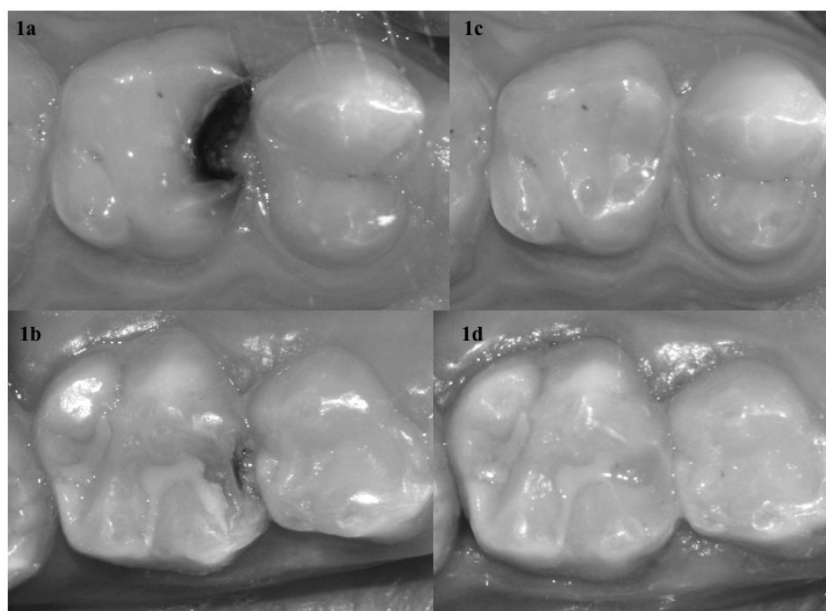


Fig. 1. *a, b*) Pre-operative views. *c*) At 24-month follow-up with composite restorations. *d*) At 24 month follow-up with SDR® restorations.

Secondary caries

There were no observed secondary caries lesions up to 24 months, at which time, in both control groups, there were three failures. The statistical analysis using Pearson's *chi*-squared test and the OR values calculated through logistic regression analysis showed no significant values for this parameter.

Plaque

The presence of plaque related to the restoration was relatively frequent in both groups. The statistical analysis using Pearson's *chi*-squared test and the OR values showed no significant values.

The multivariate model, adjusted for potential confounders (age, gender, ethnic group, income) after stratification of the study population according to gender, showed no difference in comparison to the previous analysis of unpaired data.

DISCUSSION

As shown in numerous studies (6-10), "Well designed, randomised controlled trials comparing the different types of filling materials for similar outcomes are urgently needed in dentistry". This study compared the clinical characteristics of a traditional microhybrid composite that is widely used in permanent teeth with a new flowable resin-based composite. Due to the presence of a polymerization modulator, this new material is capable of low-stress polymerization, which allows stratification into a single mass of up to 4 mm, making the material suited to modern pediatric dentistry. Early studies compared resin materials to the amalgam and glass ionomer that were commonly used and were considered the gold standard. Many authors have agreed that resin-based materials could, even in a population with high risk caries activity, be suitable alternatives to amalgam for restorations in primary teeth (11). Subsequent studies have sought a new gold standard by comparing glass-ionomer cements, compomers and traditional composites (7). A recent study showed that "The type of restorative material used did not influence the longevity of the restorations" (12). The results of this study are, therefore, in line with other studies in the literature.

The lack of a statistically significant difference between the two materials, except for the point of contact parameter, can be assumed to be related to the different nature of the filler and, thus, to the different ways of applying the material. Another interesting finding was the initial absence of a difference in surface wear and dimensional stability of the materials which, in turn, could increase to the same level for SDR and Z250. SDR, however, can be applied in a single step and in a very short time; this advantage seems to be a determining factor in the clinician's choice for pediatric patients.

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LETTER TO THE EDITOR

DEEP INFILTRATION FOR THE TREATMENT OF HYPOMINERALIZED ENAMEL LESIONS IN A PATIENT WITH MOLAR INCISOR HYPOMINERALIZATION: A CLINICAL CASE

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Received March 14, 2018 – Accepted April 12, 2018

This case report presents the technique of deep infiltration for the treatment of hypomineralized enamel lesions in a patient affected by molar incisor hypomineralization (MIH) with the purpose of obtaining an aesthetic improvement in the affected vestibular enamel. Deep infiltration treatment involves a sequence of steps starting with an initial mechanical abrasion of the outside surface of the enamel, followed by a phase of chemical erosion using 15% hydrochloric acid, penetration through the porosity of the enamel by a very fluid resin, and finally the repair of the thin layer of lost enamel using a composite. The aesthetic improvement of the elements affected by hypomineralized lesions is discernable immediately following treatment and becomes more accentuated with time. At the expense of minimal damage to surface enamel, deep infiltration enables treatment of all types of white spot enamel lesions, regardless of aetiology, offering the possibility of restoring an aesthetically pleasing smile for patients.

To the Editor,

The erosion/infiltration technique was devised following release on the market of a new product (Icon DMG, Hamburg, Germany) originally intended for the treatment of incipient interproximal caries (1). During *in vitro* and *in vivo* studies on this product to test its effectiveness for the treatment of incipient interproximal carious lesions, a positive outcome of its use was noted: following treatment, white spot lesions were seen to be less opaque and therefore less visible. This is due to the application of a resin with a refraction index of 1.46, which is very similar to the refraction index of healthy enamel (1.62) (2-4).

The technique proposed initially was surface infiltration, but this is subject to limitations regarding the treatment of lesions that involve the deeper

portion of the enamel. This is because the erosive action of hydrochloric acid alone only removes the outermost component of the tissue, preventing the penetration of the resin into the body of the lesion when this is in the inner third of the enamel, as is the case for molar incisor hypomineralization (MIH) lesions (5-6).

A variant of this method was proposed in order to overcome the limitations of surface infiltration and treat all types of white spot lesions. This is the so-called deep infiltration technique, which includes a stage of initial mechanical abrasion of the enamel before the stages already described above (1).

Case report

The patient reported no history of fixed orthodontic treatment, systemic fluoridation, or childhood traumas.

Key words: molar incisor hypomineralization, deep infiltration, restorative treatment, paediatric dentistry, biomimetic approach

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0393-974X (2018)

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Under physical examination the patient exhibited hypomineralized lesions of upper central incisors, and of the first permanent molars, and was thus diagnosed with MIH. Deep infiltration treatment was proposed for upper left incisor and surface infiltration for the upper right incisor (Fig. 1 a,b,c), and informed consent was obtained from the patient.

Following rubber dam isolation, the surface of the upper left central incisor was first cleansed with a prophylactic paste and rubber polishing, and then abraded with a finishing bur (granulometry 40 μm) on a high-speed handpiece. Mechanical abrasion was applied only to this element in order to access the limit of the hypomineralized lesion, located in the inner third of the enamel thickness. Next, a 15% hydrochloric acid based gel (Icon Etch, DMG, Germany) was applied for 2 min on the surfaces of both elements. The acid gel was removed with a water jet for 30 s, and the surface was then dried for the application of 99% ethanol (Icon Dry, DMG, Germany) for 30 s.

After rinsing and drying the surface, the lesion

appeared more accentuated. This phenomenon derives from the incorporation of water or air (which have respective refractive indexes of 1.33 and 1.01) within the porosity of the enamel. The application of ethanol to the treated surface displaces the water particles present within the enamel pores, and since its refraction index is similar to that of the resin that will be subsequently inserted, it offers an indication of the achievable final results. The colour of the lesion should change during penetration of the ethanol. If this does not happen it must be assumed that the ethanol has not reached the body of the lesion and thus the resin will not be able to correctly infiltrate the lesion. In this case it is necessary to repeat the etching procedure.

In the present case study, the etching stage followed by rinsing and application of ethanol was repeated 3 times. The resin (Icon Infiltrant, DMG, Germany) was applied and left on the surface for 3 min so that it could penetrate into the porosity of the lesions. After removal of excess resin, polymerization was

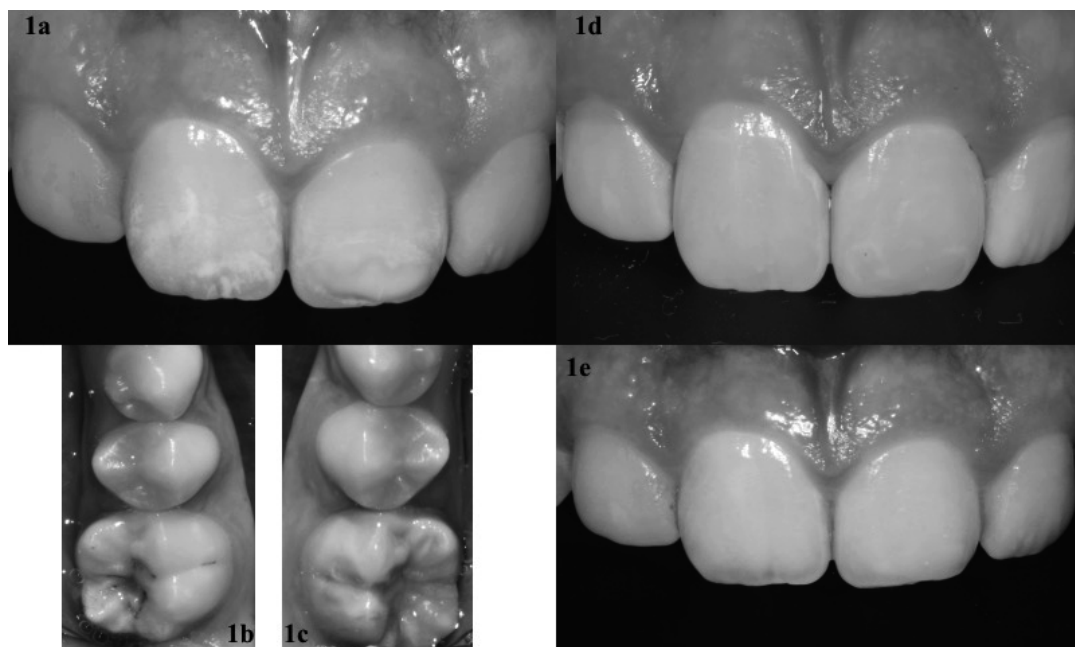


Fig. 1. *a) Initial front view. b) Hypomineralization in the molar area. c) Hypomineralization in the molar area. d) Final frontal view immediate post-op. e) Final frontal view at 24-month follow-up.*

conducted for 40 s. Resin infiltration was repeated a second time, maintaining the product on the surface for 1 min. The excess was again removed, followed by polymerization, and then the application of a bonding agent (OptiBond FL, Kerr, USA) and a very thin layer of composite (Enamel Plus Hri, Micerium, Italy) to replace the thickness of enamel lost during the initial stage. Treatment was concluded with thorough polishing using rubber heads and a goat hair brush.

On completion of the treatment, an obvious aesthetic improvement in the treated dental elements could already be observed, despite the dental elements being dehydrated due to their isolation with the rubber dam (Fig. 1d). This improvement was even more marked one month after treatment (Fig. 1e)

DISCUSSION

Deep infiltration involves very minor damage to surface enamel by intra-oral sandblasting or grinding in order to reach and correctly infiltrate a lesion body and thus attenuate its visibility. Compared to surface infiltration, deep infiltration requires a slightly greater sacrifice of tissue, but always remains within the confines of the enamel. The purpose is not the removal of a lesion by mechanical abrasion, but to reach the limit of the lesion so that resin can subsequently infiltrate its full extension (9).

While surface treatment alone has shown encouraging results for the treatment of certain types of hypomineralized enamel defects, this is not true for other enamel defects including pre-eruptive lesions (10-11). The reason for this emerges clearly in a study of histological samples of dental elements affected by MIH. Analyses with a scanning electron microscope reveal that the zone of hypomineralization starts at the amelodentinal junction and does not include the more superficial enamel layers. This is the reason why in cases of moderate MIH the hypomineralization remains confined to the internal third of the thickness of the enamel, while the two most external thirds remain relatively intact. Instead, in cases of severe MIH the entire enamel layer is involved in hypomineralization (5-6, 12).

It is advisable to precede deep infiltration treatment with a careful assessment of the medical history and

physical examination, in order to establish the least invasive form of treatment possible for each type of lesion. The technique presented above is indicated, since it involves very minor mutilation of the surface enamel in order to treat a lesion located in the inner third of the enamel, which cannot be reliably treated with surface infiltration alone.

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ERRATA CORRIGE

Withdrawal

The article below published in Volume 32 No. 2 has been withdrawn owing to discrepancies in the results found by the co-Authors.

S. Ali, C. Sola, M. Harrison, M. Tahir, A. Sheed Khan, M. Mumtaz, E. Chimara and F. Hassan. Spoligotyping analysis of *Mycobacterium tuberculosis* with TB-SPRINT technology

Authors

The Authors of the article entitled “Clinical course of severe colitis: a comparison between Crohn’s disease and ulcerative colitis” published in Volume 32 No. 2 p. 415 should read:

E. Sinagra, A. Orlando, F. Mocciaro, V. Criscuoli, L. Oliva, S. Maisano, M. Giunta, F. La Seta, G. Solina, A.G. Rizzo, A. Leone, G. Tomasello, F. Cappello and M. Cottone

