JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS
Volume 30, No. 4 (Supplement 1), September - December 2016
Italian Orthopaedic Research Society 2016
Guest Editors: G. Vadalà, R. Papalia and V. Denaro

CONTENTS

EFFECT OF NERVE GROWTH FACTOR ON CULTURED HUMAN CHONDROCYTES

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Nerve growth factor (NGF) is involved in several joint diseases. It participates in nociception and neurogenic inflammation and its concentrations increase in synovial fluid and tissue from arthritis. However, data about its role in articular cartilage are scant and conflicting. This study analysed effects of different NGF concentrations on cultured human chondrocytes by evaluating cell proliferation, cell phenotype, and gene expression. The MTT test excluded an influence on cell viability. Alcian blue and S100 staining demonstrated that NGF may induce de-differentiation of the chondrocyte phenotype. Real-time PCR showed that NGF did not influence gene expression of type I, II and XI collagen, TGF-β, IGF-1 and metalloproteinase (MMP)-13, while it reduced the expression of MMP-3. These findings show that NGF may have uncertain effects in human chondrocytes. Further investigations by wider gene expression and protein synthesis analyses are required to determine how chondrocytes may be influenced by NGF.

Nerve growth factor (NGF) is a dimeric protein made up of two identical, non-covalently bound polypeptide chains linked by intrachain disulfide bonds. It was the first neurotrophin (NT) to be isolated and is the best characterized. NGF interacts with two cell surface receptors, tyrosine receptor kinase A (trkA) and p75 NT receptor (p75NTR) (1). NGF is involved in nervous, endocrine and immune system function. It is also involved in some inflammatory and rheumatic diseases. It has been demonstrated to participate in pain initiation, inadequate nociception and neurogenic inflammation by stimulating neuropeptide overexpression and activating immune cells (2, 3). NGF has also been suggested to facilitate sensory nerve sensitization or growth in articular cartilage, linking osteochondral angiogenesis to arthritis pain (4). There are few *in vitro and in vivo studies* of NGF in normal and pathological articular cartilage, and their findings about its role are conflicting. NGF and trkA upregulation has been described in chondrocytes from osteoarthritis (OA) patients, suggesting a role for NGF in its pathophysiology. However, it is unclear whether it stimulates chondrocyte metabolism by promoting cartilage repair in OA or enhances the osteoarthritic process in some other way (5). The NGF-trkA complex expressed by OA chondrocytes might confer protection against OA development by slowing down chondrocyte differentiation (6).

The inconsistent literature about the role of

Key words: nerve growth factor, articular cartilage, chondrocytes, cell culture

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0393-974X (2016)

NGF in articular cartilage prompted the present in vitro study about the effects of different NGF concentrations on the proliferation, phenotype and gene expression of human healthy chondrocytes.

MATERIALS AND METHODS

Chondrocyte cultures

Healthy cartilage was harvested from the medial surface of the lateral femoral condyle of the knee in 6 subjects (2 males, 4 females) of similar age (40±5 years), undergoing arthroscopic procedures for acute injury. None had OA, rheumatic, autoimmune, or metabolic disease. All participants provided a written informed consent to proceed. Cartilage samples were minced into small pieces under aseptic conditions and washed in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) containing 1% antibiotics. After enzymatic digestion, cells were passed through a 30 µm nylon filter, centrifuged at 1200 rpm, suspended in DMEM/F12, seeded in 75 cm^2 flasks and allowed to expand for three weeks. Cells were used at a third passage at a concentration of 5000 cells/ml. Cultures were subdivided into three groups: two treatment groups, receiving respectively 50 ng/ml (group A) or 200 ng/ml (group B) NGF-β (Sigma Aldrich), and an untreated, control group (group C). The medium and NGF-β were replaced twice weekly. The effects of NGF exposure were evaluated in chondrocyte monolayers at 7 and 14 days.

Cell viability (MTT assay)

For viability evaluation, cells were seeded in 48-well multiplates (5000 cells/well). The culture medium was replaced with a filtered 10% sterile solution of 5 mg/ml 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) in DMEM without phenol red (both from Sigma Aldrich) and incubated at 37°C for 4 h. After removing the supernatant, the dark blue formazan crystals that had accumulated in cells were dissolved by adding 1 ml of solvent (0.01 N HCl in absolute isopropanol) and quantified by spectrophotometry (BioPhotometer Plus, Eppendorf, Hamburg, Germany) at 550 nm. The MTT test was performed at 7 and 14 days. Data are the mean of 3 assays and are expressed as optical density (O.D.) for each cell group and time point.

Cell phenotype

Cells were seeded in chamber slides at a concentration of 5000 cells/ml. At 14 days for histochemical analysis, cells were stained for 30 min with alcian blue pH 1.0 to evaluate cartilage glycosaminoglycans production. Cells for immunohistochemistry were incubated with anti-S100 polyclonal antibody**.** The reaction was detected using the Dako LSAB+ System-HRP kit according to the manufacturer's instructions. The percentage of stained cells was assessed by computerized morphometric analysis using Leica Application Suite 4.1 by examining five random fields/culture.

Real-time PCR and gene expression analysis

The expression of seven marker genes associated to chondrocyte activity and metabolism - type II collagen (COL2A1), type XI collagen (COL11A1), type I collagen (COL1A1), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), metalloprotease (MMP) 3 (MMP-3), and MMP-13 - was determined by real-time PCR (RT-PCR), 7 and 14 days into culture in monolayers, treated with the two NGF concentrations and in untreated chondrocyte. cDNA was amplified by RT-PCR using Power SYBR® Green PCR Master Mix and the specific assay designed for the genes investigated, except in the case of COL1A1, whose cDNA was amplified using TaqMan universal PCR master mix (Applied Biosystems). Gene expression levels were normalized to the expression of the housekeeping gene RPL13 and are presented as fold changes relative to expression in control cells. Results are the mean of 5 observations selected randomly. Quantification was done with the delta/delta calculation method using 7500 Software v2.0.5 (Applied Biosystems).

Statistical analysis

A non-parametric approach was used, as variables were not normally distributed. Variables were summarized using median and interquartile range (1^{st}-3^{rd} quartiles). A ranked-based non-parametric analysis for longitudinal data (7) was used to compare MTT viability data (at O.D. 550 nm) in the three cell groups at 7 and 14 days. The effect of treatment type and time and their interaction was evaluated; results are presented as a box plot. The Kruskal-Wallis test was applied to compare histochemical staining (alcian blue and S100 protein) at 14 days. Statistical significance was set at

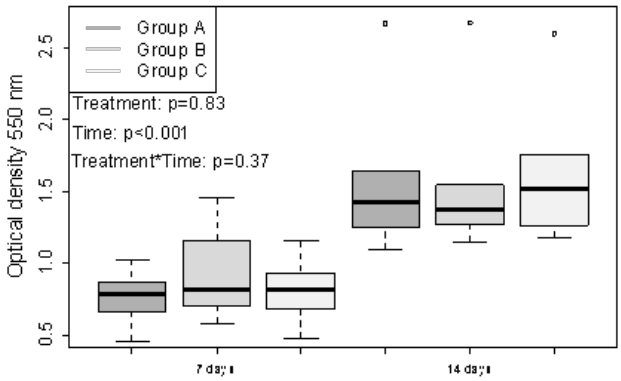

Fig. 1. *MTT test. Results are reported as optical density, which in this assay correlates directly with cell number. The values of the three groups show no significant differences.*

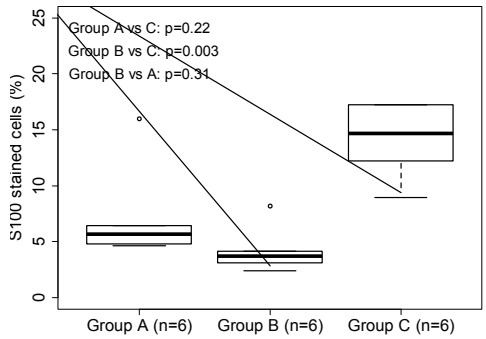

Fig. 2a

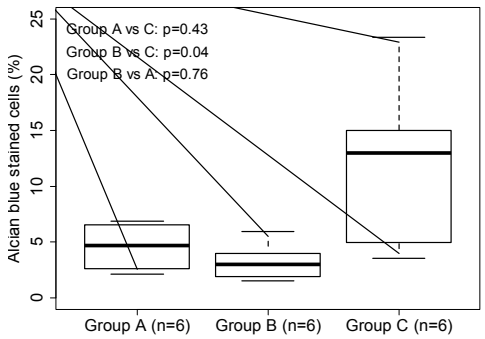

Fig. 2b

Fig. 2. *Immunohistochemical analysis. Percentage of cells staining with S100 protein antibodies and alcian blue. As shown in Fig. 2a and 2b, the median proportion of stained cells was significantly lower in Group B than in Group C (respectively p=0.003, and p=0.04).*

a level of probability of 0.05. Changes in gene expression are given as fold change; >2 fold change expression for upregulation and <0.5 fold change for downregulation were considered significant ($p<0.05$).

RESULTS

Cell viability (MTT assay)

Median O.D. readings at 7 days were 0.82 (1^{st}-3^{rd} quartiles: 0.69-0.92) in group C; 0.79 (1^{st}-3^{rd} quartiles: 0.69-0.85) in group A; and 0.82 (1^{st}-3^{rd} quartiles: 0.72-1.09) in group B. At 14 days they were 1.52 (1^{st}-3^{rd} quartiles: 1.32-1.70) in group C; 1.42 (1^{st}-3^{rd} quartiles: 1.28-1.61) in group A; and 1.37 (1^{st}-3^{rd} quartiles: 1.27-1.52) in group B. The results of non-parametric analysis are reported in Fig. 1. No significant differences in cell viability were observed in the three groups.

Histomorphometry

The median percent values of cells staining with alcian blue at 14 days were 13% in the control group (1^{st}-3^{rd} quartiles: 6.55-14.92); 4.70% (1^{st}-3^{rd} quartiles: 3.02-6.21) in group A and 3.04% (1^{st}-3^{rd} quartiles: 1.99-3.94) in group B. A significantly lower percentage of stained cells were detected in group B compared with group C (Fig. 2a). The median percent values of cells staining with S100 protein were 14.70% in group C (1^{st}-3^{rd} quartiles: 12.68-16.77), 5.71% (1^{st}-3^{rd} quartiles: 4.92-6.37) in group A, and 3.72% (1^{st}-3^{rd} quartiles: 3.22-4.08) in group B. Again, the proportion of stained cells was significantly lower in group B than in group C (Fig. 2b).

Gene expression

COL1A1, COL2A1, COL11A1, TFG-β, IGF-

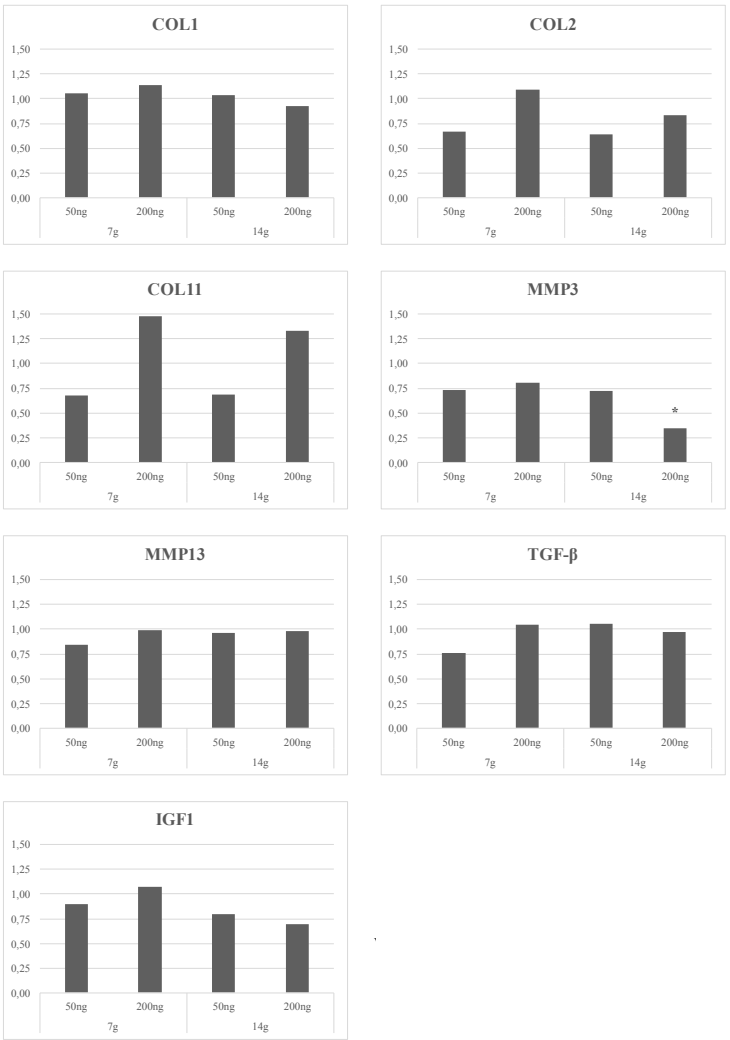

Fig. 3. *Gene expression pattern. Mean gene expression values in NGF-treated samples from 5 healthy adult individuals (Groups A and B) normalized to control values (Group C). Changes in gene expression are given as fold change; > 2 fold change expression for upregulation and < 0.5 fold change for downregulation were considered significant ($p<0.05$) (marked with *).*

1 and MMP-13 gene expression did not differ significantly in group A and B compared with group C. MMP-3 gene was significantly downregulated in group B only at 14 days. The gene expression data are reported in Fig. 3.

DISCUSSION

Some researchers of our group described the expression and topographic distribution of NGF, trkA, and p75 in hyaline, fibrous and elastic cartilage from normal adult subjects (8). These findings suggested that NGF could be involved in cartilage pathophysiology through an autocrine/paracrine mechanism. Other authors reported that chondrocytes could produce NGF under various stimuli such as IL-1β and visfatin (9) and TGF-β in an ALK5/Smad2/3-dependent manner (10). However, the exact role of NGF on chodrocyte behaviour is still debated.

According to some studies, NGF could mainly act as a pro-inflammatory factor; the notion is supported by NGF overexpression detected in synovial fluid, membrane and articular cartilage of humans (5) with OA or RA. NGF also contributes to OA pain

perception through nerve sprouting, which enhances peripheral sensitization in articular cartilage (4). Other data support a role for NGF mainly as a reparative factor. NGF-trkA upregulation in inflamed joints might provide protection against OA development, by slowing chondrocyte differentiation and inhibiting their maturation beyond the prehypertrophic stage of differentiation (6).

This is the first study describing the *in vitro* effects of NGF on proliferation, phenotype and gene expression of human articular chondrocytes in primary cultures.

In regards to chondrocyte proliferation, MTT test disclosed that NGF did not influence cell proliferation; this suggests that NGF does not act as a growth factor on articular chondrocytes, but rather similar to a cytokine.

Regarding chondrocytes differentiation, results of the present study were somewhat ambiguous: histochemical and immunohistochemical analysis of cells phenotype by alcian blue and S100 protein staining, showed a detrimental effect on chondrocyte phenotype. However, RT-PCR analysis showed that gene expression of some molecules related to chondrocyte differentiation/dedifferentiation, such as COL2A2, COL11A1 and COL1A1, were not altered by NGF administration.

Wider analyses regarding gene expression and protein synthesis are necessary to clarify this particular aspect of chondrocyte behaviour under NGF stimulation.

Among the growth factor family, TGF-β and IGF-1 have proven to play a crucial role in cartilage development, homeostasis and repair.

TGF-β has strong mitogenic action on chondrocytes (11) and induces NGF expression on chondrocytes in an ALK5-Smad2/3 dependent manner (10). NGF can stimulate TGF-β1 gene expression and protein secretion in PC12 pheochromocytoma cells. On the contrary, in the present study, NGF did not influence TGF-β gene expression in articular chodrocytes suggesting that NGF and TGF-β have not a reciprocal action on these cells. IGF-1 usually promotes mesenchymal stem cell differentiation in chondrocytes (12). In the present study, its gene expression was not influenced by NGF administration, suggesting that NGF does not influence chondrocytes behaviour via IGF-1 secretion.

Among metalloproteases, several families of these zinc dependent enzymes participate actively to development, remodeling, and subsequent pathological processes of the joints. MMP-13 degrades mainly type II collagen; its expression is minimal in healthy articular cartilage while it is upregulated in OA and in subjects with advanced cartilage disruption (13). In our cultures, its gene expression was not influenced by NGF administration.

MMP-3 (Stromelysin-1) is critical in maintaining normal ECM turnover. MMP-3 degrades proteoglycans and it is expressed by healthy articular chondrocytes (14). It has also been described in OA chondrocytes with a biphasic pattern in early and late disease stages (15). MMP-3 seems to have a dual role: to maintain homeostasis in healthy cartilage and to initiate cartilage disruption in OA (13). In the present study, its expression was significantly downregulated in cells treated with NGF at 14 days. Considering that our study was performed on healthy chondrocytes in primary cultures, MMP-3 downregulation could represent a negative effect for chondrocytes homeostasis inducing ECM alteration.

Taken together, our findings suggest that NGF may have an uncertain effect on human chondrocytes. NGF did not influence chondrocytes proliferation and gene expression of collagens, TGF-β, IGF-1 and MMP-13. However, some data indicate that NGF may adversely affect differentiated human articular chondrocytes: histochemical and immunohistochemical analysis showed a detrimental effect on chondrocyte phenotype. Moreover, MMP-3 expression was downregulated. Further investigation through a wider and more specific gene expression pattern analysis is required to determine how articular chondrocytes may be influenced by NGF.

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HYBRID COMPLEXES OF HIGH AND LOW MOLECULAR WEIGTH: EVALUATION USING AN *IN VITRO* MODEL OF OSTEOARTHRITIS

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Hyaluronan (HA) is central in joint and cartilage functions and to restore synovial fluid viscosity. In patients with osteoarthritis (OA), molecular weight (MW) and concentration of hyaluronic acid (HA) are reduced, diminishing joint lubrication. IL-1β treatment was used to mimic osteoarthritis in a chondrocytes based *in vitro* model. The aim of our research, using this model and human chondrocytes was to assess the anti-inflammatory effect of H/L-HA hybrid complexes (SINOVIAL-HL®) in comparison with HA at high (H-HA) and low molecular weight (L-HA) separately used, through the evaluation of specific biomarkers involved in cartilage degradation and correlated to osteoarthritis. Specifically, TNF-α and IL-6 mRNA were evaluated by qRT-PCR. Cytokines levels were measured using Bio-plex assays and COMP-2 through immunofluorescence staining and western blot. H/L-HA significantly reduced inflammation biomarkers respect to both L-HA or H-HA separately considered at transcriptional and protein level.

Osteoarthritis (OA), is recognized as a metabolically active, dynamic process of degradation and synthesis involving joint, cartilage, bone, synovia, ligaments and muscle (1) that has affected thousands of old and even young people worldwide each year. The main pathological characteristics of OA result from failure of the repair process of the synovial joints that is activated by different damages such as loss of chondrocytes and cartilage matrix leading to biomechanical joint failure (2). The etiology of OA is not completely understood, but considered as a long-term degenerative process and based on different factors such as inflammation, mechanical and physical injury and other metabolic causes (3). It has also been observed that OA presents an important inflammatory component, driving cytokines production into the synovial fluid (4, 5). Pro-inflammatory cytokines such as TNF-α and IL-6 are crucial factors involved in the development and progression of this degenerative pathology (6). Cartilage Oligomeric Matrix Protein (COMP) is an important degradation protein complex of articular cartilage and was investigated as marker of OA (7). As degenerative joints disease, OA progression is correlated to the production of inflammatory factor and to the enzymes responsible either for degradation or for the reduced synthesis of HA. Pharmacological treatments are based on chondroprotective agents that can stimulate the synthesis of collagen and proteoglycans by chondrocytes and production of HA by synoviocytes, also preventing fibrin formation in the subchondral and synovial vasculature. Compounds that show some of these characteristics are endogenous molecules of the articular cartilage, including HA, glucosamine, and chondroitin sulfate (8). In OA, as the cartilage wears down, the level of the HA responsible for the viscoelastic properties of synovial fluid also decrease in the joints. Actually,

Key words: Osteoarthritis, joints diseases, hyaluronan, hybrid cooperative complexes, inflammation, chondrocytes based in vitro model

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0393-974X (2016)

in clinical practice, to alleviate pain and restore/improve tissue functions, not only during acute phases but also as maintenance therapy, intra-articular injections of corticosteroids or similar drugs are used, it is a diffused viscosupplementation procedure based on hyaluronan gels. In particular, this last clinical practice is based on the rheological/mechanical features of HA and proves to re-establish the synovial joints function. We recently reported that HA have a short half-life *in vivo* (e.g enzymatic digestion, free radicals, etc.) (9); however, chemically cross-linked HA have been suggested to improve its *in situ* permanence and long-term stability. Here we present our experimental research where we tested a novel formulation based on H/L-HA of L-HA and H-HA obtained through a patented NAHYCO™ technology. The rheological properties of H/L-HA make it possible to double the amount of HA delivered to the injured sites, without increasing the volume injected and using very thin needle bores (e.g. up to 29-30 gouge) (10). In our model, human chondrocytes were pre-insulted with IL-1β, in order to mimic osteoarthritis *in vitro*. On these cells we assayed the efficacy of this new formulation compared those to the sole L-HA or H-HA. Biomarkers such as TNFα, IL-6 and COMP-2, both at transcriptional and protein level, were evaluated permitting an extensive multifactorial comparison.

MATERIALS AND METHODS

High- (H-HA; MW 1200±200 kDa Mw/Mn=1.4 Intrinsic viscosity?) (0.16 % w/w) and low- (L-HA: MW=100±20, kDa Mw/Mn=1.4, Intrinsic viscosity (22.6±0.10 dL/g) (0.16% w/w) molecular weight hyaluronic acid were kindly provided by Altergon S.r.l., Italy. Altergon HA is a fermentative HA from *Streptococcus equi ssp. equi*, at pharmaceutical grade (e.g. purity >95%, water content <10%, EU/mg <0.05). For all samples, pH and osmolarity were measured in order to perform experiments in physiological conditions (pH=6.5-8.0 and osmolality=300mOsm). Hybrid complexes SINOVIAL-HL® were obtained through NAHYCO™ patented technology, (starting material H-HA MW 1400 kDa and kDa and L-HA MW 100 kDa). The final concentration used in these experiments was 32g/L: 32 mg H-HA+32 mg L-HA in 2 mL volume, provided in prefilled syringes. Interleukin-1β (IL-1β 50nq/mL) was purchased from Sigma Aldrich (Milan, Italy).

Rhelogical measurement SEC-TDA Viscotek

The hydrodinamic characterization was performed using the SEC-TDA (Size Exclusion Chromatography-Triple Detector Array) equipedded by Viscotek ((Malvern Instruments™ Ltd, UK)). A full description of the SEC-TDA system and experimental conditions are extensively described elsewhere (11).

Chondrocytes in vitro cultures expansion: phenotype characterization and proliferation

The study was approved by the Medical Ethics Committee of the Second University of Naples (AOU-SUN registration No 0003711/2015), (10). Human chondrocytes (3.0×10^4 cells/cm^2):

i) Un-treated (CTR) , ii) treated with H-HA 1400kDa (0.16% w/v), iii) L-HA 100kDa (0.16% w/v) and iv) hybrid complexes (0.16% w/v) were seeded into 24-well tissue plates in order to perform proliferation experiments using Time Lapse Video Microscopy (Okolab, Italy). Cell isolation and TLVM station are described in previous papers (10, 12). Proliferation analyses were performed using Image-Pro® Plus 5.1, a platform for cell image analysis and includes a full range of qualitative and quantitative tools for analyses.

Osteoarthritis like chondrocytes in vitro model

The experiments were conducted using human chondrocytes pre-treated with Interleukin-1β (IL-1β) to mimic *in vitro* OA-damaged chondrocytes. The cells were seeded into 24-well tissue-plates and at the confluence were incubated in FBS free medium containing IL-1β (50 ng/mL). Two hours later, chondrocytes were treated with H-HA (1400kDa, 0.16% w/v), L-HA (100kDa, 0.16% w/v) and hybrid complexes (H/L-HA 0.16% w/v) for 24 h.

RNA evaluation and quantitative real-time PCR

Total RNA was extracted from non-treated cells (control) and treated chondrocytes (5×10^3 per well) using TRIzol® Reagent (Invitrogen, Milan, Italy), following manufacturer's instructions. RNA concentrations were measured using a Nanodrop Instrument (Celbio, Milan Italy) and 1μg of total RNA was reversely transcribed into

cDNA with Reverse Transcription System Kit (Promega, Milan, Italy) according to the manufacturer's instructions. Quantitative real-time PCR was performed using IQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Milan, Italy) to analyze the expression of TNF-α and IL-6 genes. The primer sequences and amplification protocol are reported in Table I. All reactions were carried out in triplicate and the expression of specific mRNA relative to the control was determined after normalization with respect to the HPRT housekeeping gene. The results were expressed as normalized fold expression, calculated by the ratio of crossing points of amplification curves of the specified genes and internal standard using the comparative threshold method (ΔΔCt =difference of ΔCt between treated cells and non-treated cells used as controls) (10).

Bio-Plex cytokines assay

Bio-Plex pro-human cytokine was used to quantified cytokines profile in supernatants of chondrocytes treated as described in the previous section. Cell supernatants were collected, centrifuged (3,000 rpm for 10 min at 4°C) and then stored at -80°C until analyzed. Three independent experiments in duplicate were performed for each sample. Human cytokine 8-plex assay (Bio-Rad Laboratories s.r.l., Milan, Italy) including IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, and TNF-α, was used for our experiments. Standards and samples were determined using a Bio-Plex array reader (Luminex, Austin, TX). The analytic concentrations were calculated using a standard curve according to the manufacturer's instructions.

Immunofluorescence staining

For immunofluorescence staining, chondrocytes were grown on chamber slides (BD Falcon, Italy). After 24 h of treatment, the cells were fixed with 4% w/v paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and permeabilized in 0.2% Triton X-100 v/v in PBS for 1 h. Non-specific sites were blocked using blocking buffer solution (PBS containing 10% v/v bovine serum and 1% w/v BSA). The cells were then incubated with anti-COMP antibody (Abcam) overnight at 4°C followed by a corresponding secondary antibody for 2 h at room temperature. Nuclei were stained with 20-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2.50-bi-1H-benzimidazole trihydrochloride hydrate, bisBenzimide (Hoechst) and actin filaments were stained using phalloidin tetramethylrhodamine B isothiocyanate

Table I. *Primer sequences used for qRT-PCR analysis.*

Gene	Primer sequence	Thermal Cycles
HPRT	Forward: 5' TGACCTTGATTTATT TTGCATACC 3' Reverse: 5' CGAGCAAGACGTTCAGTCCT 3'	95 °C 10 s, 55°C 30 s, 72 °C 3 min, 40 cycles
Tumor necrosis factor TNF-α	Forward: 5'CGAGTGACAAGCCTGTAGC 3' Reverse: 5'GGTGTGGGTGAGGAGCACAT 3'	95 °C 10 s, 55°C 30 s, 72 °C 3 min, 40 cycles
Interleukin-6 IL-6	Forward: 5'GCCAGAGTCATTCAGAGCAA3' Reverse: 5'CAAACGGGGAAATACTGTGG3'	95 °C 10 s, 55°C 30 s, 72 °C 3 min, 40 cycles

Table II. *TNF-α, IL-6, IL-4 and INFγ levels in supernatants (pg/mL) and relative fold decrease (FD) for each sample respect to IL-1β treated cells (Bioplex cytokines assay).*

Samples	TNF-α (pg/mL)	FD	IL-6 (pg/mL)	FD	IL-4 (pg/mL)	FD	INFγ (pg/mL)	FD
IL-1β	28.3±2.1	1	21955±1000	1	7.0 ±0.5	1	138.1 ±2	1
H-HA	21.5 ±1.5	0.76	11511±900	0.52	5.8 ±0.4	0.83	117.5±2	0.85
L-HA	25.4 ±1.1	0.89	11995±1000	0.54	6.4 ±0.8	0.91	126.4±3	0.91
H/L-HA hybrid complexes	18.1±1.1	0.64	3470±200	0.16	4.8 ±0.3	0.68	91.4±2	0.66

(Sigma-Aldrich, Milan Italy). Fluorescence images were captured using fluorescence microscopy system (Nikon, Tokyo Japan).

Immunoblotting

Chondrocytes were lysed in RIPA buffer (Cell Signaling Technology). Protein concentration was determined using the Bradford method (13) and 60 µg of intracellular proteins were loaded and resolved using 8% SDS–PAGE. The separated proteins were then transferred to nitrocellulose membrane and blocked in 5% milk, Tris-buffered saline and 0.05% Tween-20. Primary antibody to detect COMP-2 (Abcam) was used at 1:250 dilutions. Immunoreactive bands were detected by chemiluminescence using corresponding horseradish peroxidase-conjugated secondary antibody (Santacruz Biotechnology; 1:5000 dilutions) and reacted with an ECL system (Chemicon-Millipore). Protein levels were normalized with respect to the signal obtained with anti actin polyclonal antibody (Santacruz Biotechnology; 1:500 dilutions). The semi-quantitative analysis of protein levels was carried out by the Gel Doc 2000 UV System and the Gel Doc EZ Imager, using quantity one software (Bio-Rad Laboratories).

Statistical analysis

All experiments were performed in triplicate. Student *t*-test was used for statistical analyses. The level of significance was set at $p<0.05$.

RESULTS

Chondrocytes proliferation assay

Time Lapse Video Microscopy was exploited for cell growth curves in presence of H-HA, L-HA and H/L-HA. Chondrocytes proliferation (Fig. 1), reported that both H-HA and L-HA promoted proliferation compared to the untreated cells, however H/L-HA exerted a superior effect. In fact, at 24 h, H-HA and L-HA treated wells presented a cell number increase compared to CTR, respectively of 30% and 18%, while hybrid complexes showed a cell density increase of up to 50% ($p<0.05$). The average doubling time of chondrocytes was of approximately 72±7 h for CTR, 44.5±7h for H-HA, 63±5h for L-HA and only 35.6±4h for H/L-HA.

OA in vitro model: TNF-α and IL-6 expression on IL-1β treated human primary chondrocytes

Gene expression analysis was performed using quantitative real-time PCR. TNF-α and IL-6 (Fig. 2) were analyzed on chondrocytes pre-treated with IL-1β for 2h to resemble OA damage (10). Successively, hyaluronic acid was added to wells (H-HA, L-HA and H/L-HA 0.16% w/v) for 24 h. Our results showed that the treatment with IL-1β was very effective in increasing inflammation activating to the TNF-α and IL-6 expression 13- and 40-fold respectively.

Interestingly, in presence of H-HA, L-HA and H/L-

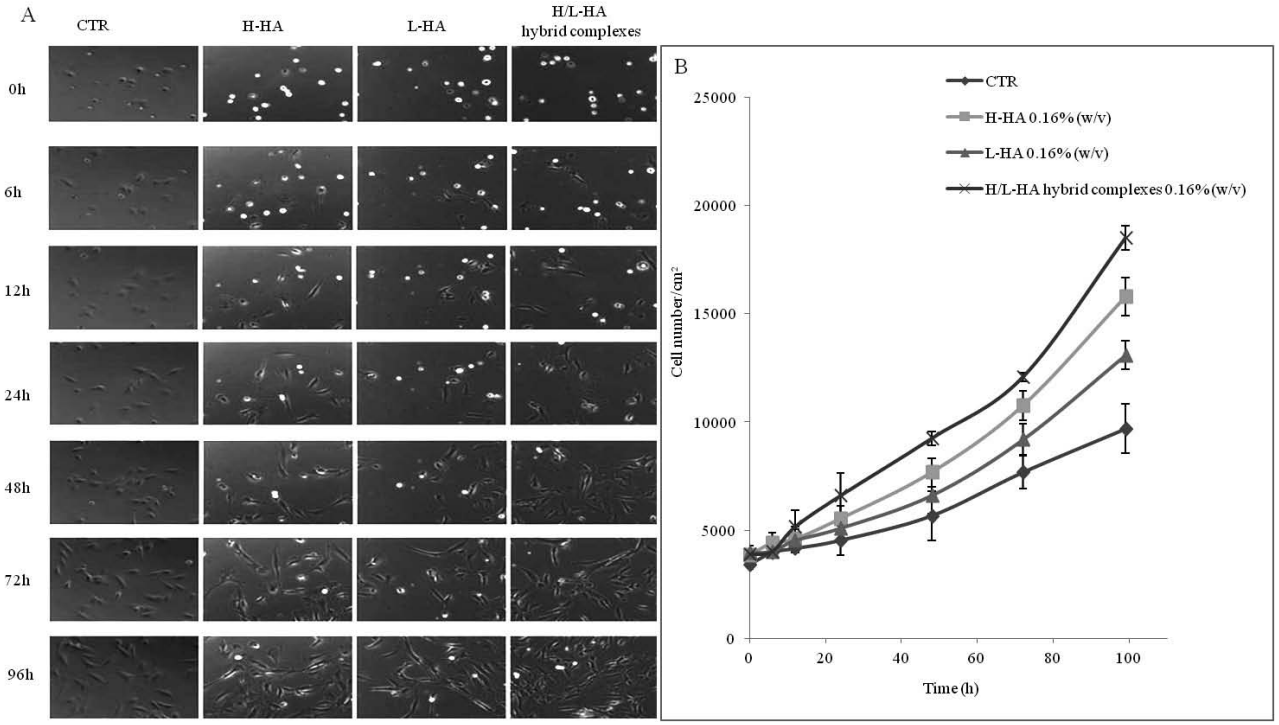

Fig. 1. *Human chondrocytes proliferation assay. **A**): Representative micrographs pictures relative to time lapse experiments at 0, 6, 12, 24, 48 ,72 and 96 h of cell incubation with H-HA (1400kDa), L-HA (100kDa) and H/L-HA hybrid complexes, compared to un- treated cells (control); **B**): Cells growth curves were obtained counting, four field of view of three different wells for each treatment. Proliferation experiments were repeated three times. Data shown are means±SD.*

HA the TNF-α expression was reduced by 2, 8, 5.2 and 9-fold compared to IL-1β (positive control) ($p<0.05$). Similarly, IL-6 expression levels was reduced ($p<0.05$) in the presence of H-HA or L-HA of 3.3- and 3.0-times, while an outstanding decrease of 15-fold was recorded when using H/L-HA (Fig. 2).

Bio-Plex cytokines assay

To evaluate how the diverse HA forms affect cytokine production, bioplex assay (Fig. 3) was performed to measure the cytokine levels (pg/mL) on treated chondrocytes. Among the 8 cytokines evaluated, only IL- 4, IL-6, IFN-γ and TNF-α were statistically different compared to the positive control (IL-1β). The results proved that H-HA, L-HA and H/L-HA significantly reduced the inflammatory effect obtained through IL-1β treatment ($p<0.05$). In particular, TNF-α was reduced by 36% compared to positive control (Fig. 3) only when the cells are treated with H/L-HA. IL-6 decreased by 45% for H-HA, by 48% for L-HA and by 85% for H/L-HA respectively; IFNγ was reduced by 15% and by 34% for L-HA and H/L-HA, respectively. A similar trend was found for IL-4 outcomes (17% and 34% of reduction, respectively).

Cartilage Oligomeric Matrix Protein-2 (COMP-2): protein evaluation of IL-1β treated samples with addition of diverse hyaluronans

a) immunofluorescence imaging

For immunofluorescence staining, the different hyaluronan treatments were added and cells were incubated for 7 days to evaluate cartilage oligomeric matrix protein (COMP-2) expression.

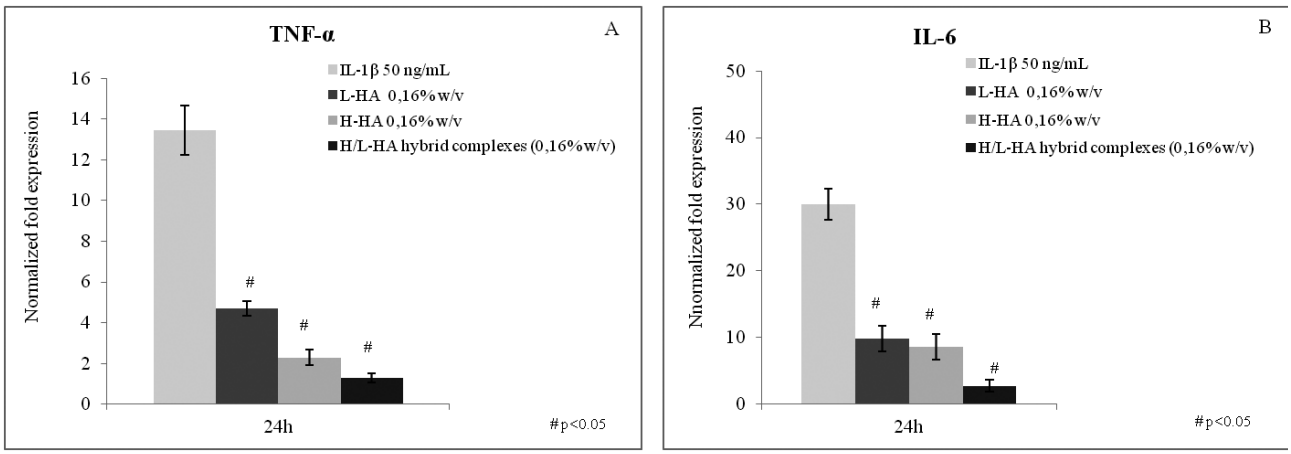

Fig. 2. *Osteoarthritis in vitro model (IL-1β treated human chondrocytes). TNF-α and IL-6 fold expression values analyzed by quantitative real time PCR (qRT-PCR) on primary chondrocytes, pre-treated with IL-1β 50ng/mL and following treated with H-HA, L-HA and H/L-HA, compared to control after 24 h. H/L-HA significantly reduced TNF-α and IL-6 gene expression. Data are reported as mean values±S.D. of three independent experiments. Significance of differences was tested using Student's t-test and is indicated. #: p<0.05.*

The localization of COMP-2 was analyzed through immunofluorescence staining. The images clearly showed an increase of the fluorescence for IL-1β treatment that correspond to an increase of COMP-2 expression and normally occur during cartilage damage. Instead, as reported in figure 4, in presence of H-HA, L-HA and H/L-HA, COMP-2 expression was significantly reduced compared to the positive control.

b) Western blotting analysis

Western blotting analysis relative to COMP-2 after 7 days of treatment was reported in Fig. 5. The results revealed a significant increase in COMP-2 protein level in the IL-1β treated chondrocytes sample, compared to untreated cells, as expected. Besides, the level of COMP-2 significantly reduced with hyaluronan, but it was clearly lower in presence of H/L-HA (densitometry analysis obtained by respect to Actin housekeeping protein). In particular IL-1β treatment increased expression by 2.6-fold compared to CTR and a reduction of 15%, 52% and 61% occurred in presence of H-HA, L-HA and H/L-HA respectively when compared to the positive control.

DISCUSSION

Primary human chondrocytes were treated with H-HA (1400kDa) or L-HA (100KDa) and with the H/L-HA obtained through patented NAHYCO™ technology, the latter showed a faster growth rate (2-fold) compared to control. In our experimental research, we used an *in vitro* model based on IL-1β treated chondrocytes (12, 14) in order to mimic osteoarthritis and thus assess the anti-inflammatory effects of the diverse hyaluronan formulations. In fact, there is a scientific and clinical agreement that osteoarthritis (OA) is a degenerative joint disease, associated with aging and inflammation, leading to pain and increasing disability (15). It was demonstrated that in many cases intra-articular injections of HA could be a valuable alternative to analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) (16). OA also occurs with reduced concentration and molecular weight of HA in the synovial and modification of permeability. In this respect, viscosupplementation may permit to restore the rheological (viscoelastics) properties of synovial fluid (9, 16, 17).

Intra-articular treatments with HA (un-cross linked and cross-linked) were used to manage symptoms and as alternative to surgical procedures (18, 19). However, un-crosslinked HA is quickly degraded by digestive enzymes and free radicals compared to crosslinked HA, but the last may induce a cytotoxic

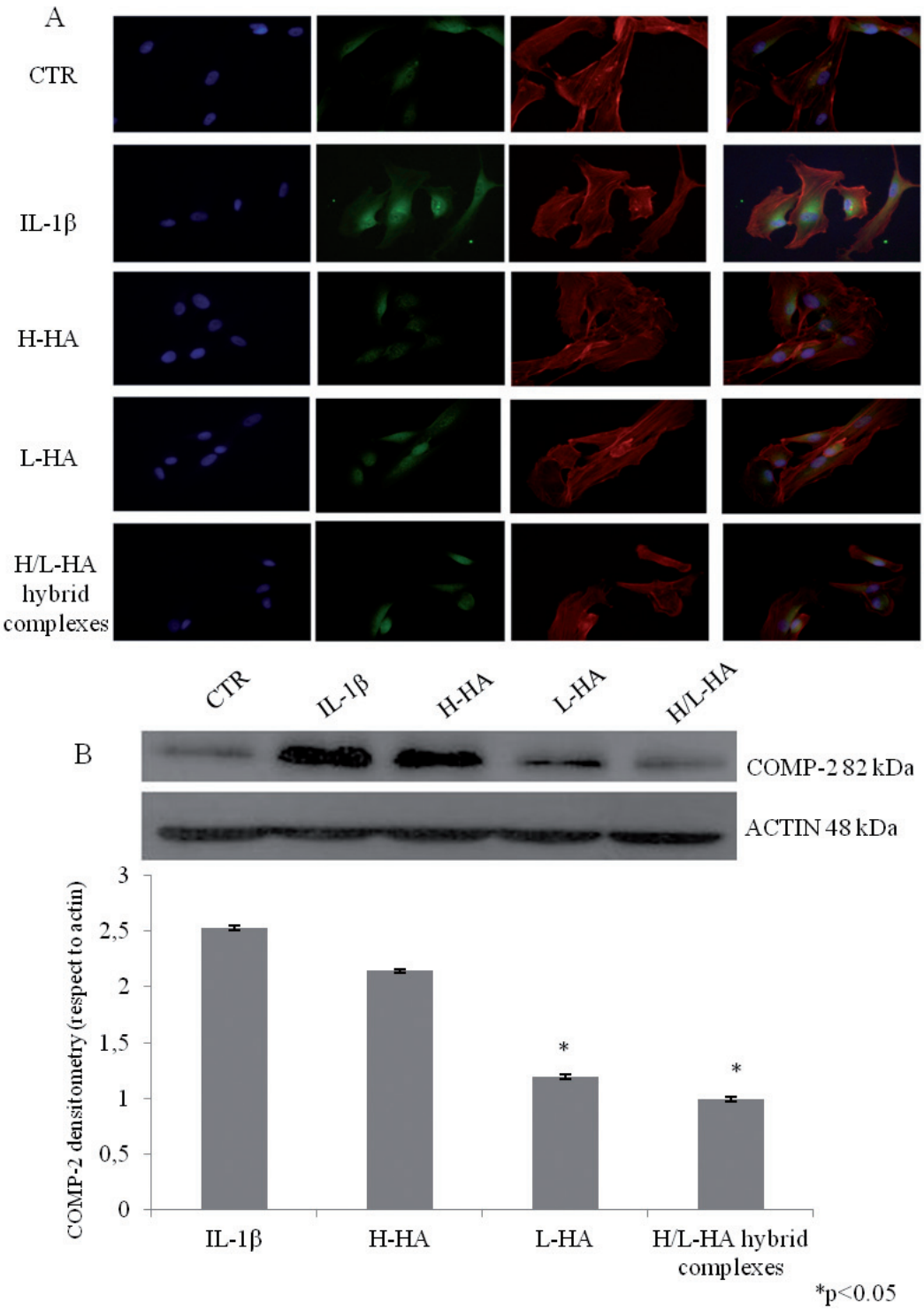

Fig. 3. A): *Immunofluorescence staining. Cytoplasmatic distribution of COMP-2 (green) in human primary chondrocytes treated with H-HA, L-HA and H/L-HA, was determined by immunofluorescnce staining as described above. DAPI and phalloidin were used to stain nuclei and cytoskeleton respectively;* ***B):*** *Western blotting analysis of COMP-2 and actin protein levels in chondrocytes treated with H-HA, L-HA and H/L-HA after 7 days of treatment; Densitometric analysis of the intensities of specific bands relative to the protein of interest, are measured using commercially available software (Image J software). The histogram reported the mean of three different experiments performed in duplicate ($p<0.05$).*

response related to non-natural chemical structures introduced for the HA chains crosslinking (20). In this respect, the H/L-HA, represent a new medical concept (10) that could be applied for osteoarthritis/cartilage repair applications. In the framework of this research, we have demonstrated that both H-HA and L-HA and above all, H/L-HA, reduced inflammatory levels on IL-1β treated chondrocytes. However, several studies focused the attention on the profile of systemic and local inflammatory molecules in OA patients that have serum, synovial fluids and synovial membranes highly enriched with plasma proteins, complement components and cytokines (21, 22). Furthermore, TNF-α IL-6 and IL-1β are increased in synovial fluids of patients affected by joint tissues injuries and may induce the production of degrading extracellular matrix enzymes (23). Hence, in our study, TNF-α and IL-6, were evaluated as key biomarkers and found significantly reduced at transcriptional level in presence of HA. These results were confirmed by a significant reduction of pro-inflammatory cytokines release measured in multiplex array. COMP-2 is a recognized clinical biomarkers of cartilage degeneration and osteoarthritis (24). In our *in vitro* model, COMP-2 expression was higher when the chondrocytes were treated with only IL-1β, confirming that an inflammatory response was elicited, but after seven days of treatment, its expression was significantly reduced as highlighted by immunofluorescence and western blotting evaluations.

Overall, the results obtained showed that the GAGs tested on human primary chondrocytes cultures, reduced the inflammatory process, particularly we underlined a better performance of the H/L-HA respect to H-HA and L-HA single treatments. By reducing the localized inflammatory state, patients have reduced pain. In addition motion will be improved for a prolonged period as H/L-HA is degraded more slowly than HA (25) thus performing their mechanical and viscoelastic function replacement/substitution in the synovia.

CONCLUSIONS

In vitro studies are powerful tools to obtain a comparative evaluation of pharmaceutical preparations and medical devices. When the primary cells are contemporarily treated following scientifically sound protocols, they could generate a set of data that are consistent with the translational concept.

In this study, a full array of biochemical outcomes may then support a potential regeneration of cartilage itself. It is well know that cartilage does not have self-healing properties, however the findings of this study with increased proliferation rate and the reduction of COMP-2 expression, may concur to a more physiological and functional state of the joint itself.

ACKNOWLEDGEMENTS

This research work was funded by MIUR PONPE_60_00007. SinovialH/L was a gift of IBSA Italia, and H-HA and L-HA were offered from Altergon Italia. The authors acknowledge Dr Anna Virginia Adriana Pirozzi for helping in western blotting analyses.

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COMPARING HIBRYD HYALURONIC ACID WITH PRP IN END CAREER ATHLETES WITH DEGENERATIVE CARTILAGE LESIONS OF THE KNEE

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Cartilage lesions are very common causes of chronic knee pain in athletes. Current treatment options consist in conservative strategies, such as viscosupplementation and platelet-rich plasma injections. This randomized controlled trial aims to investigate the effect of intra-articular Hybrid Hyaluronic Acid injections compared to PRP for the treatment of cartilage lesions among athletes at the end of their career. Since March 2015, 48 professional soccer players were randomized into two groups: 24 patients received 3 injections of HHA and 23 patients received 3 intra-articular injections of PRP. All patients achieved a statistically significant clinical improvement from preoperative to postoperative time in both groups. Patients in the HHA group showed a significant superiority compared to PRP group at 3 and 6 months. Intergroup differences decrease gradually until loss of significance at 12 months follow-up. Athletes with chronic degenerative cartilage lesions of the knee responded positively both to HHA and PRP until last follow up.

Osteoarthritis (OA) is the most common joint disease and one of the most common causes of disability. Indeed, patients with OA have a low quality of life that leads to an important loss of productivity. In literature, OA is recognized as a metabolically active and dynamic process, which involves the degeneration of cartilage and all joint tissues (1). Articular cartilage of athletes is continually solicited during their physical activity; this process may lead to its premature degeneration with subsequent exposure of subchondral bone particularly rich in nociceptive receptors. Cartilage damage also leads to the release of a number of detrimental factors for the joint environment resulting in a progressive breakdown of the articular surface (2).

Metabolic and biochemical changes related to sport activity are similar to the changes described in early stages of OA and contribute to the progressive joint degeneration that occurs in end career athletes (3). The high demands on the joint, proper of impact athletes, lead to a high incidence of cartilage degeneration in asymptomatic athletes. Joint pathologies, such as meniscal tear, ACL o PCL injury or valgus-varus axis deviation, can cause symptoms and lead to a rapid progression of cartilage injury (4). Among conservative strategies, the HA viscosupplementation is currently widespread in clinical practice, with good results reported in several studies (2, 5).

HA is a highly viscous polysaccharide physiologically present in the extracellular matrix.

Key words: hybrid hyaluronic acid, platelet rich plasma, end career athletes, cartilage lesion, knee

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0393-974X (2016)

Specifically located in the synovial fluid at a concentration of about 2.5-4.0 mg/ml. In pathological conditions, a lowering of both the concentration (which decreases to 1-2 mg/ml) and the molecular weight occurs; this leads to a reduced elasticity and viscosity of the synovial fluid, which causes an increase in friction between the articular surfaces (6). Intra-articular HA injections (or viscosupplementation) are universally accepted for the treatment of pain associated with OA.

The purpose of viscosupplementation is to reduce pain and restore the visco-elastic properties of the synovial fluid (7, 8, 9). In addition to the mechanical function described, the intra-articular HA also plays a biological role of chondroprotection that reduces disability and improves the function of the affected joint (10) . The Hybrid Hyaluronic Acid (HHA) (Sinovial HL; IBSA) has been proved to have a superior capacity of stimulating ECM production *in vitro*, due to the presence of a low molecular weight HA fraction. (11). PRP (Platelet Rich Plasma - Platelet Rich Plasma) has recently gained great importance as an alternative treatment for musculoskeletal disorders. It is defined as a volume of autologous plasma containing a concentration of about 1,000,000 platelets/ul in 5 ml of plasma and is obtained by centrifugation of a sample of human autologous whole blood anticoagulated, from which the part of the plasma containing a high concentration of platelets is extracted (12).

According to the technique used for the production, PRP may contain, in addition to the high concentration of platelets, variable amounts of plasma, red blood cells (RBC), white blood cells (WBC) and platelets (PLT) (13). The effects of PRP are mainly attributed to the presence of platelets that, once activated, release growth factors that have healing properties (13, 14, 15). In this randomized controlled trial, we compared the effect of hybrid hyaluronic acid (HHA) to platelet-rich plasma (PRP) injections for treatment of cartilage lesions among athletes at the end of their career.

MATERIALS AND METHODS

This study was performed at the Department of Orthopedic and Trauma Surgery and the Immunohematology Transfusion Medicine Unit of Campus Bio-Medico University Hospital. The Local Ethics Committee approved the study and all participants provided written informed consent prior to enrollment. The lesions were classified according to the Kellgren-Lawrence radiographic system (1, 2) and only patients with symptomatic unilateral pain condition, unresponsive to non-injective conservative therapies (rehabilitation, cryotherapy, rest, NSAIDs) and without previous surgery on the knee were included. Patients presenting excessive axial deformity (>5°), knee instability and osteochondral lesion were not included in the study. Hematologic and general health status exclusion criteria were also applied (Table I). Since March 2015, 48 professional soccer players with clinical and radiographic evidence

Table I. *Contraindications to the use of PRP.*

Absolute contraindications	**Relative Contraindications**
Platelet dysfunction syndrome	Consistent use of NSAID's within 48 h of procedure
Critical thrombocytopenia	Local or systemic use of corticosteroids within 2 weeks of procedure
Hypofibrinogenemia	Recent fever or illness
Hemodynamic instability	Cancer- especially haematopoietic or of bone
Septicaemia	Active history or history of *Pseudomonas, Enterococcus* or *Klebsiella infection*
	HGB<10 g/dl
	Platelet count less than 105/

of degenerative changes to the knee were enrolled in the study (Fig. 1). Patients were randomized into 2 groups: 24 patients (HHA Group) received 3 injections of HHA (3.2%, 64mg/2ml, 32 mg High-MW 1100-1400 kDa+32 mg Low-MW 80-100 kDa; IBSA) and 24 patients (PRP Group) received 3 intra-articular injections of 5.5 mL PRP.

Hyaluronic acid

HHA (Sinovial HL, 3.2%, 64mg/2ml, IBSA) has been used for this study. It is composed of 32 mg of high-molecular weight (1100-1400 kDa) hyaluronan and 32 mg of low-molecular weight (80-100 kDa) hyaluronan. Its rheological and biological behavior has been extensively evaluated (11).

PRP production

Eight milliliters of the patients' whole blood was collected in one RegenLab THT tubes® containing a special gel. The tube was centrifuged at 3100 rpm for 9 min. After centrifugation, the gel separated red blood cells from PRP. After shaking, the PRP was aspirated with an 18-gauge needle and directly infiltrated. Details of the content of each treatment session (especially with regard to postoperative injections) and any adverse effects were reported on standardized forms and given to the medical assistant. All co-interventions during the follow-up period were discouraged, but the prescription of pain medication if necessary, was allowed.

Clinical assessment

International Knee Documentation Committee (IKDC), Knee injury and Osteoarthritis Outcome Score (KOOS) and Visual Analogue Scale (VAS) were administered to all patients before starting the treatment (baseline), at 3, 6 and 12 months from the end of the management. A different and independent investigator, unaware of the previous measurement to ensure a blinded status, recorded each measurement.

Injection procedure

An orthopaedic surgeon in the outpatient clinic injected the product. Knee injection was performed with lateral approach. The upper pole of dorsal margin of the patella and the femoral condyle were considered as landmarks to identify the point of least resistance. After the sterile dressing of the skin with a 22-g parallel needle to the surface of the bed and tilted toward the articular surface of the patella, operator penetrates through the skin and releases the product after lateral subluxation of patella. Immediately after the injection, the affected knee underwent passive flexion and extension for 3 times, followed by 10 min of resting in supine position.

Statistical analysis

Statistical analyses were blinded. The analyses were performed using SPSS version 16.0.1 (SPSS Inc., Chicago, Illinois). Primary end point was the postoperative difference in the IKDC and KOOS scores and VAS scale compared to baseline. Paired *t*-test was used to perform this analysis and unpaired *t*-test to compare the postoperative results between the 2 groups. A P value of 0.05 was set as a threshold for statistical significance. Data were presented with average value and standard deviation (SD).

RESULTS

Demographic and basal characteristics

From the initial population study, 47 patients (all men) were analyzed with one patient lost at follow up. The median age was 37.2 years (range 34-39). No statistically significant differences in terms of sex, age, and lesion degree and baseline clinical outcome value (IKDC, KOOS and VAS) between HA and PRP group after randomization were found. No adverse injection-related events during the treatment or follow-up period were observed. One patient treated with PRP was lost at follow-up.

Clinical and pain outcomes

A statistically significant clinical improvement from baseline to follow-up was recorded in both groups. Statistical intragroup significance ($P<0.05$), was obtained since first follow-up set point and maintained until 12 months when compared to baseline. IKDC increased from 38.8±3.9 to 59.8±5.8 at 12 months follow up for HHA group and from 39.8±1.2 to 57.3±7.6 at 12 months follow up for PRP group. KOOS showed an improvement from 43.4±1.9 to 52.8±3.2 at 12 months follow up for HHA group and from 43.1±1.2 to 51.9±2.4 at 12 months follow up for PRP group. VAS ranged from a baseline score of 7.5±1.1 to a final value of 3.2±0.7

Table II. *IKDC*

		Av	SD	P Intergroup difference
IKDC baseline	HHA	38.8	3.9	.286
	PRP	39.8	1.2	
IKDC 3 months	HHA	53.1	1.9	.001
	PRP	51.4	1.4	
IKDC 6 months	HHA	60.4	6.3	.018
	PRP	56.3	7.8	
IKDC 12 months	HHA	59.8	5.8	.125
	PRP	57.3	7.6	

Table III. *KOOS*

		Av	SD	P Intergroup difference
KOOS baseline	HHA	43.4	1.9	.579
	PRP	43.1	1.2	
KOOS 3 months	HHA	52.1	1.9	.002
	PRP	50.1	1.4	
KOOS 6 months	HHA	53.1	2.1	.006
	PRP	51.7	2.2	
KOOS 12 months	HHA	52.8	3.2	.099
	PRP	51.9	2.4	

Table IV. *VAS*

		Av	SD	P Intergroup difference
VAS baseline	HHA	7.5	1.1	.841
	PRP	7.4	1.2	
VAS 3 months	HHA	3.8	1.1	.000
	PRP	5.0	1.4	
VAS 6 months	HHA	2.9	0.8	.043
	PRP	3.3	0.9	
VAS 12 months	HHA	3.2	0.7	.570
	PRP	3.4	0.8	

at 12 months in HHA group and from baseline score of 7.4±1.2 to a final follow up of 3.4±0.8 in PRP cohort. Three months intergroup analysis, showed a statistical significant superiority in terms of IKDC (0.001), KOOS (0.002) and VAS (0.000) scale for patients treated with HHA compared to PRP group. Significant intergroup difference was maintained at 6 months follow-up for IKDC (0.018), KOOS (0.006) and VAS (0.043). Last follow-up analysis showed a loss of intergroup significance for both clinical score and pain scale (IKDC 0.125, KOOS 0.099, VAS 0.570). (Table II, III and IV).

DISCUSSION

The authors conducted a prospective controlled trial to investigate the efficacy of HA viscosupplementation compared to PRP injections in an athletic population. It is well demonstrated that professional soccer players are particularly susceptible to articular cartilage damage and early development of OA because of the repetitive high-level impact, stress and trauma due to running, jumping, acceleration, kicking and direction changing (16) . Therefore, athletes, at the end of their career configure a relatively young, high demanding patient that practices high-level sport, yet complains from pain and functional impairment of the knee.

This prospective controlled trial shows an improvement in all clinical scores tested from the basal evaluation at follow-up of 3, 6 and 12 months, in both athletes treated with HHA and PRP injections. However, at intergroup analysis, HHA group showed a better outcome only at 3 and 6 months follow-up compared to PRP. The selection criteria were established based on similar studies. Only one patient was lost from PRP group once the study was underway, which represented a significantly lower loss rate than in previous studies (17, 34).

Injection therapies with PRP for knee OA aroused great interest over the last years, leading to a large production of studies, although to the best of our knowledge there are no high-level evidence reports based on sports population. Recent studies have shown that PRP is as effective as HA in the treatment of knee OA, showing more effectiveness for patients with early stages of OA, being potentially safe and efficient in improving function and reducing pain (18). Sanchez et al (19) showed that patients who underwent PRP injection had a better reduction of pain compared to those treated with HA injection at six months follow-up. Similarly, Vaquerizo et al (20) compared 3 injections of PRGF-Endoret (BTI Biotechnology Institute, Vitoria, Spain) versus one single intra-articular injection of Durolane hyaluronic acid (HA), showing a significant clinical improvement in patients treated with platelets concentrate, with pain reduction and stiffness improvement at 24 and 48 weeks follow-up. Cerza et al (21) conducted a randomized controlled trial on 120 patients affected by knee OA treated with autologous conditioned plasma (ACP) or HA in the short term follow up (2, 12 and 24 weeks). They showed significant outcomes in the group treated with platelets concentrates even in grade 3 OA, unlike the previously published literature (22). Patel et al (23) carried out a randomized trial comparing PRP versus saline solution in 78 patients affected by bilateral knee OA.

This study showed better clinical outcomes in PRP group compared to placebo group. In contrast with the reported outcomes, a recent randomized controlled trial was performed by Filardo et al. (22) on a total of 192 patients treated with 3-weekly intra-articular injection of PRP or HA and followed up at 6 and 12 months. However, the clinical outcomes did not report a significant difference between the two treatments. In the present study HHA group showed significantly higher scores at 3 and 6 months after injections. This may be due to several reasons. Indeed, some authors have reported that patients treated with PRP experienced improvement of clinical scores at six months from treatment (17).

The HHA featuring both the low and high MW HA may show the benefits of both components having an anti-inflammatory, antiapoptotic, analgesic and biomechanical function at the same time. Indeed, it has been proven that the therapeutic effectiveness of HA is not only related to its biomechanical properties, whose usage is to increase the viscosity of the synovial fluid, but is also associated to biological features that may explain the observation

that clinical results exceed the life span of the HA exogenously administered into the joint (24). Indeed, the HA has important cellular effects such as the inhibition of prostaglandin E2 (PGE2) and nitric oxide (NO) synthesis which is induced by interleukin-1 (IL-1), protection against proteoglycan depletion, protection against cytotoxicity induced by oxygen-derived free radicals and apoptosis induced by NO and Fas stimulation, modulation of leukocyte adherence, proliferation, migration and phagocytosis and suppression of cartilage matrix degradation by fibronectin fragments (24, 25).

All of these features are strictly related to the MW of HA, which plays a crucial role in the biological effects on articular chondrocytes. In particular, the low MW HA has an anti-inflammatory and antiapoptotic activity while the high MW HA is the key player in maintaining viscoelastic properties and analgesia. The rheological and biological properties of HHA with and without PRP have been extensively evaluated.

The HHA has proven to stimulate ECM production, possibly due to the presence of the low MW HA fraction. These effects, together with its viscoelastic properties, may translate in better clinical outcomes. This is the first clinical study that evaluates the HHA on a homogenous, strictly selected and athletic population. Moreover, it compares the clinical effects of HHA to PRP instead of using a placebo as control group. However, a higher number of patients enrolled in the study are still needed and the evaluation of the optimal number and interval of injections need to be assessed.

CONCLUSION

In this RCT both treatments showed to be effective in relieving patients' symptoms. HHA showed higher effectiveness in terms of pain reduction and function compared to PRP, although its superiority was limited by time. Nevertheless, considering athletes, yet at the end of their career as a peculiar patient population looking for fast and effective treatments, HHA seems to fit their expectation better than PRP.

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COMPARISON OF THREE NOVEL BIPHASIC SCAFFOLDS FOR ONE-STAGE TREATMENT OF OSTEOCHONDRAL DEFECTS IN A SHEEP MODEL

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In the last years, several tissue engineering techniques have been applied to develop different kinds of osteochondral substitutes to overcome the scarce reparative properties of this tissue. The aim of this study was to generate and compare three biphasic scaffolds in an osteochondral lesion in a large-animal model. A critical osteochondral defect was generated in the medial femoral condyle of 18 skeletally mature sheep. Three defects were left untreated, the remaining lesions were divided into three groups: 5 lesions were treated with a biphasic scaffold made of collagen type I and small cylinders of Magnesium Hydroxyapatite; 5 lesions were treated with a biphasic substituted formed by collagen type I and Wollastonite, 5 lesions were treated with a scaffold made of collagen type I and small cylinders of Wollastonite/Hydroxyapatite. Animals were sacrificed after 3 months and samples were analyzed by CT and MRI, macroscopic evaluation and histology. Our study demonstrated that one of these novel biphasic scaffolds possesses the potential for being applied for one-stage procedures for osteochondral defects.

In the synovial joint, articular cartilage and subchondral bone form a load-bearing system that provides a large range of joint motion with excellent lubrication, stability and uniform distribution of high acting loads (1). Articular cartilage and subchondral bone frequently undergoes degeneration as a result of traumas or disease (2). The osteochondral tissue has a poor healing potential; thus great debate persists about the best available treatment for osteochondral defects. Current surgical procedures (i.e. microfractures or mosaicplasty) lead to the formation of a fibrocartilaginous tissue, which does not exhibit the wear characteristics of native hyaline cartilage (3). Recently, the application of the tissue engineering approaches for the repair of osteochondral defects has received an increasing

Key words: cartilage, osteochondral lesion, scaffold, tissue engineering, animal models

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0393-974X (2016)

interest (4-6). Because of the extremely different nature of cartilage and bone, when a mono-phasic scaffold is used, the natural environment is not well duplicated and the new tissue is not properly formed (5, 7). To overcome this issue, modern approaches focus on the design and development of scaffolds combining distinct layers mimicking the natural cartilage and bone tissues. In literature, several bi- or three-layered osteochondral scaffolds made of different materials are described (8-11). The soft part, mimicking the cartilage, is usually collagen while the bottom part, mimicking the subchondral bone, is prevalently Hydroxyapatite (HA) (12, 13). Some studies reported results on bi-layered in which, instead of the collagen, chitosan (14), PLGA (15), PVA (16) were used.

In our previous work, we have tested a new three-dimensional biphasic scaffold in osteochondral lesions in pigs. The results showed promising outcomes as the biphasic scaffold well integrated with the native tissue and it promoted the formation of a fibrocartilaginous tissue, which filled the defect (11). Subsequently, we decided to improve the biomechanical and morphological characteristics of the scaffold by changing its architecture. Thus, in this new project, we tested and we compared three biphasic scaffolds with a slight different structure in sheep. Two substitutes were fabricated combining a layer of Collagen type I for the cartilaginous phase and small columns of Hydroxyapatite or Hydroxyapatite/Wollastonite for the osseous phase; the third scaffold was developed as previously described (11), that is, a layer of collagen type I and a small cylinder of Hydroxyapatite/Wollastonite. Our results showed the potential reparative capacities of one of these novel osteochondral substitutes in a large-animal model.

MATERIAL AND METHODS

Fabrication of the three biphasic scaffolds

Two different scaffold configurations, both including a collagen and a bioceramic part, have been designed, Bi-layer (BWS) and Honey (in the two versions HWS and HMG), where the name Honey was chosen as this architecture resembles the configuration of a beehive. The BWS substitute was fabricated as described in our previous work (11). For the Honey configuration, four small bioceramic pillars, inserted in four slots of a cylindrical collagen scaffold, replaced the cylindrical bioceramic part of Bi-layer. Briefly, the collagen type I slurry was synthetized as previously described (11) and it was then poured in specific moulds with a honeycomb architecture generated by 3D printing (Fig. 1). The inorganic component, both for the Bi-layer and for the Honey configurations, was synthesized by the foam replication method (17) and composed of Mg doped Hydroxyapatite (Mg-HA, HMG group) or Wollastonite/Hydroxyapatite (WS/HA, HWS group) mixtures. Mg-HA was prepared substituting a 10% molar of calcium ions, keeping the (Ca+Mg)/P=1.67. WS/HA scaffold was prepared by sintering WS/HA mixtures powders in a 1/1 ratio. The bioceramic pillars were subsequently embedded in the collagen type I layer to form the biphasic scaffold. All biphasic substitutes were sterilized in oven under vacuum at 160°C for 2-4 h before implantation.

Animal study

Eighteen sheep (100 kg of body weight) were anesthetized as previously described (11), and placed in the supine position. A longitudinal paramedian incision was made in the medial aspect of the right knee. Subsequently, the articular capsule was opened and a critical osteochondral defect was generated in the medial femoral condyle. In particular, the size of the lesion was 11 mm in diameter and 9 mm in depth and it was created with custom-made instruments.

Three defects were left untreated (CTRL), whereas the remaining 15 lesions were divided into 3 groups: 5 lesions were treated with a biphasic scaffold made of collagen type I for the cartilaginous phase and small columns of Magnesium Hydroxyapatite embedded in collagen type I for the osseous component (HMG); 5 lesions were treated with a biphasic substitute formed by collagen type I and Wollastonite (BWS); 5 lesions were treated with a scaffold made of collagen type I and small columns of Wollastonite/Hydroxyapatite embedded in collagen type I (HWS). All scaffolds were implanted by using a press-fit technique. The capsule was sutured and the wound was then closed in layers in standard fashion. Postoperative care included pain control and prevention of infection using antibiotics. The animals were allowed

to stand and to walk freely.

12 weeks after surgery, animals were sacrificed under anesthesia with a lethal injection of KCl. The right knees were then harvested and further analyzed as described below.

Radiological evaluation

CT scans and MRI were performed on the harvested knees at the Department of Veterinary Medicine (DIMEVET), Ospedale Didattico Universitario Az. Polo Veterinario di Lodi, University of Milan. In particular, for the CT scans a GE BrightSpeed 16 slice CT machine was used. The acquisition protocol was performed as follows: helical mode, slice thickness 1,25 mm, 120 kVp, 200mA, pitch 0,93, convolution kernel "bone plus". For the MRI scans, aVet-MR, Esaote 0,18T was used with the following acquisition protocol: dual phased array coil for elbow study, TSE80, Repetition Time 2520 msec, Echo Time 80 msec, slice thickness 4 mm, gap 0.4 mm, matrix 256x256.

Macroscopic evaluation

The skin and the articular capsule were opened and macroscopic assessment of the knees was performed by using the International Cartilage Repair Society (ICRS) Macroscopic score, which evaluates these specific parameters: levelling, stiffness, integration, degree of repair and overall macroscopic aspect of the defects. Three different observers independently scored the samples.

Histological evaluation

The repaired tissue, together with healthy cartilage and bone, was manually removed by means of a saw and fixed in 10% phosphate-buffered Formaldehyde. It was then processed for paraffin embedding. Samples were dehydrated in a graded 50% (v/v), 70% (v/v), 95% (v/v) and 100% (v/v) ethanol series, embedded in paraffin and cut into 10-mm-thick sections. Subsequently, some slides were stained with Hematoxylin & Eosin following standard protocols to analyze cell morphology. Other sections were stained with Safranin O Fast Green to evaluate the presence and distribution of Glycosaminoglycans in the reparative tissue (10, 11).

Statistical analysis

For each experimental group, means were assessed

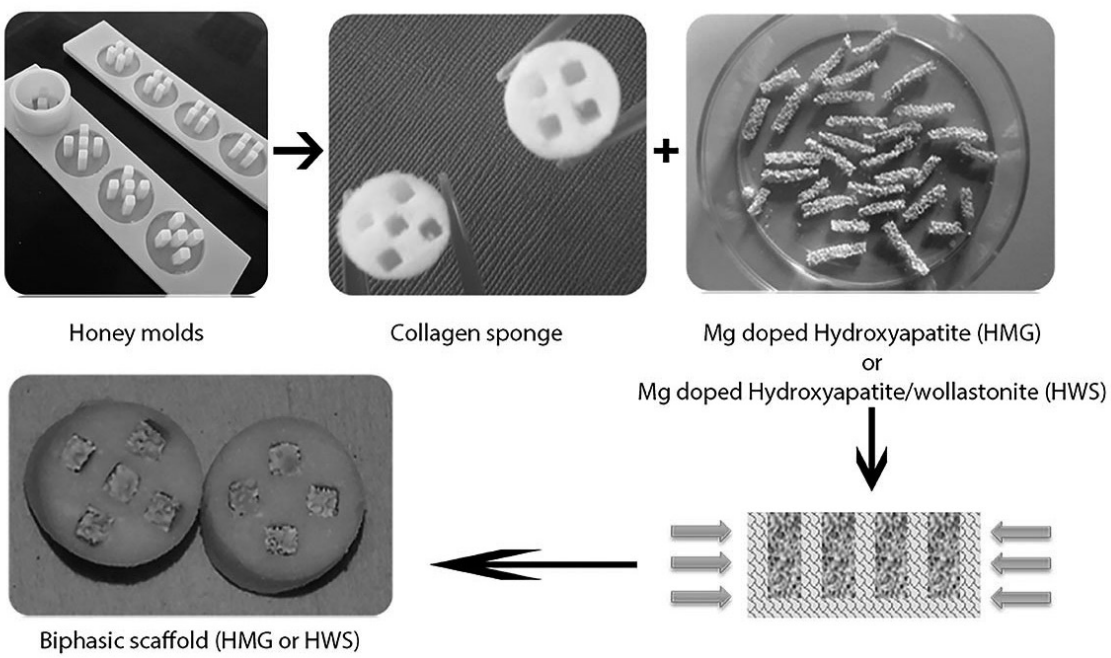

Fig. 1. *Fabrication of the biphasic scaffold (Honey configuration). Biphasic scaffold made of collagen type I for the cartilaginous phase using a specific mold and small columns of Magnesium hydroxyapatite or Magnesium Hydroxyapatite/ Wollastonite embedded in collagen type I for the osseous component (**HMG** and **HWS**, respectively). The Bi-layer biphasic substitute fabrication has been already described. (11).*

by descriptive statistics. Each experimental group was compared with the control (CTRL) group and standard Student's *t*-test was performed. Statistical significance was defined as a p-value of <0.05.

RESULTS

All animals survived the surgery and did not sustain any major post-surgical complication. After 3 months, animals were sacrificed and the right knees immediately underwent a radiological evaluation. CT scans showed the absence of any reparative process of the subchondral bone in the CTRL group (Fig. 2A).

In the other groups, the presence of the ceramic material (Hydroxyapatite or Hydroxyapatite/Wollastonite) was still evident, however the bony phase of the scaffolds apparently looked well integrated with the surrounding subchondral bone (Fig. 2A). MRI evaluation displayed a big osteochondral defect in the CTRL group on both the frontal and the sagittal planes (Fig. 2B). Conversely, the experimental groups were characterized by the presence of a reparative tissue; nevertheless, its quality was scarce especially in the BWS group (Fig. 2B). Moreover, the integration of the HWS scaffold with the surrounding tissue looked insufficient. On the other hand, the HMG group displayed a good reparative tissue filling the defect, but the complete restoration of the lesion was not yet achieved.

At the end of the radiological evaluation, the knee joints were opened and the overall status of the reparative tissue was evaluated. In the CTRL group, the defect was substantially unchanged: the surface of the lesion was indeed coated by a thin layer of fibrocartilaginous tissue. Macroscopically, all osteochondral scaffolds were still in place and no noticeable signs of synovitis were present. The level of filling was different among the experimental samples; however, all of them were characterized by the presence of a certain amount of reparative tissue. The ICRS macroscopic assessment showed a significant difference between the CTRL and the HMG group (Fig. 3Ae): in particular, few parameters (integration, degree of repair and the overall macroscopic analysis) were significantly higher in the HMG group. No significant difference was found between the CTRL and the BWS or the HWS groups. Of notice, the integration of these two latter scaffolds with the healthy tissue was much lower than the HMG group.

The samples were subsequently processed and sectioned for histological evaluation. H&E stain displayed the absence of a valid reparative tissue in the CTRL group. Safranin O stain confirmed these data, as no GAGs were detected in this group (Figure 3B). On the other hand, the experimental samples were characterized by the presence of a reparative tissue within the defects. In all three experimental groups, the ceramic material was still there, but it was well integrated with the healthy subchondral bone. The reparative cartilaginous layer was constituted mainly of elongated, fibrous-

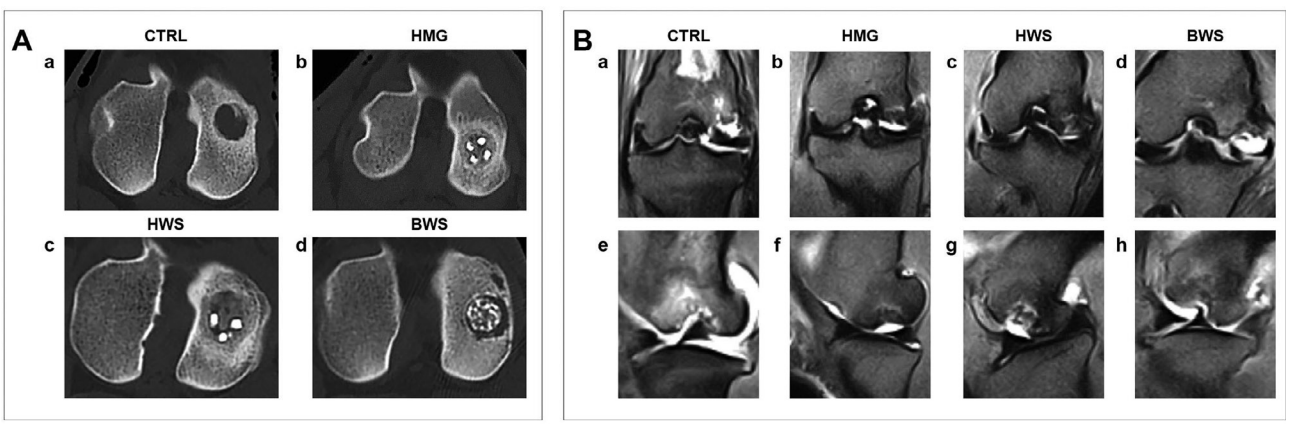

Fig. 2. *Radiological assessment of the three biphasic scaffolds.* ***A****): microCT evaluation of the osteochondral lesions at 3 months. A representative scan for each experimental group is shown: CTRL (**a**), HMG (**b**), HWS (**c**), BWS (**d**).*
B*). MRI evaluation of the osteochondral lesions at 3 months. Representative scans (High resolution Turbo Spin) for each experimental group are shown: CTRL (**a, e**), HMG (**b, f**), HWS (**c, g**), BWS (**d, h**).*

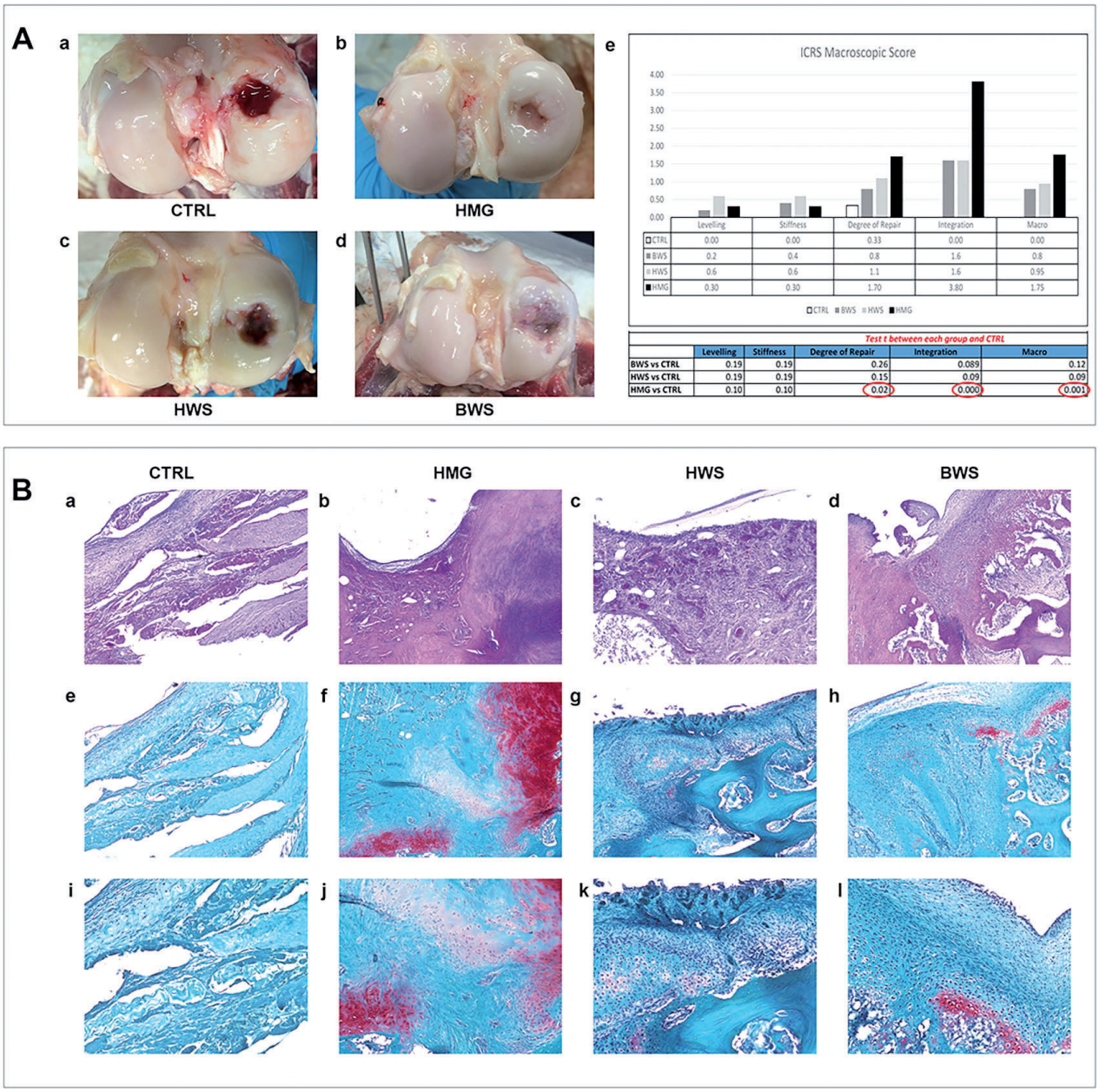

Fig. 3. *Macroscopic and microscopic assessment of the three biphasic scaffolds.* ***A****): Macroscopic pictures of the experimental groups; A representative lesion for each experimental group is shown. CNTRL: (****a****); HMG: (****b****); HWS: (****c****); BWS: (****d****). ICRS macroscopic assessment is shown in (****e****). The average values of the different parameters for each experimental group have been plotted in a graph. T-test values are reported below (p<0,05).* ***B****): Histological evaluation of the osteochondral lesions; Representative H&E (****a-d****) and Safranin O (****e-l****) pictures for each experimental group are shown: CTRL: (****a, e, i****); HMG: (****b, f, j****); HWS: (****c, g, k****); BWS (****d, h, l****); Magnification: 5X: (****a-h****); 10X: (****i-l****).*

like cells, especially in the superficial region in all sections. However, the Safranin O stain showed some important differences between the groups: in the HWS samples, almost no GAGs were detected, suggesting that the cells invading the defects did not undergo a chondrogenic differentiation.

In the BWS group, positive Safranin O stain was spotted in the transition layer between the cartilaginous and the bony phase. This result suggests that an endochondral bone reparative process was active in

these samples. Finally, the HMG group displayed the presence of GAGs both in the deep and in the superficial layer of most of the samples. Moreover, several spots in the HMG samples were characterized by the presence of round, chondrocyte-like cells.

DISCUSSION

In this study we analyzed three different, cell-free, biphasic scaffolds for the repair of osteochondral lesions. In our previous study (11), we compared a biphasic substitute seeded with chondrocytes and a cell-free scaffold in a swine orthotopic model. Surprisingly, we proved the superiority of the unseeded scaffold: cell-free samples were characterized by the presence of fewer cells but with a chondrocyte-like phenotype. However, the repair of the osteochondral lesions in this model was far from being completed. Thus, we decided to change the architecture of the biphasic scaffold in order to improve the reparative potential and the integration of the substitute with the surrounding healthy tissue.

In this new project, we compared three biphasic scaffolds with different design and composition: the aim was to improve the integration of the scaffold with the healthy tissue and to favor the acquirement of a chondrocytic phenotype of the migrated cells. It is known that the composition and the porosity of the material may indeed influence cell migration and differentiation (12, 13, 18). Thus, we tested two different scaffold configurations: the Bi-layer and the Honey. The Bi-layer design had already been used (11), however, in this new study we added Wollastonite to the ceramic composition, with the purpose of strengthening and improving the integration of the bony part with the subchondral bone. Conversely, the Honey design represents a new osteochondral scaffold configuration fabricated with the aim of increasing the scaffold radial compliance and reducing the amount of inorganic material.

Our preliminary data successfully proved the biocompatibility of all substitutes and they demonstrated the superiority of the construct made of small columns of Hydroxyapatite (HMG group). This scaffold allowed for a better integration and for a slightly better cartilaginous restoration. The other two experimental groups did not show promising results in terms of cellular migration and differentiation. The presence of a new ceramic material (Wollastonite) in the construct could probably interfere with cell differentiation.

Despite the better results achieved with the HMG scaffold, we are still far from obtaining a good cartilaginous tissue, as shown by histology. We can speculate different reasons for this scarce outcome. First, we generated a bigger osteochondral defect (11 mm x 9 mm) in order to avoid the occurrence of the natural reparative process, which may have confounded our results. In fact, this larger lesion might have inhibited the migration of the cells in the whole scaffold. Moreover, the increased distance between the two sides of the native cartilage might have interfered with the migration of growth and nutritive factors within the scaffold. Thus, the migrated cells might have lacked the stimuli required for cell differentiation.

Finally, it is possible that the age of the animal contributed to these unsatisfactory results: it is well known that chondrocytes undergo senescence and that they progressively lose their ability to proliferate with age (19, 20).

In conclusion, we have demonstrated the biocompatibility of three cell-free, biphasic scaffolds in an osteochondral defect. The novel "Honey" configuration of the scaffold with Hydroxyapatite allowed for a better reparative process. Nevertheless, we are still far from obtaining a complete restoration of the defect. Therefore, in the future we will try the HMG scaffold in smaller lesions. The addition of active molecules or growth factors will be considered in order to stimulate the migration of the endogenous reparative cells and to favor the reparative process, thus proving the potentials of this new cell-free, biphasic substitute for the repair of osteochondral defects.

ACKNOWLEDGEMENTS

This work was supported by the PON RINOVATIS grant (No.02_00563_3448479) from Ministero dell'Istruzione, della Ricerca e dell'Università (MIUR). IRCCS Istituto Ortopedico Galeazzi received "Ricerca Corrente" funds from

Ministero della Salute.

The authors gratefully acknowledge Prof. Antonio Crovace, Dr. Luca Lacitignola together with the students of the veterinary medical school and the PhD program in Tissue and Organ Transplantation and Cellular Therapy for their outstanding professional help and support in handling the different steps of the animal surgery.

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TRANEXAMIC ACID EFFECTS ON CARTILAGE AND SYNOVIAL TISSUE: AN *IN VITRO* STUDY FOR A POSSIBLE SAFE INTRA-ARTICULAR USE

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The possible toxic effects of intra-articular tranexamic acid (TA) are still debated. The aim of this study was to evaluate TA effects on human cartilage fragments and synovial biopsies. Explant culture of minced articular cartilage underwent prolonged TA exposure. Histological analysis, immunofluorescence and colorimetric assay for quantification of s-GAG and DNA were performed at the end term. Synoviocytes were cultured for 48h in presence of TA. Light microscopy and flow cytometry analysis were performed at the end of the exposure to TA and one week after the treatment. TA exposure did not influence i) the chondrocyte outgrowth and migration, ii) the expression of chondrogenic and proliferative markers and iii) the s-GAG/DNA ratio. TA treatment did not affect synoviocytes' morphology and treated cells were phenotypically similar to control cells. This study demonstrated that TA does not negatively affect chondrocytes and synoviocytes cultured in vitro. Thus, our findings may be clinically relevant in order to validate the intra-articular TA administration during orthopedic procedures.

Blood loss is a common event in orthopedic surgery and it is usually treated with blood transfusions. Tranexamic acid (TA) is an anti-fibrinolytic drug, which has been introduced to prevent excessive bleeding (1-3). The direct application of TA in the joint fluid has been described since the '70s and it was initially proposed for the treatment of joint bleedings in hemophiliac patients during intra-articular operations (4). Currently, TA is routinely used in joint replacements (5) and more recently, in ACL reconstruction (6).

TA is a synthetic analog of the amino acid lysine. TA prevents the formation of plasmin through the inhibition of the proteolytic activity of plasminogen activators. Furthermore, at high concentrations, it is also a noncompetitive inhibitor of plasmin. Thus, TA inhibits tissue fibrinolysis, it prolongs thrombin effects and it ultimately enhances and stabilizes the clot formation.

Intra-articular injection of TA could have different advantages: it would allow for an optimal concentration at the site of bleeding and it would reduce systemic absorption, thus, it would reduce any possible systemic side effects, such as vascular occlusive events (7). Several clinical studies have proven the safety of topical TA administration during joint replacement surgery, however, the effects of this drug on synovium and cartilage are still under

Key words: tranexamic acid, chondrocytes, synoviocytes, in vitro culture

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0393-974X (2016)

investigation. Recently, a potential toxicity of TA on bovine and murine articular chondrocytes has been shown in-vitro (8). On the contrary, an anti-arthritic effect has been described for intraarticular TA administration in a preclinical rabbit model (9) and this observation has also been confirmed in subsequent preclinical rat and rabbit models, when TA was orally or intramuscularly administered (10, 11).

The aim of this *in vitro* study was to evaluate the effects of TA on human cartilage explants and human synovial biopsies. In particular, the possible negative effects of TA were assessed on chondrocytes migrated from cartilage fragments, harvested from young donors (<40 years) and on synovial biopsies obtained from the same patients.

MATERIAL AND METHODS

Approval from the Ethical Committee of Mauriziano Hospital was obtained for the use of human tissue. Informed consent was obtained from each human donor.

Human cartilage biopsies were harvested during trochleoplasty from the intercondylar notch of young donors (15 patients) undergoing anterior cruciate ligament reconstruction and synovial biopsies were obtained from the same patients. Patients between 14 and 40 years-of-age without major osteoarthritic disease were included in the study (Kellgren-Lawrence classification grade 0-1).

Cartilage fragment construct culture

Tissue fragments were processed within 2 h following the biopsy. Samples were rinsed, placed on a Petri dish and manually minced into small cuboidal fragments (less than 1 mm^3), with a sharp scalpel in sterile conditions. For construct preparation, 10-15 mg of cartilage fragments were evenly seeded into the plate and coated with 200 µl of fibrinogen and thrombin (100 U.I./ml) (Tissucol-Tisseel, Baxter) Thrombin had been previously diluted 1:4 with calcium chloride. Constructs were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 21% O_2. Culture medium contained DMEM high glucose (4500 mg/l) with 10% fetal bovine serum, HEPES (10 mM), non-essential amino acids (0,1 mM), L-proline (20 ng/ml), ascorbic acid (50 µg/ml), penicillin/streptomycin (100 u/ml each) (Invitrogen, Life Technologies, Gaithersburg, MD).

Culture conditions were set as follows: 2 weeks in culture without TA, 2 weeks of culture with TA (Ugurol®, Rottapharm S.p.A.), at a concentration of 7mg/ml, and two weeks without TA. Cultures without TA were used as control.

At the end of the *in vitro* culture, some samples were processed and sectioned for routine histology and other samples were subjected to biochemical analysis.

Synoviocyte culture

Synovial biopsies were processed within 2 h after the harvesting. The tissue was digested in collagenase (Collagenase from clostridium histolyticum, Sigma-Aldrich, Milan, Italy) in sterile DMEM supplemented with 1% penicillin-streptomycin in a shaking incubator at 37°C. Samples were sheared using a sterile syringe, filtered using a fine sterile gauze, washed and resuspended in DMEM containing 10% FBS and 1% penicillin streptomycin. Cells were counted and seeded into 6-well tissue culture plates (2.5×10^4 cells/cm^2) and left overnight to allow cell adhesion. Cells were then washed three times with PBS and fresh DMEM supplemented with 10% FBS, 1% penicillin-streptomycin after which, 5 ng/ml basic fibroblast growth factor (b-FGF; Peprotech, Rocky Hill, NJ, USA) was added.

Synoviocytes type A and B were cultured for 48 h with TA (concentration 7mg/ml). Cultures without TA were used as control. Synoviocytes were assessed by light microscopy and flow cytometry analysis immediately after TA exposure and 1 week after from TA exposure.

Chondrocyte migration assessment

Chondrocyte migration and outgrowth were assessed by light microscopy (hematoxylin/eosin). Samples were fixed, embedded in paraffin and sectioned following standard protocols. Sections were stained for hematoxylin/eosin histology and examined under light microscopy. Cell counting was performed to quantify migration and outgrowth of chondrocytes from cartilage fragments. Three different areas in two different sections per construct were examined by two independent observers using light microscopy. The areas were examined at 20x magnification. Mean numbers of migrating cells from every area were statistically compared and graphed with GraphPad Prism®.

Scaffold immunofluorescence technique

SOX9 (Merck Millipore, Milano, Italy), collagen

type II (Merck Millipore, Milano, Italy), β-catenin (Santa Cruz Biotechnology, Dallas, TX, USA) and PCNA (Santa Cruz Biotechnology, Dallas, TX, USA) expression was assessed by immunofluorescence. The primary monoclonal antibodies were diluted in 1% PBS-BSA and sections were incubated for 2 h at room temperature. The secondary dylight 488 antibody (KPL, Kirkegaard & Perry Laboratories, Maryland, USA), diluted 1:200, was added for 1 h at room temperature. The stained sections were analyzed with an Apotome fluorescence microscope (Zeiss). Digital images were collected using a ×20 dry lens within 0-5 days after labelling.

Evaluation of fluorescence intensity

Fluorescence intensity between different cartilage constructs was evaluated using ImageJ program. Ten cellular fields were randomly chosen among the different areas of migrated chondrocyte in each slide. Brightness values for each image were calculated as the arithmetical mean of all values in all fields recorded from that image. For each construct, mean fluorescence intensity of each marker was calculated and plotted as a graph. Statistical analysis was carried out with the statistical software package GraphPad Prism 5.0.

Biochemical analysis

Cartilage samples were directly digested in papain, as described by Hoemann et al (12). Fibrin glue-cultured samples were then heated to 70°C for 10 min to melt the fibrin glue, vortexed and centrifuged at 21.000g for 1 min at room temperature. The supernatant was transferred to a fresh tube and immediately submitted to the Hoechst fluorescent DNA assay. A dimethyl methylene blue (DMMB) assay was conducted to evaluate GAGs content, according to previously described methods (13). A VMax microplate reader (Molecular Devices, Sunnyvale, CA) spectrophotometer was used. The GAGs value obtained was normalized on the DNA content.

Immunophenotypic characterization of synoviocytes

Immunophenotyping characterization of synoviocytes was performed by flow cytometry. The following antibodies were used:

- CD14, CD68 and CD163 (Miltenyi Biotec S.r.l, Bologna, Italy) for macrophagic-like cells (type A cells).
- CD106 and CD55 (Miltenyi Biotec S.r.l, Bologna, Italy) for fibroblast-like cells (type B cells).

Analysis was performed on a FACScan (Becton Dickinson (BD), Buccinasco, Italy) for at least 10.000 events and using CellQuest software (BD).

Statistical analysis

Statistical analysis was carried out with the statistical software package GraphPad Prism 5.0.

RESULTS

Cartilage fragment construct culture

Chondrocyte migration and outgrowth into the constructs was evident after 6 weeks of culture in both experimental conditions. The average of migrated cells was 72.20 (SD±2.557) in samples exposed to

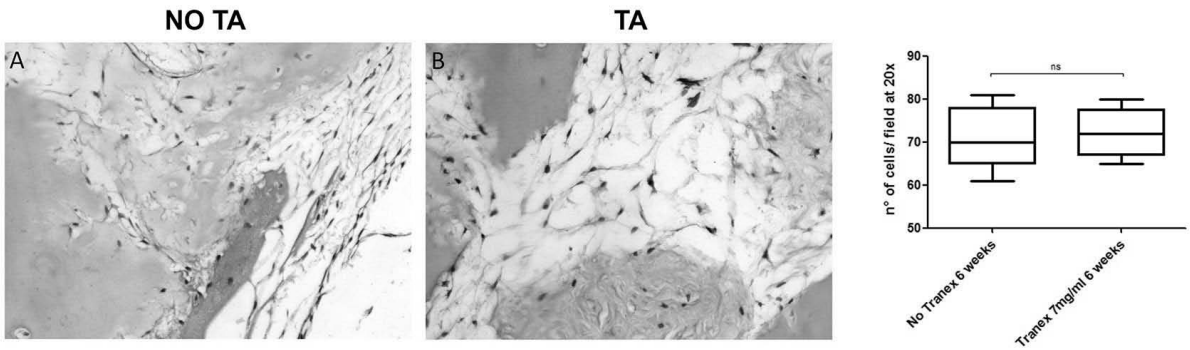

Fig. 1. *Light microscopy and quantification of migrated cells on paraffin sections (haematoxylin/eosin) of cartilage fragment cultures without TA treatment (**A**) and with TA treatment (**B**).*

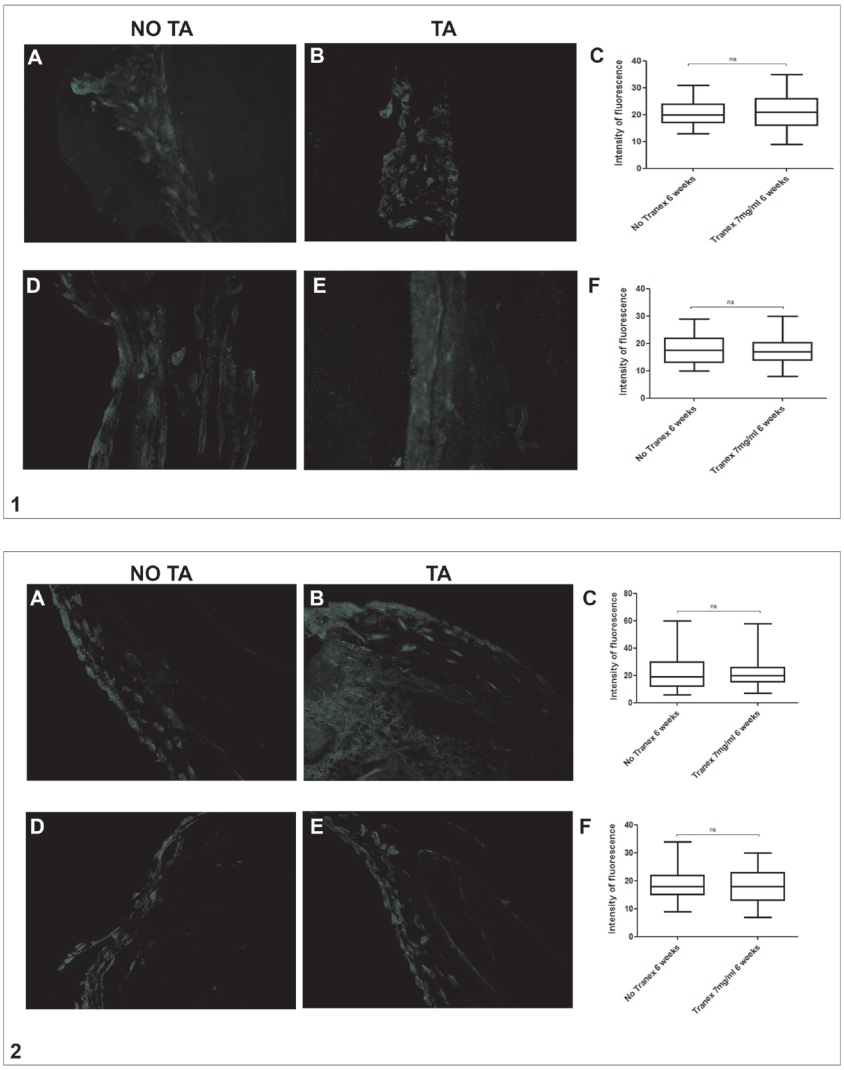

Fig 2. ***1)****: Immunofluorescence analysis for SOX9 and collagen type II expression without TA treatment (****A, D****) and after TA treatment (****B, E****). Quantification of fluorescence intensity for SOX9 and Collagen type II expression is shown in (****C****) and (****F****), respectively.* ***2)****: Immunofluorescence analysis for β-catenin and PCNA expression without TA treatment (****A, D****) and after TA treatment (****B, E****). Quantification of fluorescence intensity for β-catenin and PCNA expression is shown in (****C****) and (****F****), respectively.*

TA and 71.20 (SD±3.323) in samples without TA. Thus, no significant difference was found between the two experimental groups (p value >0.05) (Fig. 1).

Construct immunofluorescence analyses

SOX9 expression was present in both the experimental groups. The mean fluorescence value was 21.10 (SD±0.3709) for cultures with TA exposure and 20.81 (SD±0.3877) for cultures without TA exposure. The same pattern was observed for collagen type II expression. In particular, the mean fluorescence intensity was 17.39 (SD±0.2835) for cultures after TA exposure and 17.63 (SD±0.4183) for cultures without TA exposure. Similarly, positive signal for proliferative marker β-catenin was found in cultures both with and without TA exposure. The intensity of fluorescence was 21.42 (SD±0.4551) for cultures after TA exposure and 22.45 (SD±1.047) for

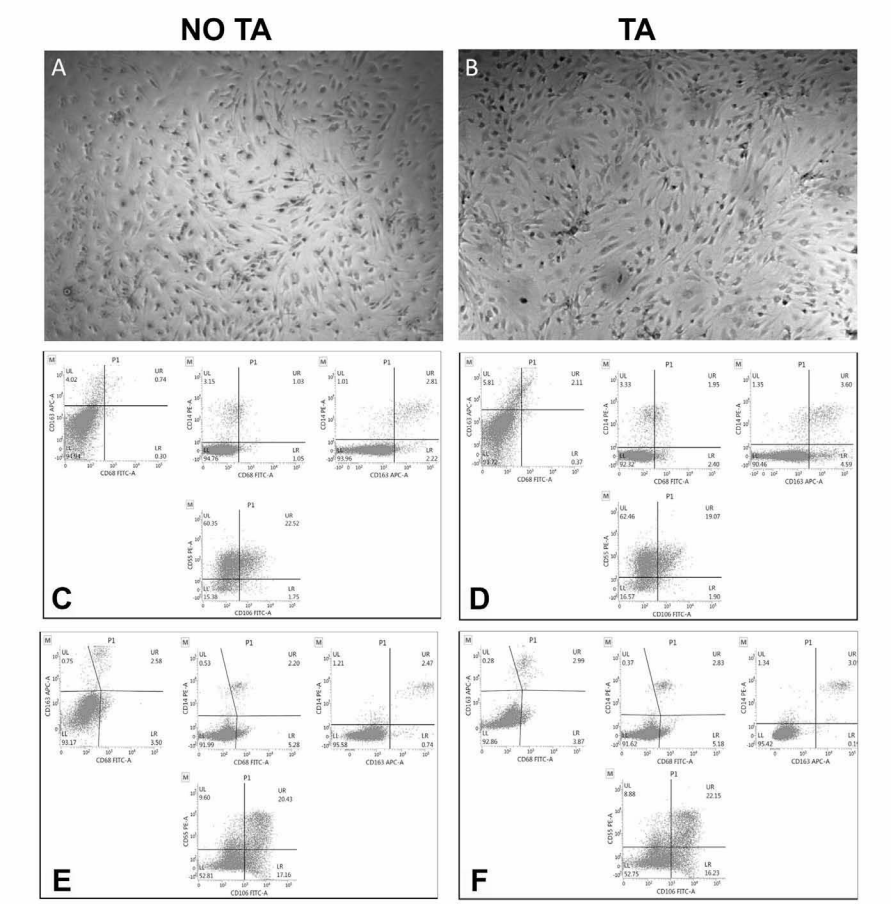

Fig. 3. ***A,B)*** *Light microscopy of synoviocyte primary culture. (**A**): untreated cells after 48h of culture; (**B**): cells exposed to TA for 48h (7mg/ml). In primary culture typical macrophagic (type A cells) and fibroblast-like type **B** adherent cells were observed.* ***C-F)*** *Synoviocyte flow cytometry for CD68, CD163, CD14, CD55, CD106. (**C**): after 48h of TA exposure (7mg/ml); (**D**): after 48h of monolayer culture without TA treatment; (**E**): 1 week after TA exposure; (**F**): after 9 days of monolayer culture without TA treatment.*

cultures without TA exposure. Finally, expression of PCNA was also present in both groups, with a mean value of fluorescence intensity of 17.78 (SD±0.5521) for cultures after TA exposure and 18.66 (SD±0.4914) for cultures without TA exposure. No significant difference in fluorescence intensity was found in any of these markers (Fig. 2; Panel 1, 2: C, F).

Biochemical Analysis

The average s-GAG/DNA ratio at 6 weeks was 0.3757 (SD±0.09872) for the cultures with TA exposure and 0.3220 (SD±0.09821) for the cultures without TA exposure. No significant difference was noted between the two groups ($p>0.05$) (data not shown).

Morphologic and immunophenotypic characterization of synoviocytes

Synoviocytes were qualitatively assessed by light microscopy. In primary cultures, typical macrophagic cells (type A) and fibroblast-like cells (type B) were observed. Results showed that TA treatment did not affect the synoviocytes morphology, and treated cells appeared morphologically similar to untreated cells both after 48 h of treatment and 1 week after TA exposure (Fig. 3A, B). Synoviocyte phenotype was then analyzed by flow cytometry at the two experimental time points. Data from one representative experiment are reported (Fig. 3C-F).

Results showed that TA exposure for 48 h did not affect synoviocyte phenotype. The expression of synoviocyte markers (CD163, CD68, CD14, CD55

and CD106) was unchanged in cells exposed to TA for 48 h. Most of the collected synoviocytes showed a positive expression of type B markers, CD55 and CD106, but a lower expression of type A markers, CD163, CD68 and CD14.

Similarly, flow cytometry results 1 week after TA exposure showed that expression of synoviocyte markers was comparable in the two experimental conditions (with and without TA treatment). Notably, 1 week after TA exposure, the expression of CD163, CD14 and CD55 was lower than the values observed after 48 h of TA treatment, while the expression of CD106 was higher than the values after 48 h of TA exposure.

DISCUSSION

A long-term exposure at low concentration of TA (7mg/ml) did not exert any influence on chondrocyte outgrowth and behavior in constructs obtained from human cartilage fragments embedded in fibrin glue. Moreover, TA did not show any significant effect on human synoviocytes after a brief exposure (48 h) to the same TA concentration. These findings suggest that TA does not negatively affect chondrocytes and synoviocytes during *in vitro* culture.

To our knowledge, this is the first study providing *in vitro* data on TA toxicity on human cartilage tissue and synovial explants.

In an *in vivo* study, Butler et al (9) analyzed the effects of TA intraarticular injections at a high concentration in a rabbit knee. These authors observed the absence of major toxic effect of TA towards the intraarticular microenvironment and they also reported a positive anti-osteoarthritic activity of the drug.

A recent *in vitro* study (8) analyzed bovine explants and murine chondrocytes as a model of joint environment. They observed early cytotoxic effects of a single administration of TA in culture at a concentration of more than 25 mg/ml. The authors suggested that similar effects of TA might be exerted on different component of the joint microenvironment such as the synovial cells.

Lower concentrations of TA were also tested in a recent clinical study (14). The authors advocated the effectiveness of these concentrations in preventing blood loss during knee replacement. They also affirmed that a low dose of local TA has the advantage of very low systemic absorption in view of an uncompromised effect in reducing blood loss. Thus, all these studies show that a proper intra-articular TA concentration is still a matter of debate.

However, the positive effect of TA in reducing hemarthrosis is advisable in common clinical practice, as demonstrated in a recent clinical trial (6). The authors observed an early postoperative improvement in pain and range of motion in patients who underwent ACL reconstruction and who received preoperative and postoperative intravenous TA administration. Nevertheless, in this study a high dose of TA was used. This specific intravenous TA protocol may arise again the question about the need of a safer and easier method to administer TA.

In our study, an *in vitro* model of intraarticular prolonged (2 weeks) TA exposure was assessed for cartilage explants following previous *in vitro* protocols for minced cartilage (15, 16). In parallel, a brief (48 h) TA exposition was assessed for type A and type B synoviocytes cultures from synovial biopsies.

Tissue samples were obtained from a homogeneous population of young donors, who may represent the ideal target for reconstructive orthopedic procedures as ligament reconstructions and cartilage repair. In this subpopulation, the use of TA is still highly discussed. Indeed, few studies in literature deal with TA application in a subpopulation of young patients, and the clinical work of Karaaslan et al (6) may be considered a pioneer work in this field. We purposely used a low TA concentration in our project: it is indeed well below the threshold suggested by a recent *in vitro* work (8) and it is still compatible with clot-stabilizing properties as indicated by a previous clinical study (14). The prolonged TA exposure allowed for a long-term evaluation of the possible effects of TA on cartilage behavior, such as chondrocyte migration.

As shown in previous studies (15, 16), the expression of cartilage and proliferative markers after 6 weeks of tissue culture represented the landmarks of migrating chondrocyte "performance" and their presence was considered a standard reference

to identify an active cell population. It has been previously shown that migrating chondrocyte may express both chondrocyte and proliferative markers, representing a peculiar subpopulation of cells, which share similar features with chondroprogenitor cells (16, 17).

The main finding of our study is that TA did not affect neither the chondrocyte ability to migrate and to proliferate in culture nor the production of cartilage matrix. Indeed, the number of migrating cells after 6 weeks of culture did not show any qualitative difference or any significant quantitative difference between samples exposed to TA and controls. Furthermore, the immunofluorescence analysis did not show any significant difference in the expression of chondrogenic markers (namely SOX9 and collagen type II) and in the level of proliferative markers (namely β-catenin and PCNA). Moreover, s-GAG/DNA ratio remained unchanged after exposure to TA.

A similar TA effect was observed in synoviocytes culture. Treated cells appeared similar to untreated cells at two different time points. This was observed by qualitative light microscopy evaluation and by semi-quantitative flow cytometry. Specifically, we measured the expression of markers of both synoviocytes type A (CD14, CD163 and CD68) and synoviocytes type B (CD106 and CD55) (18). As a collateral observation (Fig. 3), we detected a greater presence of type B synoviocytes at 7 days compared to that after 48 of exposition. This feature was similar in both culture condition and it was unaffected by the presence of TA.

The lack of any significant morphological and phenotypical change in cultured chondrocytes and synoviocytes at different time points may suggest a possible use of TA intra-articular injections to prevent excessive bleeding.

The first limitation of our study is the low number of donors (15 patients). A second drawback of this work is the single low TA concentration used in our *in vitro* cultures. However, this low concentration has already been validated in previous studies (8, 14).

In conclusion, the results of our study certainly do not allow for promoting an immediate routine use of intra-articular TA to prevent hemarthrosis during orthopedic reconstructive procedures. Nonetheless, they suggest the urgent need for clinical phase I and II studies to investigate the potential *in vivo* safety and effectiveness of this approach. Indeed, our findings are clinically relevant in order to validate the intra-articular TA administration as a prophylactic measure during orthopedic surgical procedures at high risk of haemarthrosis, such as ACL reconstruction and cartilage repair.

Moreover, the stabilizing properties of TA on the fibrin clot (19, 20) may exert a secondary positive effect both on i) the ACL graft integration and ii) the repair process (clot) resulting from traditional cartilage repair procedures as microfractures or nanofractures.

These final hypotheses also may deserve future prospective studies to allow for a better understanding of the potential positive effects of intra-articular TA administration.

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INTRAOPERATIVE APPLICATION PLATELET RICH FIBRIN, POSTOPERATIVE INJECTIONS OF PRP OR MICROFRACTURE ONLY FOR OSTEOCHONDRAL LESIONS OF THE KNEE: A FIVE-YEAR RETROSPECTIVE EVALUATION

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Cartilage lesions are the most common cause of chronic knee pain. Micro-fracturing is reliable, effective, easy to perform and inexpensive. We propose a novel approach to cartilage lesions where microfractures are performed contextually to intra-operative or post-operative administration of platelet concentrates. We retrospectively evaluate 48 patients divided in 3 groups. Group 1: 15 patients underwent microfractures and intraoperative administration of PRF (PRF group); group 2: 16 microfractures and postoperative injections of PRP (PRP group); group 3: 17 patients with isolated microfractures (Microfractures group). Clinical scores (IKDC, VAS pain) were administered at 2 and 5 years postoperative and MRI was performed to evaluate the lesions of patients according to the MOCART criteria (2006). Patients treated with platelet concentrates achieved better clinical results compared to patients treated with microfracture only. The PRF group showed better results than the PRP group at 2 years, with loss of significance at 5 years. At MOCART score, PRF group obtained better results earlier than the other two groups.

Cartilage lesions of the knee occur in 60% of patients complaining of knee pain. (1, 2). They affect athletes as a consequence of traumatic injuries and sedentary patients, as part of the spectrum of degenerative joint disease and are very painful (3).

Cartilage can tolerate a great amount of stress but avascularity and its low mitotic activity make it less suitable for intrinsic healing or repair process. In fact, normal hyaline cartilage has little or no potential to heal spontaneously when injured (4, 5).

As primary end-points, the short-term aim is symptom relief and resuming daily activities and sports, while prevention of degenerative changes is wished over time. In the management of grade II-III cartilage lesions, restorative procedures seem to be encouraging. Micro-fracturing is reliable, effective, easy to perform and inexpensive, however, the repaired tissue is fibro-cartilage, which is less resilient and stiff than hyaline cartilage (6), Autologous Chondrocytes (ACI) have been successfully implanted in young physically active patients (7) although this procedure is relatively invasive, two stage, and expensive.

A new approach is to administer platelet

Key words: platelet rich plasma, cartilage lesions, microfractures, osteoarthritis, platelet rich fibrin

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0393-974X (2016)

concentrates rich in autologous growth factors. Once injected, results are not univocal (8-10). In fact, preparations may be different, the number and timing of injection are still unknown, and procedure criteria are undefined (11-12).

We propose a novel approach to cartilage lesions. Microfractures are performed contextually to intra-operative administration of platelet rich fibrin. Theoretically, the rationale is that fibrin would allow to concentrate and lock growth factors onto the injury site, potentiating their action. In addition, it would also improve the healing effects of microfracturing. The present study hypothesized that patients undergoing platelet concentrates administration fare better than those undergoing microfractures only, in terms of pain, clinical and functional outcomes at long-term follow-up.

MATERIALS AND METHODS

Patient selection

We performed a retrospective analysis of the medical records (MR) of Campus Bio-Medico University Hospital, and identified 78 patients who consulted the participating orthopaedic surgeons for knee cartilage complaints (Table I) and were diagnosed with knee cartilage lesion. We included patients in whom a grade II-III outerbridge cartilage lesion had been detected (average 401±27 mm^2, range 200-600 mm^2). All patients included had a platelet count >150 000/mcl, no hypofibrinogemia, Hb>10mg/dl before starting any treatment. We imposed no age limits. Patients who had, in the past or present, a history neoplastic disease, severe cardiovascular disease, neurologic disorders or decompensated diabetes mellitus, were excluded from the study.

Finally, data on the use of NSAIDs and anticoagulant by the patients was suspended at least 7 days before surgery. Furthermore, MR had to report at least a visit at 5 years from the end of treatment and records had to report clinical evaluation and IKDC and VAS scores of the patients. Forty-eight patients satisfied these inclusion criteria and were divided in 3 groups, according to the treatment they underwent. Fifteen patients underwent microfractures and intraoperative administration of PRF (PRF Group);

Table I. *Patients characteristics.*

	PRF GROUP	PRP GROUP	MICROFRACTURE GROUP
N°	15	19	17
Sex	M=9; F=6	M=9; F=10	M=6; F=11
Mean age (± SD)	53 ± 9.8	52 ± 11	53 ± 9.8
Lesion site	13 MFC 1 LFC 1 FT	15 MFC 4 FT	13 MFC 4 FT
Lesion degree	IV =15	III=7 IV=12	III=6 IV=11
Associated procedures	MM=8 ACL Reconstruction=1	MM = 12 LM=2 ACL Reconstruction=1 Lateral Release=1	MM=9 LM=1 ACL Reconstruction=2 Lateral Release=2
complications	No	No	No

***MFC**: medial femur condyle; **LFC**: lateral femur condyle; **FT**: Femur throclea; **MM**: medial meniscectomy; **ACL**: anterior cruciate ligament; **LM**: Lateral Meniscectomy.*

16 patients microfractures and postoperative injections of PRP (PRP Group), and 17 patients isolated microfractures (Microfractures Group).

Study protocol

An assistant who had not been previously involved in the management of the patients checked all selection criteria in the medical records and identified 48 patients who satisfied the inclusion criteria. The local medical ethics committee approved the protocol.

Methods of treatment, "VivostatPRF" preparation and operative use

The Vivostat System™ is a medical device for the perioperative preparation and application of platelet gel in the operating room. The system is fully automated and microprocessor controlled and is composed of three components: an automated processor unit, an automated applicator unit and a disposable single-patient-use unit, which includes a preparation set and the endoscopic applicator.

The anaesthetist in the operating room took 120 ml of the patient's whole blood from the antecubital vein and was collected in Vivostat System™ device in about 30 min. Approximately 5ml of Platelet Gel was produced from 120ml of the patient's own blood. Details of the biochemical process of the Vivostat System™ have been reported by others (13-15). The entire process, from taking the patient's blood sample until the PRF is ready for use, was fully automated and microprocessor controlled.

"Regen PRP" preparation and injection

Eight millilitres of the patient's whole blood were collected in a Regen Lab THT tube® containing a special gel. The tube was centrifuged at 3100 rpm for 9 min. After centrifugation, the red blood cells were separated from the platelet rich plasma. The PRP was then aspirated with an 18-gauge needle and injected in the knee.

Details of the content of each treatment session, especially in regards to postoperative injections and any adverse effects were reported on standardized forms and given to the medical assistant.

Micro-fractures technique

The surgical procedure was performed under regional anesthesia with the patient supine and with the knee to be operated on put in a leg holder. Standard antero-medial and antero-lateral arthroscopic portals were used. All associated procedures (including partial meniscectomy (32), ACL reconstructions (4), patellar realignment (3) were carried out before microfractures.

Cartilage lesions were clearly identified and shaved. Microfractures were performed using a surgical awl (16) to let the subchondral bone bleed.

Postoperative care

All patients followed a standard post-operative rehabilitation program (39). Partial weight bearing was allowed from 4 to 6 weeks, then full weight bearing was achieved gradually at 8 weeks. Compliance to the program was verified through the medical records.

Outcome assessment

The subjective International Knee Documentation Committee (IKDC) score (17) and VAS pain (as recommended by the International Cartilage Repair Society) were considered as the main tools for clinical evaluation and scores reported in the medical records of the patients were retrieved and analyzed.

Radiologic evaluation

All patients received a Magnetic Resonance Imaging (MRI) scan pre-operatively and at 5 years from the index procedure. We used a standard knee MRI protocol that incorporate proton density and fast spin-echo acquisitions for cartilage evaluation. All MRI were examined by 2 independent and fully trained musculoskeletal radiologists.

MRI classification system: MOCART (2006)

To describe the repair tissue we used the MOCART classification (18) which includes nine morphological variables to compare the structure and signal intensity to the adjacent native cartilage. The repair was considered complete when the repair tissue appeared as thick as the adjacent native cartilage, with complete integration of the margins and a smooth articular surface that reproduced the original articular contour with no adhesions and an intact subchondral bone plate and marrow. The signal intensity of the repair tissue was separately determined in fast spin-echo (dual T2-FSE) and fat-suppressed gradient-echo (3D- GE-FS) sequences. A complete repair was graded as isointense if it appeared as intense as the adjacent native cartilage.

Statistical analysis

Clinical improvement over time, was assessed for each group using analysis of variance (ANOVA) with repeated-measures analysis. The significance of the results obtained from comparison among treated groups were statistically analyzed by unpaired Student's *t*-test. A P-value of <0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0p (SPSS Inc., Chicago, USA).

RESULTS

Clinical Assessment

The IKDC score showed no pre-operative significant difference among the groups. At 2-year follow-up, all groups showed significantly better results than before athroscopy (P<0.05 for all scores). Evaluating reported IKDC scores, patients in the PRF and PRP groups achieved significantly better results than patients in the microfracture group (PRF *vs* microfracture P=0.001; PRP *vs* microfracture P=0.012). Comparing the IKDC scores of the two types of platelets concentrate, patients treated with intraoperative application of PRF fared better than those who underwent postoperative injection (P=0.035) (Table II).

At 5 years, patients treated with platelet concentrates (PRF and PRP groups) achieved significantly better IKDC values than those observed in the microfractures group (P<0.05), although it was noted that the outcomes worsen compared to 2-year follow-up. Moreover, there was no statistically significant difference between the results of PRF and PRP groups at later follow-up (Table II). Patients treated with microfractures only, showed a non-significant difference in clinical outcomes compared to preoperative time (P>0.05) and 5 patients underwent prosthetic surgery. No serious complications were reported.

Pain had been measured using a 10-point Visual

Table II. *Clinical outcome assessment.*

2 Years Follow-up						
Mean (SD)				P (< 0.05)		
Outcome measures	PRF Group	PRP Group	Microfracture Group	PRF vs PRP group	PRF vs Microfracture group	PRP vs Microfracture group
IKDC	85.3 (15.6)	73.9 (14.3)	62.7 (9.8)	0.035	0.001	0.012
VAS	1.35 (1.8)	3.5 (1.7)	5.2 (1.6)	0.001	0.001	0.007
5 years Follow-up						
Mean (SD)				P (< 0.05)		
Outcome measures	PRF Group	PRP Group	Microfracture Group	PRF vs PRP group	PRF vs Microfracture group	PRP vs Microfracture group
IKDC	78.4 (13.9)	76.5 (14.9)	62.8 (12.8)	0.701	0.002	0.007
VAS	3.4 (1.8)	3.1 (1.51)	5.5 (1.5)	0.542	0.001	0.001

Analogue Scale (VAS). Improvements from the pretreatment level were statistically significant in all groups at 2-years follow-up (all P<0.05) but at 5 years postoperatively, the microfracture group showed no significant differences compared to preoperative time (P>0.05). The PRF and PRP groups achieved significantly better results than patients from the microfractures group (all P<0.05) (Table II). Furthermore, patients treated with intraoperative application of platelet concentrate showed a significant better pain score at 2-year follow-up (P<0.001). However, at later follow-up, no significant differences were reported between PRF and PRP groups (P=0.542), as with the IKDC score.

Pre-operatively, MRI showed marked changes in the subchondral bone edema at the lesion site and thinning of the articular cartilage in all patients. The results of the MOCART score are reported in Table III.

Imaging evaluation of cartilage repair

The MRI scans performed 5 year after the index intervention showed significantly better MOCART scores for the PRF and PRP groups when compared with the control group. The MOCART scores were significantly better in the PRF group, with 8 patients (53%) who had complete cartilage coverage of their lesions *vs* 2 patients in PRP group (13%), while in the microfractures group no patients presented complete cartilage coverage. In the PRP group, 10 patients (63%) had greater than 50% cartilage coverage, which was significantly higher than in the Microfractures group, in which only 4 patients (23%) had similar results. In the microfractures group, 3 (17%) patients had subchondral bone exposure. Significantly better integration of the regenerated cartilage was found in 67% (10 of 15) of patients in the PRF group, with complete integration of the regenerated cartilage to the border zone, and in 31% (5 of 16) of the patients in the PRP group, whereas 70% of the control group showed incomplete integration with visible defects (Table III).

DISCUSSION

Microfractures can be an effective treatment for severe cartilage lesions of the knee at 2 years obtaining significantly better results compared to preoperatively. This effectiveness disappears at long term follow-up. However, intraoperative (through the application of fibrin-rich gel) or postoperative (3 intra-articular injections) administration of platelet concentrates improves the results at 5 years after treatment.

The choice to administer three injections of PRP was made in accordance with clinical experience at our institution and with the contemporary literature (8, 19). However a recent study demonstrated that multiple injections of PRP were more effective compared to a single injection of PRP (20).

Considering the state of vascularization and innervation of the cartilage, the methods by which these lesions are treated are still developing. Many strategies have been proposed, but the latest evidence promote the application of products containing mesenchymal cells or autologous growth factors. The rationale of the microfracture in subchondral bone, after exposure of the cartilage lesion, lies in the fact that the microfractures allow hematopoietic stem cells and autologous growth factors to reach the site of the injury, inducing a local healing response (16). Microfractures are effective in the short term, but the effectiveness in the long term is somewhat lacking (21).

Despite the fact that evidence regarding the PRP application is quite variable in terms of indications, application criteria, product administration and results, we decided to conduct this study.

Emerging models require the direct application of these factors to the site of injury, in the context of membranous-like structures (9). In the PRF group, we applied a new preparation of platelet concentrate, made of fibrin gels (solid) following the Vivostat PRF preparation method. In this way, the growth factors can be concentrated and located at the lesion site, reducing the product dispersion.

From the comparison of the two groups treated with platelet concentrates, the one treated with PRF obtained significantly higher clinical outcomes in the short term leading to a faster recovery. However, there were no statistically significant differences after 5 years.

In agreement with other evidence (22), the short-

Table III. *MOCART MRI evaluation of repair tissue, by number and percentage.*

	PRF GROUP (N=15)	PRP GROUP (N=16)	MICROFRACTURE GROUP (N=17)
1. DEGREE OF DEFECT REPAIR AND FILLING OF THE DEFECT	**N°(%)**	**N°(%)**	**N°(%)**
Complete (on a level with adjacent cartilage)	8(53)	2(13)	0
Hypertrophy (over the level of the adjacent cartilage)	0	3 (19)	2 (11)
Incomplete (under the level of the adjacent cartilage; underfilling)			
>50% of the adjacent cartilage	5 (33)	10 (63)	4 (23)
<50% of the adjacent cartilage	2(13)	1 (6)	8 (47)
Subchondral bone exposed (complete delamination or dislocation and/or loose body)	0	0	3 (17)
2. INTEGRATION TO BORDER ZONE			
Complete (complete integration with adjacent cartilage)	10(67)	5(31)	1 (5)
Incomplete (incomplete integration with adjacent cartilage)	4 (27)	4 (25)	2 (11)
Demarcating border visible (split-like)	1 (7)	4 (25)	2 (11)
Defect visible			
<50% of the length of the repair tissue	0	2 (13)	7(41)
>50% of the length of the repair tissue	0	0	5 (29)
3. SURFACE OF THE REPAIR TISSUE			
Surface intact (lamina splendens intact)	8 (53)	9 (56)	4 (23)
Surface damaged (fibrillations, fissures and ulcerations)			
<50% of repairtissuedepth	3 (20)	4 (25)	7 (41)
>50% of repair tissue depth or total degeneration	4 (27)	1 (6)	6 (35)
4. STRUCTURE OF THE REPAIR TISSUE			
Homogenous	11 (73)	11 (69)	8 (47)
Inhomogenous or cleftformation	4 (23)	5 (31)	9 (53)
5. SIGNAL INTENSITY OF THE REPAIR TISSUE			
T2-FSE			
Isointense	13 (87)	11 (69)	6 (35)
Moderatelyhyperintense	2 (13)	2 (13)	4 (23)
Markedlyhyperintense	0	3 (19)	7 (41)
3D-GE-FS			
Isointense	1 (7)	0	0
Moderatelyhypointense	14 (93)	12 (75)	6 (35)
Markedlyhypointense	0	4 (25)	11 (64)
6. SUBCHONDRAL LAMINA			

6. SUBCHONDRAL LAMINA			
Intact	13 (87)	12 (75)	4 (23)
Notintact	2 (13)	4 (25)	13 (76)
7. SUBCHONDRAL BONE			
Intact	3 (20)	3 (19)	2 (11)
Edema	12 (80)	9 (56)	10 (58)
granulationtissue, cysts, sclerosis	0	1 (6)	5 (29)
8. ADHESIONS			
No	13 (93)	14 (88)	10 (58)
Yes	2 (13)	2 (13)	7 (41)
9. EFFUSION			
No	12 (80)	12 (75)	6 (35)
Yes	3 (20)	5 (31)	11 (64)

term improvement observed in the two PRP- treated groups could be the result of a reduction in the local inflammation. In the context of degenerative knee cartilage disease, PRP appears to be superior compared to administration of hyaluronic acid (8).

Our study partially agrees with previously published study evidence (23) in which two groups of patients with cartilage lesion sized less than 4 cm^2 and early osteoarthritis, underwent only arthroscopic microfracture or arthroscopic microfracture and PRP. The patients were prospectively evaluated at 24 months showing a significant improvement in clinical results compared to preoperative evaluation in both groups; furthermore, clinical results showed significantly better outcomes in patients treated with platelet concentrates at final follow-up, as in this study. However, in the study above mentioned, PRP was administered as a single intra-operative injection, while we preferred a intraoperative application in a more solid form to reach an elevated concentration of growth factors at the site of interest. Recently, combined therapy with microfractures and three post-operative PRP injections have been investigated by Manunta and Manconi (24). The authors found a greater improvement in IKDC scores in that patients treated by combined therapy when compared with the group of patients treated by microfracture alone. A similar result has been also reported for cartilage defects of the talus, when a single post-operative injection (24h after surgery) had been performed after microfracures (25).

After 5 years of follow-up, we observed that all the scores showed a superiority of the platelet concentrates treated-groups when compared to those of patients treated with microfractures alone, but the results gradually decrease over time.

Furthermore, patients who had undergone microfractures treatment alone showed no significant difference at 5 years follow up compared to preoperative time, and 5 of 17 patients underwent a knee prosthesis, while no patients treated with platelet concentrates required further interventions. Platelet concentrates seem to help to slow the degenerative cascade of osteoarthritis, although it is not possible to provide definitive evidence.

As strong points, we must consider that all the procedures were performed by a single surgeon, who applied in all cases a standard protocol for the administration of the products. From a radiological healing point of view, patients treated with Vivostat PRF showed better outcomes than other patients did. This is probably because the application of gelled suspension containing concentrated autologous platelet can produce a more suitable environment for the repair and filling of the lesion. Furthermore, the results were broadly comparable between the

PRP and PRF treated groups after 5 years. Hence, it seems that PRP injections need a longer time to accomplish its potential reparative. However, we can not yet provide conclusive evidence based on this preliminary study.

CONCLUSION

The association between platelet concentrates and microfractures is a valid alternative to the treatment with microfractures alone. In addition, where feasible, the best treatment is the intra-operative application as it allows faster and less painful recovery. Additional data, more patients and longer follow-up are needed to assess the long term effectiveness of platelet use in osteochondral lesions of the knee.

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PAIN REDUCTION AND IMPROVEMENT OF FUNCTION FOLLOWING ULTRASOUND-GUIDED INTRA-ARTICULAR INJECTIONS OF TRIAMCINOLONE HEXACETONIDE AND HYALURONIC ACID IN HIP OSTEOARTHRITIS

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The scientific literature has shown positive results regarding intra-articular injections of hyaluronic acid in osteoarthritic joints. When injecting in the hip joint, the guidance of ultrasound can provide higher injection accuracy and repeatability. However, due to the methodological limitations in the current available literature, its recommendation in the current practice is still controversial. This study shows that ultrasound-guided intra-articular injections of triamcinolone hexacetonide and hyaluronic acid can improve pain, function and quality of life in patients with symptomatic and radiographic hip osteoarthritis. In addition, the administration of triamcinolone hexacetonide and hyaluronic acid to the hip joint in these patients can delay the need for interventional surgery.

Osteoarthritis (OA) is one of the most common causes of functional disability and loss of autonomy in the elderly population worldwide, causing a massive socio-economic burden. Hip OA prevalence estimates ranges from 3.9% to 6.1% in persons aged between 40 and 75 years, being the second most frequent form of OA, slightly lower than knee OA (1).

The etiology of OA has known to be multifactorial and currently is not completely understood (2), and includes non-genetic (age, gender, body mass index, joint mechanical stress, sedentary lifestyle, joint trauma, participation in sports or work-related activities) and genetic (modified gene expression patterns of the cartilage and subchondral bone) risk factors (3). This systemic and chronic joint disorder involves a slow and progressive deterioration of the articular cartilage, including changes in the underlying subchondral bone and in the lubricating properties of the synovial fluid, leading to a gradual increase on pain and loss of function (3-5). In this sense, several conservative therapeutic approaches have been developed, aiming for the reduction of pain and inflammation, improvement of joint function and reducing the progression of OA (3,6,7).

Key words: hyaluronic acid, viscosupplementation, osteoarthritis, hip, ultrasound-guided

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0393-974X (2016)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

The triamcinolone hexacetonide (HT) is glucocorticoid with a synthetic analog with anti-inflammatory effects and the most commonly used and preferred corticosteroid (8). It is the least soluble injectable steroid commonly used for intra-articular purposes and yields the most durable effect (9). It decreases the inflamed joints' synovial blood flow (10) by shutting down the gene expression within the synovial lining, subsequently decreasing the inflammatory response (11). It has been suggested that corticosteroids may be able to block the cartilage-degrading features of the neutral proteoglycanases (12).

Hyaluronic acid (HA) is an organic polysaccharide, present throughout the human body, especially in the connective tissue. It is produced in the synovial membrane by the chondrocytes and synoviocytes and dissolved in the synovial liquid, forming part of the extracellular matrix of hyaline cartilage (13). It has been suggested that the stimulation of endogenous HA, the direct anti-inflammatory effect and the inhibition of the tissue nociceptors and matrix metalloproteinases activity are associated mechanisms of pain reduction following an intra-articular injections of HA (14). These mechanisms act to decrease the mechanical, chemical or thermal noxious stimuli of the synovial joint innervated tissues, reestablishing the normal homeostasis and leading to a reduction in pain and stiffness and improved function (15).

HA is known to have an outstanding safety and tolerability, with very few reported adverse effects (13, 15, 16). Repeated intra-articular injections of HA that are performed under the guidance of fluoroscopic or ultrasound devices are an effective and alternative conservative treatment for the OA of the hip (16-19). Additionally, the use of ultrasound guidance allows an enhanced accuracy and repeatability of the intra-articular HA injections (20).

Despite the several benefits and positive results reported throughout the scientific literature, the role of HA injections in the management of hip OA still remains controversial. Moreover, the combined use of HT and HA seems to improve the HA outcomes by providing a faster and more durable effect in the osteoarthritic knee joints, without deleterious effects on joint structure (21). Nevertheless, there are no studies in the literature evaluating the effect of combined therapy (HT+HA) in patients with hip OA. Therefore, this prospective cohort study aims to access the effectiveness of intra-articular hip injections of HT+HA under ultrasound guidance for pain reduction and functionality in patients with hip OA.

MATERIALS AND METHODS

Patient selection

This prospective cohort study was performed in order to establish the effectiveness of intra-articular injections of HA on hip OA, between January 2013 and December 2015. The Institutional Ethical Committee (IRB 0005/0012) approved the present study and all patients signed a written informed consent before entering in the clinical trial, authorizing all the procedures involved.

Patients with symptomatic hip OA were identified through regular consultations at our institution and invited to participate in the study. In order to participate in the clinical trial, the patients needed to be in accordance with the following inclusion criteria: presence of symptomatic hip OA, and with radiological findings according the Kellgren and Lawrence classification (22); no previous HT+HA therapy or concomitant systemic or oral therapy.

The exclusion criteria was as follows: previous extensive surgery of the hip joint; excessive deformity of the hip joint; associated rheumatologic diseases (for example, connective tissue diseases); *protrusio acetabuli*; concentric femoral head migration; poor quality of radiographic imaging; any open or arthroscopic within the last 3 months (such as, debridement or lavage procedures); suspected or known hip joint infection; suspected joint fracture; extensive skin pathology in the area of injection; uncontrolled high blood pressure; severe congestive heart failure; allergy to the HA.

At the initial consultation, the grade of symptomatic hip OA was independently identified by the same two independent senior investigators (JPA, NL), experienced sports and medicine/physical medicine and rehabilitation clinicians. The grade of OA was classified using digitized radiographs according to Kellgren and Lawrence classification (22). All radiographs were taken at our institution by the same imaging technicians, using anteroposterior and mediolateral projections.

Injection technique

All the injections were performed by the same two independent senior sports medicine clinicians/physical medicine and rehabilitation clinicians (JPA, NL). Following the eligibility screening, the patients were scheduled to receive 3 intra-articular injections, within a 1 week of interval between them. The first intra-articular injection was composed of 2ml of HT (Hexatrione®, Ethypharm, Saint Cloud, France), concentrated at 20mg/ml. The second and third intra-articular injections comprised 2ml of HA (Suplasyn®, Mylan Institutional, Zurich, Switzerland), concentrated at 10mg/ml.

On the day of each injection, patients were assessed for potential local and systemic side effects. Patient were placed in a supine position with the heels together and with the legs slightly rotated externally. The examination was performed with a low frequency (3.5-7.5 MHz) linear array probe or a convex one (2.5-5 MHz) for obese and muscular patients. The anterior approach was used and after locating the femoral neuro-vascular structures, the probe was displaced laterally (longitudinal view) to see the hip joint placing and the transducer parallel to the axis of the femoral neck. After that, the skin was disinfected with an antiseptic solution and local anesthetic (lidocaine 5 ml) was administered. A 21-gauge spinal needle was inserted obliquely through the skin, fascia, muscle and joint capsule, until bone (femoral neck) was reached. The HA was gradually injected under ultrasound guidance, taking care to not feel resistance (Fig. 1). After each procedure, the hip was mobilized within the 4 quadrants and patients were allowed to immediate full weight-bearing and to carry out their dayli routines, while advised to prevent sports or physical activity for the following 2 weeks. After 2 weeks, individual programs of strengthening, stretching and cardiorespiratory reconditioning were provided to patients for the monthly follow-ups.

Outcome measures and follow-up

All patients were assessed at baseline and at 6 and 13 months of follow-up. The severity of pain was primary assessed by a 10 points Visual Analogue Scale (VAS) concerning the level of hip pain in the last 24 hours. Additionally, all patients filled out the Hip Disability and Osteoarthritis Outcome Score (HOOS) in order to assess the pain, function and quality of life (23). Moreover, the success rate was estimated based on the need for interventional surgery within the 12 months following the last HA injection.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences version 24.0® (SPSS,

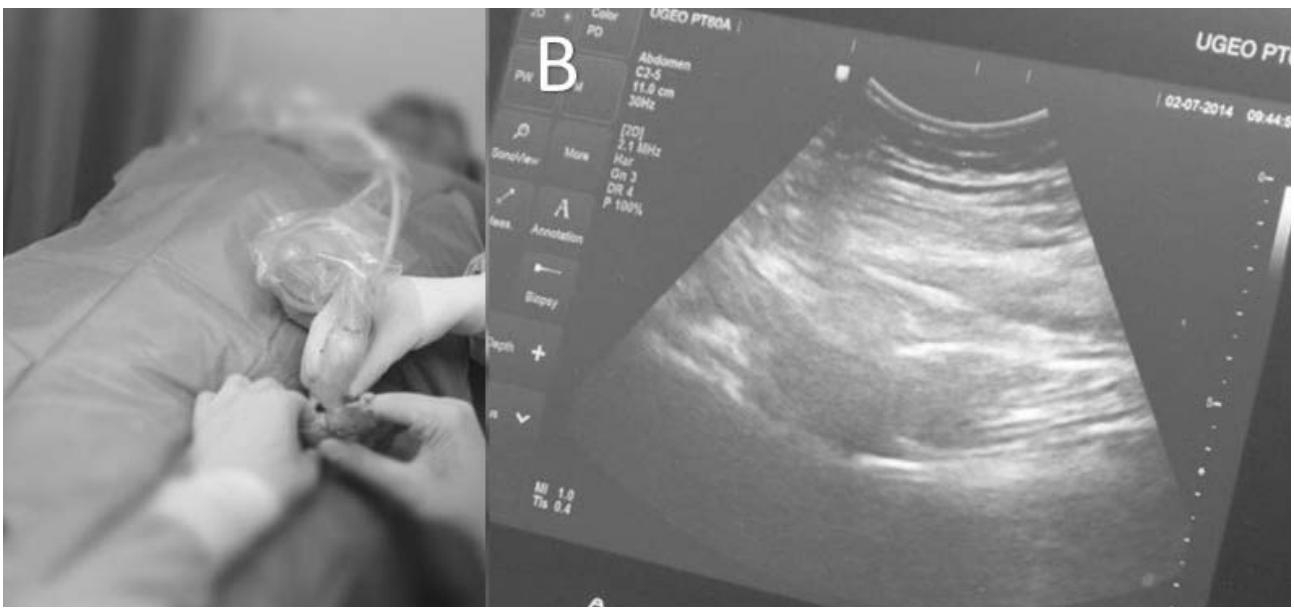

Fig. 1. ***a)****: Application of intra-articular injection of HA in the hip joint under guidance of ultrasound;* ***b)****: The accuracy of the injection was confirmed by direct visualization under ultrasound guidance.*

Inc., Chicago, Illinois). All data was screened and tested for normal distribution using visual inspection of graphics and normality plots. Categorical variables were presented as counts and percentages and continuous variables as median and interquartile amplitude. The pain intensity (VAS) and the HOOS were assessed according to the total sample and stratified by the radiological grade of hip OA. Chi-square test was used to compare the categorical variables. For the survivorship analysis, Kaplan-Meier was used within the different groups to determine the cumulative therapy success rate and the log-rank test to assess statistical significant differences in the cumulative therapy success rate. The non-parametric Friedman test was used for paired samples (VAS and HOOS) throughout (0, 6 and 12 months) and the Wilcoxon test to identify significant differences within paired samples. Mann-Whitney U-test was used to assess significant differences between the two independent samples. Kruskal-Wallis for independent samples was used to compare the VAS and HOOS scores according the different OA grades. Additionally, correlations between body mass index (BMI), age, VAS and HOOS were calculated through Spearman's rho. For all tests, a P value under 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Sixty-seven patients (31 male and 36 female) with symptomatic hip OA who met the inclusion criteria, were enrolled on the present study and received ultrasound-guided intra-articular hip injections of HT+HA. The patients were 58.4±15.6 years old at the time of the first injection and weighted 22.3±3.7 kg/m2. The baseline sample of participants was homogenous in regards to gender and side of lower limb affected ($p>0.05$). All clinical and demographical characteristics are summarized in Table I.

The right hip was the most affected (58%), however 5 patients (7%) had bilateral hip OA. Overall, the most common OA grade was the grade II (33%) and grade III (50%).

Cumulative therapy success rate

Of the 67 patients included in this study, 8 patients (12%) required hip arthroplasty without occurence of infection 6 were considered drop-outs (4 at 6 months, and 2 at 12 months), resulting in an overall success rate of 86.9%. When sub-grouped, OA grade I patients were the most successful, with 100% rate of success (Table II). Males with grade II OA and females with grade III OA also presented excellent success rate (90% and 93.3%, respectively). No statistical significant differences were found regarding the hip OA degree, gender and gender sub-grouped by OA degree.

Pain intensity

There was a statistically significant reduction of pain intensity over time for hip OA grades II and III, and when considering the total sample ($p<0.001$; Table III). In addition, these three conditions were also statistically significant between all assessment points ($p<0.05$), with the exception of grade II between 6 and 12 month assessment points ($p=0.157$). In each assessment point (0, 6 and 12 months) there were no statistical significant differences between the 4 grades of hip OA ($p>0.05$).

When comparing the patients that required surgery against the ones who did not, there was statistically significant lower pain intensity for grade II hip OA at baseline ($p<0.05$), for grades II and III and the global sample at 6 months follow-up ($p<0.05$; Table IV). In regards to gender, there were no statistically significant differences between genders in the VAS score over time, nor at baseline or at 6 and 12 months follow-up ($p>0.05$). The only exception was grade III hip OA at baseline, where the female participants presented higher pain intensity ($p<0.05$; Table V).

The intensity of pain was positively correlated with age at all assessment time points ($p<0.05$) and with the BMI at the baseline ($p<0.05$; Table VI).

Hip function

There was a statistically significant improvement of the hip function (HOOS) over time for hip OA grades II and III, and when considering the total sample ($p<0.001$; Table VII). Moreover, in the same line as pain assessment, in these three conditions the difference was also statistically significant between all assessment points ($p<0.05$), with the exception of grade II and III between 6 and 12 month assessment points ($p=0.123$ and $p=0.118$, respectively). In each

Table I. *Baseline clinical and demographical characterization of the samples in the study.*

Gender:	
Female, n (%)	36 (53.7)
Male, n (%)	31 (46.3)
Age (years), mean±SD	58.4±15.6
BMI (kg/m^2), mean±SD	22.3±3.7
Injured lower limb:	
Right, n (%)	42 (58.3)
Left, n (%)	30 (41.7)
Radiographic grade of OA:	
Grade I, n(%)	5 (6.9)
Grade II, n(%)	24 (33.3)
Grade III, n(%)	36 (50.0)
Grade IV, n(%)	7 (9.7)

***BMI**: Body mass index; **OA**: Osteoarthritis.*

Table II. *Treatment success rate after 12 months of follow-up.*

			$n_{baseline}$	$n_{surgeries}$	$n_{drop-out}$	Cumulative success rate (%)*	p**
Global sample			67	8	6	86.9	-
OA grade	I		5	0	0	100.0	0.380
	II		23	3	2	85.7	
	III		32	3	3	89.7	
	IV		7	2	1	66.7	
Gender	F		36	4	5	87.1	0.961
	M		31	4	1	86.7	
Gender by OA grade	I	F	2	0	0	100.0	-
		M	3	0	0	100.0	
	II	F	13	2	2	81.8	0.602
		M	10	1	0	90.0	
	III	F	17	1	2	93.3	0.508
		M	15	2	1	85.7	
	IV	F	4	1	1	66.7	1.000
		M	3	1	0	66.7	

: Kaplan-Meier analysis; **: Log-Rank test; **OA: osteoarthritis; **F**: female; **M**: male; $n_{baseline}$: baseline number of participants; $n_{surgeries}$: number of participants that required surgery; $n_{drop-out}$: number of participants that drop out from the study before the 12 month follow-up for reasons other than surgery.*

Table III. *Pain intensity at 0, 6 and 12 months of follow-up, according the degree of OA.*

OA grade	VAS med (IQR)			p*
	Baseline	6 months	12 months	
Grade I	5.0 (4.0-6.5) (n=5)	2.0 (0.0-4.0) (n=5)	2.0 (0.0-6.0) (n=5)	0.174
Grade II	5.0 (5.0-7.0) (n=21)	1.0 (0.0-3.0) (n=20)	0.0 (0.0-5.0) (n=19)	<0.001
Grade III	6.0 (5.0-7.0) (n=32)	2.0 (0.0-3.0) (n=30)	2.0 (0.0-6.5) (n=29)	<0.001
Grade IV	6.0 (5.5-7.5) (n=5)	1.5 (0.0-5.3) (n=4)	3.5 (0.0-7.8) (n=4)	0.135
Grade I,II,III & IV	5.0 (5.0-7.0) (n=63)	2.0 (0.0-3.0) (n=59)	2.0 (0.0-6.0) (n=57)	<0.001

: Friedman test; **VAS: Visual analogue scale; med: median; **IQR**: Interquartile range (percentile 25 to 75).*

Table IV: *Comparison of pain intensity between participants that required surgery and the ones who did not, at 0 and 6 months of follow-up, according the degree of OA.*

OA grade	VAS med (IQR)					
	Baseline			6 months		
	Without surgery	With surgery	p*	Without surgery	With surgery	p*
Grade I	5.0 (4.0-6.5) (n=5)	-	-	2.0 (0.0-4.0) (n=5)	-	-
Grade II	5.0 (5.0-7.0) (n=21)	7.0 (n=3)	0.041	1.0 (0.0-3.0) (n=20)	5.5 (n=2)	0.035
Grade III	6.0 (5.0-7.0) (n=32)	6.0 (5.3-6.8) (n=4)	0.903	2.0 (0.0-3.0) (n=30)	5.0 (4.5-5.5) (n=3)	0.011
Grade IV	6.0 (5.5-7.5) (n=5)	6.5 (n=2)	1.000	1.5 (0.0-5.3) (n=4)	5.0 (n=2)	1.000
Grade I,II,III & IV	5.0 (5.0-7.0) (n=63)	7.0 (6.0-7.0) (n=9)	0.096	2.0 (0.0-3.0) (n=59)	5.0 (4.0-6.0) (n=7)	0.004

**: Mann-whitney test; OA: osteoarthritis; VAS: Visual analogue scale; med: median; IQR: Interquartile range (percentile 25 to 75).*

Table V. *Comparison of pain intensity between female and male genders at 0, 6 and 12 months of follow-up, according the degree of OA.*

OA grade	VAS med (IQR)								
	Baseline			6 months			12 months		
	Female	Male	p*	Female	Male	p*	Female	Male	p*
Grade I	4.0 (n=2)	5.0 (4.5-6.5) (n=3)	0.800	1.0 (n=2)	2.0 (1.0-4.0) (n=3)	0.800	1.0 (n=2)	2.0 (1.0-6.0) (n=3)	0.800
Grade II	5.0 (5.0-6.8) (n=12)	6.0 (5.0-7.0) (n=9)	0.219	2.0 (0.0-3.0) (n=11)	0.0 (0.0-3.5) (n=9)	0.603	1.5 (0.0-3.5) (n=10)	0.0 (0.0-7.0) (n=9)	1.000
Grade III	7.0 (5.0-7.5) (n=17)	5.0 (5.0-6.0) (n=15)	0.044	2.0 (0.0-3.0) (n=15)	2.0 (0.0-4.0) (n=15)	0.870	2.0 (0.0-5.0) (n=15)	4.0 (0.8-7.0) (n=14)	0.290
Grade IV	7.0 (5.5-7.0) (n=3)	6.5 (n=2)	0.800	1.5 (0.0-3.0) (n=2)	3.0 (n=2)	0.667	3.5 (0.0-7.0) (n=2)	4.0 (n=2)	0.667
Grade I,II,III & IV	5.5 (5.0-7.0) (n=34)	7.0 (6.0-7.8) (n=29)	0.577	2.0 (0.0-3.0) (n=30)	2.0 (0.0-4.0) (n=29)	0.855	2.0 (0.0-4.5) (n=29)	2.0 (0.0-7.0) (n=28)	0.285

***: Mann-whitney test; **OA**: osteoarthritis; **VAS**: Visual analogue scale; med: median; **IQR**: Interquartile range (percentile 25 to 75).*

Table VI: *Correlation between age, BMI and VAS score, at 0, 6 and 12 months.*

Variable	VAS		
	Baseline	**6 months**	**12 months**
Age	0.239 (p=0.044) (n=72)	0.378 (p=0.002) (n=66)	0.291 (p=0.028) (n=57)
BMI	0.251 (p=0.033) (n=72)	-	-

***VAS**: Visual analogue scale; **BMI**: Body mass index.*

Table VII: *Hip function (HOOS) at 0, 6 and 12 months of follow-up, according the degree of OA.*

OA grade	HOOS med (IQR)			p*
	Baseline	6 months	12 months	
Grade I	60.6 (50.0-61.6) (n=5)	78.1 (53.5-94.1) (n=5)	78.1 (47.8-94.1) (n=5)	0.174
Grade II	52.5 (48.1-55.6) (n=19)	85.6 (65.0-91.9) (n=19)	86.3 (48.8-91.9) (n=19)	<0.001
Grade III	53.2 (49.4-58.4) (n=30)	70.7 (64.1-88.4) (n=30)	73.1 (50.0-88.2) (n=29)	<0.001
Grade IV	55.4 (52.4-60.6) (n=4)	69.7 (64.7-87.4) (n=4)	65.4 (49.4-87.4) (n=4)	0.092
Grade I,II,III & IV	53.8 (48.6-59.4) (n=58)	73.5 (64.9-90.0) (n=58)	78.1 (50.0-90.0) (n=57)	<0.001

** Friedman test; HOOS - Hip Disability and Osteoarthritis Outcome Score; med: median; IQR: Interquartile range (percentile 25 to 75).*

Table VIII. *Comparison of hip function (HOOS) between participants that required surgery and the ones who did not, at 0 and 6 months of follow-up, according to the degree of OA.*

OA grade	HOOS med (IQR)					
	Baseline			6 months		
	Without surgery	With surgery	p*	Without surgery	With surgery	p*
Grade I	60.6 (50.0-61.6) (n=5)	-	-	78.1 (53.5-94.1) (n=5)	-	-
Grade II	52.5 (48.1-55.6) (n=19)	40.6 (32.8-42.5) (n=3)	0.003	85.6 (65.0-91.9) (n=19)	49.4 (37.2-49.7) (n=3)	0.003
Grade III	53.2 (49.4-58.4) (n=30)	40.4 (30.4-43.3) (n=4)	<0.001	70.7 (64.1-88.4) (n=30)	46.0 (38.1-51.9) (n=4)	<0.001
Grade IV	55.4 (52.4-60.6) (n=4)	37.5 (24.4-50.6) (n=2)	0.133	69.7 (64.7-87.4) (n=4)	46.0 (15.6-76.3) (n=2)	0.800
Grade I,II,III & IV	53.8 (48.6-59.4) (n=58)	40.6 (26.5-43.9) (n=9)	<0.001	73.5 (64.9-90.0) (n=58)	49.4 (31.3-51.9) (n=9)	<0.001

**: Mann-whitney test, HOOS: Hip Disability and Osteoarthritis Outcome Score; med: median; IQR: Interquartile range (percentile 25 to 75).*

Table IX: *Comparison of hip function (HOOS) between female and male genders at 0, 6 and 12 months of follow-up, according the degree of OA.*

OA grade	HOOS med (IQR)								
	Baseline			6 months			12 months		
	Female	Male	p*	Female	Male	p*	Female	Male	p*
Grade I	60.6 (n=2)	59.4 (50.0-61.0) (n=3)	0.800	83.1 (78.1-88.1) (n=2)	75.6 (53.5-87.8) (n=3)	0.800	83.1 (78.1-88.1) (n=2)	75.6 (47.8-87.8) (n=3)	0.800
Grade II	52.9 (47.2-55.2) (n=10)	52.5 (48.1-61.3) (n=9)	0.780	72.5 (60.8-90.9) (n=10)	90.0 (66.3-95.0) (n=9)	0.315	80.7 (57.4-90.9) (n=10)	90.0 (4603-95.0) (n=9)	0.720
Grade III	53.8 (45.6-58.1) (n=15)	52.5 (50.0-59.4) (n=15)	0.567	72.5 (64.4-89.4) (n=15)	68.8 (61.9-88.1) (n=15)	0.838	78.1 (50.0-89.4) (n=15)	67.6 (47.5-87.5) (n=14)	0.652
Grade IV	57.9 (53.8-61.9) (n=2)	54.4 (51.9-56.9) (n=2)	0.667	69.1 (64.4-73.8) (n=2)	78.8 (65.6-91.9) (n=2)	0.667	60.4 (46.9-73.8) (n=2)	74.4 (56.9-91.9) (n=2)	0.667
Grade I,II,III & IV	53.8 (46.9-59.1) (n=29)	52.5 (50.0-59.4) (n=28)	0.652	73.1 (64.4-89.1) (n=29)	75.6 (65.3-91.9) (n=28)	0.402	78.1 (55.2-89.1) (n=29)	79.1 (47.7-91.4) (n=27)	0.917

: Mann-whitney test; ***HOOS: Hip Disability and Osteoarthritis Outcome Score; ***med***: median; ***IQR***: Interquartile range (percentile 25 to 75).*

Table X: *Correlation between age, BMI and VAS score, at 0, 6 and 12 months.*

Variable	HOOS		
	Baseline	6 months	12 months
Age	-0.326 (p=0.007) (n=67)	-0.455 (p<0.001) (n=67)	-0.371 (p=0.005) (n=57)
BMI	-	-	-

assessment point (0, 6 and 12 months) there were no statistically significant differences between the 4 grades of hip OA ($p>0.05$).

The patients who did not required surgery had statistically significant higher HOOS figures at baseline and 6 months for all the OA grades, except grade IV ($p<0.05$; Table VIII). Conversely, no statistical significant differences were found in the HOOS figures between female and male genders at 0, 6 and 12 months follow-up, neither according to the grade of OA, or when comparing global sample ($p>0.05$; Table IX).

The HOOS score had a weak and negative correlation with age at baseline ($p<0.01$), at 6 months ($p<0.001$) and at 12 months ($p<0.01$).

DISCUSSION

The main findings of this prospective study are that intra-articular injections of HT+HA are effective in reducing the pain intensity and enhancing function and quality of life in patients with hip OA, with a good overall success rate (86.9%).

The HA injections led to statistically significant improvements on the reported intensity pain in patients with grade II and III of hip OA, obtained at 3 months follow-up which lasted until the 12 month follow-up.

The use of intra-articular injections of HT alone showed to be effective approach to decrease pain intensity and improve hip function in patients with hip OA, lasting up to 3 months (24).

A study performed by Migliore et al. (25) showed that intra-articular injections of HA could lead to decrease on the analgesic drugs intake and pain relief that lasted at least 18 months. Along the same lines, Migliore et al. (26), in a prospective, double-blinded, randomized trial, achieved statistically significant pain reduction at 3 and 6 months of follow-up in patients with hip OA. The pain relief is estimated to be around 40 and 50% in most of the available studies (13). Additionally, Migliore et al. (27) also found significant pain relief after intra-articular HA hip injections in younger patients after a 12 month follow-up.

In this study, it was found that the administration of HT+HA led to significant improvement of the hip function in patients with grade II and III of hip OA, which held for the 12 months of follow-up. These findings are also in line with the ones reported in the scientific literature showing significant improvements in the hip function and quality of life following intra-articular hip injections of HA alone ((28, 29).

This study's results suggest that the combined use of HT+HA may improve the outcomes in patients with hip OA, with higher efficacy than those reported for HT or HA alone.

In the present study an 86% overall success rate was achieved after 12 month follow-up, with significant rates for grade I (100%), grade II (86%) and III (90%) of hip OA. These findings suggest that the administration of intra-articular HT+HA may delay the need for arthroplasty surgery, while providing a pain-free quality of life to these patients. Likewise, Michel et al. (30) reported that the administration of intra-articular HA delayed the need for surgical intervention in 45% of their patients for at least 6 months.

Although good results are reported in scientific literature, the intra-articular injection of HA in hip OA still remains controversial due to the difficulty in reaching deeper localizations of the joint and to the proximity of neurovascular structures. There is a lack of published studies regarding HA effectiveness in symptomatic and radiographic hip OA, and no studies were found regarding the combined use of HT+HA in patients with hip OA. Nonetheless, it is still important to acknowledge the limitations in comparing the results among the available different studies, due to the heterogeneity on the injection technique, the HA concentration and molecular weight, the number of injections, time-periods chosen for the HA administration, outcomes measures used and different follow-up periods. It is referred in the scientific literature that intra-articular injections of corticosteroids may increase the risk of hip infection during hip arthroplasty procedures (31), which has not happened in this subset of patients. Most likely due to the fact that all the hip arthroplasties were performed with more than 6 months of interval between the HT intra-articular injection and the reconstructive surgery.

The major limitation of the present study was the absence of a control group, which would have allowed the comparison of the results against other

type of injections, or, a group without any intervention which could have therefore calculated the effective size achieved with the application of intra-articular HT+HA injections in this subset of patients with hip OA.

Future research should focus on applying a standardized and prospective approach (same number and periods of HT+HA injections, with the same concentration and molecular weight) with longer follow-up times to assess the duration of the HT+HA effects and estimate the success in delaying the need for arthroplasty surgery. In addition, the application of validated and standardized outcome measures (at the same follow-up points), including patient-reported pain, functionality and quality of life measures.

In conclusion, this study showed that intra-articular injections of HT+HA under ultrasound guidance is a safe method which may ameliorate pain and improve function and quality of life in patients with symptomatic and radiographic hip OA, over a period of 12 months. Moreover, it delays, for at least 12 months, the need for interventional surgery in 86% of the patients, making it a relevant option in patients that are not willing to or cannot undergo surgery. These satisfactory results are encouraging for the use of HT+HA in patients with hip OA.

ACKNOWLEDGMENTS

The authors would like to sincerely acknowledge André Silva for his valuable help regarding the acquisition of illustrations.

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HOMOLOGOUS PLATELET-RICH PLASMA FOR THE TREATMENT OF KNEE INVOLVEMENT IN PRIMARY SJÖGREN'S SYNDROME

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Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterized by dry eyes, dry mouth, and other clinical manifestations. The most common extraglandular manifestation of pSS is articular involvement and to date their management is unclear. The aims of the current pilot study were to assess the safety and the outcomes of homologous platelet-rich plasma (HPRP) injections in pSS cohort affected by knee arthralgia/arthritis at short-term follow up. This pilot study provides the first evidence that HPRP injections are a safe treatment and induce a short-term clinical improvement. Although the lack of a control group, randomization and long-term follow up prevents the assessment of the real effectiveness of this treatment, further studies are needed to confirm these findings and to determine the mechanism of action, biological changes and disease-modifying properties of PRP.

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease, characterized by a focal lymphocytic infiltration of exocrine glands, with a higher incidence in female patients (9:1) and a prevalence of 0.5% in the general population. It is usually diagnosed between the ages of 40 and 60, but it also affects children and the elderly (1). SS is best defined as the triad of dry eyes, dry mouth, and evidence of an autoimmune process mediating these and other clinical manifestations. The most widely accepted classification criteria are those proposed in 2002 by the American-European Consensus Group (AECG) (2). SS is termed "secondary"when it occurs with another systemic rheumatic disease and "primary (pSS)" when such a second disease is absent..

The spectrum of pSS extra-glandular manifestations is broad and includes fatigue, vasculitis, peripheral neuropathy, kidney involvement, interstitial lung disease, lymphoproliferative disease, immunological abnormalities and joint involvement (3). Articular manifestations seem to affect both genders similarly, and are some of the most common extra-glandular pSS manifestations, with a frequency range of 15-90% (4). Joint symptoms can precede the onset of sicca symptoms (17%), occur simultaneously (52%) or also develop after pSS diagnosis with a mean period of 51 months (31%) (5). The most common symptoms reported are arthralgia involving equally small and large joints (6) and intermittent symmetrical non-erosive poliarthritis characterized by tenderness, swelling or effusion affecting mainly the small joints (7).

Recurrent monoarthritis or oligoarthritis are also reported in 10-20% of cases (8). Despite the fact that joints involvement has been reported in the last 30 years, their treatments have been paradoxically poorly studied. Joint symptoms are not adequately controlled by the wide spectrum of pharmacologic options used in clinical practice and chronic therapy

Key words: primary Sjogren's syndrome, homologous platelet, knee involvement, synovial membrane

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0393-974X (2016)

of non-steroidal anti-inflammatory drugs (NSAIDs), steroid and/or disease-modifying anti-rheumatic drugs (DMARDs) such as hydroxychloroquine, methotrexate, leflunomide, cyclosporine, TNF antagonists, and rituximab can result in important adverse events. Platelet Rich Plasma (PRP) came under the spotlight because of its regenerative potential and promising preliminary clinical results in several conditions (9). Platelets contain many important bioactive proteins and growth factors (GFs) that are able to regulate tissue repair. The GFs stimulate chondral anabolism and slow its catabolic processes, influence overall joint homeostasis, reduce synovial membrane hyperplasia and modulate cytokine levels (10). Autologous PRP (APRP) is the safest and most frequently used. However, if it is prepared from a patient with autoimmune diseases, it might have highly concentrated pro-inflammatory cytokines and accelerate the progressive cartilage degeneration (11). Homologous PRP (HPRP) is obtained from healthy, screened and habitual blood donors. In a recent cohort study we used HPRP to treat elderly patients with knee OA who were not candidates for autologous PRP treatment (12). HPRP had an excellent safety profile and offered a short-term clinical improvement. However, to date there are no published studies that evaluate the effect of HPRP intra-articular injections for knee arthralgia and/or arthritis in pSS. The primary aim of the current pilot study was to assess the safety of HPRP injections in patients with pSS. The secondary aim was to evaluate the outcomes of HPRP therapy in the pSS cohort at short-term follow up.

MATERIALS AND METHODS

This prospective open-label, single-center, uncontrolled, pilot study was conducted with the highest respect for individual participants. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the eighth revision of the Declaration of Helsinki, Seoul 2008. Before the beginning of any study-related activities, each study participant signed informed consent. Inclusion criteria were: pSS, history of chronic (>4 months) pain or knee swelling, limitation of daily activities, and radiographic imaging findings of degenerative joint changes (Kellgren-Lowrence grade I-IV). Exclusion criteria were: systemic cardiovascular or neurologic disorders, diabetes, septicemia or fever, cutaneous infections in the area to be injected, use of corticosteroids for two to three weeks before the procedure, use of NSAIDs in the 3 weeks before treatment, anticoagulation use 5 days before the procedure, and previous knee surgery. Standard weight-bearing anterior-posterior radiographs with knees in full extension were performed to determine the OA grade. All patients were treated in the Azienda Ospedaliero-Universitaria Ospedali Riuniti of Ancona (Italy), following the same inclusion criteria and were prospectively evaluated at 7 days, 30 days and 90 days. The production of HPRP followed the SIMTI recommendations on blood components for non-transfusional use (13).

Every patient received three injections of 5 ml of HPRP poor in leukocytes, containing a concentration of 1200-1600×103/μl, at 14 day intervals and no further injection during the follow-up period. Before the injection, 10% calcium chloride (Ca++ 0.22 mEq per dose) was added to HPRP to activate coagulation. The skin was sterilely dressed and the infiltration was performed through a classic lateral approach with a 22-gage needle under ultrasound guidance (7.5 MHz transducer). All patients received a series of instructions after every injection. In case of knee pain during the treatment cycle, cold therapy and rest was recommended for at least 24 hours. Mild activities and a gradual resumption of normal sport or recreational activities were allowed as tolerated (14). Each patient was monitored for 90±3 days after the intra-articular injections, through periodic visits, the first at enrollment and injection and then after 7 days, 30±3 days and 90±3 days after injections.

During each visit, each patient was assessed by measurement of the subjective knee pain, rated on a visual-analogue scale of 100 mm (EQ VAS). The assessment of patient status was evaluated through: questioning if treatment was considered satisfying (YES/NO) with respect to all the daily activities; the intensity of pain and the physical disability; global assessments of the treatment by both patient and investigator through a score from Excellent (4) to No (0); algo-functional Lequesne index for knee osteoarthritis (from 0 to 24). Adverse effects were also recorded. The Student's *t*-test

was performed to compare preoperative and postoperative values. Data are expressed as a mean, standard deviation (SD) and $p < 0.05$ was considered significant for one-tailed tests. The statistical software SPSS (version 17.0) was used for biometric analysis.

RESULTS

From October 2011 to April 2016, 18 patients were assessed for eligibility. The study sample consisted of 16 women and 2 men, mean age 47±6.92. Four subjects were suffering from unilateral knee OA, while 14 subjects had bilateral knee OA. In the latter patients, the treatment was performed in the symptomatic side. All patients completed the follow-up at 90 days. The radiological classification, carried out according to the Kellgren and Lowrence criteria (KL) by two experimenters, allowed to stratify the sample of the study: 12 patients (66.67% of the sample) had I degree, 4 patients (22.22% of the sample) II degree and 2 patients (11.11%) III degree. No systemic adverse event was observed. Three patients reported only minor side effects, such as transitory intra-articular burning sensation immediately after the injection or mild articular pain for a few days. No patients considered their status satisfying before treatment. Fourteen patients (77.78%) considered their status satisfying during or after the treatment. Three patients considered their status satisfying only at 7 days follow-up while one patient never considered her status satisfying. In regards to the overall effectiveness of the treatment, 13 patients (72.22%) reported good (3) results for the duration of follow-up, 2 patients reported little or no (0-1) effectiveness throughout the follow-up and 3 patient reported good (3) scores only at 7 days follow-up. There were statistically significant differences in pre- and post-treatment values of EQ VAS and algo-functional Lequesne index ($p<0.005$). The values of mean ± SD of EQ VAS at baseline were 79±9, at 7 days follow-up 49±17, at 30 days follow-up 46±15 and at 90 days follow-up 47±14.

The values of mean ± SD of algo-functional Lequesne index at baseline were 13.1±3.2, at 7 days follow-up were 7.9±4.6, at 30 days follow-up were 7.3±4.4 and at 90 days follow- up were 7.3±4.4.

DISCUSSION

The clinical study of pSS has traditionally been based on a diagnostic and therapeutic approach predominantly focused on glandular involvement, even though pSS is undeniably more than a sicca disease. The most common extra glandular manifestation of pSS is articular involvement, with a high prevalence of arthralgia (joint pain without inflammatory signs) observed in 48% of patients, and a lower prevalence of arthritis (defined as "articular swelling and/or joint effusion" due to synovitis) present only in 15% (15). The knee is one of the most affected joints, but metacarpophalangeal (MCP), proximal interphalangeal (PIP) joints, wrists, ankle, shoulder and hips can be involved as well (16). Articular manifestations are usually treated by symptomatic measures or conventional DMARDs in an off-label setting; however, the efficacy of these treatments is uncertain. This is the first study in pSS cohort investigating the effects of HPRP on joint involvement. In regards to the primary aim of the study, we observed no severe complications related to the injections during the treatment and follow-up period, but only minor side effects also common to HPRP therapy (14).

Regarding the secondary aim, 77.78% of the population considered their status satisfying during or after the treatment, while 72.22% reported good results for the duration of follow-up. There were statistically significant differences in pre- and post-treatment values of EQ VAS and algo-functional Lequesne index, demonstrating a marked reduction of pain and improvement of knee function.

In the present study, we can only hypothesize the mechanism of action of HPRP based on previous studies in literature. HPRP obtained from healthy, screened and habitual blood donors presents several advantages such as ease of preparation, almost unlimited availability and limited costs. Recent clinical trials demonstrated that multiple intra-articular PRP injections are a safe treatment for knee OA, able to reduce pain and to recover articular function (17). Furthermore, in an our recent study conducted in elderly patients (aged 65-86 years) affected with unilateral knee OA and degenerative

joint changes (Ahlbäck grade I to III), HPRP treatment resulted in a statistically significant improvement in IKDC, KOOS and EQ VAS scores at 2- and 6-month follow-up. Moreover, three patients were affected by systemic lupus erythematosus (SLE) and all of them showed the same trend with no adverse effects recorded (12).

APRP, obtained from the patient blood, is the safest and most frequently used (18). On the other hand APRP could differ from a patient to another according to physiological or pathological conditions. For this reason, APRP prepared from patients with autoimmune diseases, such as rheumatoid arthritis, SLE or pSS, might have high concentration of pro-inflammatory cytokines that could negatively influence joint homeostasis (11). Furthermore, the majority of studies available in literature considered platelet disorders and autoimmune diseases as exclusion criteria for APRP treatment (19-21). For these reasons, we preferred to use HPRP. Our results provide the first evidence that HPRP injections are a safe treatment and induce a short-term clinical improvement in patients with pSS affected by knee arthralgia/arthritis. Although these findings are encouraging, the lack of a control group, randomization and long-term follow up prevents the assessment of the real effectiveness of this treatment. The marked improvement could be due to a placebo effect and therefore the efficacy of HPRP is not ultimately demonstrated at this pilot stage. Therefore, further studies are needed to confirm these findings and to determine the mechanism of action, biological changes and disease-modifying properties of PRP.

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BONE MARROW CONCENTRATED CELLS AND STROMAL VASCULAR FRACTION CELLS INJECTIONS FOR OSTEOARTHRITIS TREATMENT: A SYSTEMATIC REVIEW

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The aim of this systematic review is to examine current clinical evidences supporting the intra-articular injection of bone marrow concentrate cells (BMC) and adipose-derived stromal vascular fraction cells (SVF) for the treatment of osteoarthritis (OA). The research was performed on PubMed (Medline), EMBASE and Cochrane Library considering the English literature. Only clinical trials have been included. The systematic research identified twelve clinical trials. Articles included in the study, were one of level II, four of level III, six of level IV and one level V. Among clinical trials, none were randomized, four were comparative, seven were case series, and one was a case report. Seven studies were focused on the use of SVF (1332 patients) and five on the use of BMC (963 patients), with preliminary interesting findings in the OA treatment. Despite the growing interest in this biological approach for OA, knowledge on this topic is still preliminary. Randomized controlled trials are needed to support the potential of BMC and SVF injections and to evaluate advantages and disadvantages with respect to the available treatments.

Osteoarthritis (OA) is the most common joint disease, affecting about 10 to 25% of the population. It is characterized by an increasing incidence in western world because of the longer life expectancy and obesity (1). OA is characterized by functional impairment and clinical symptoms such as pain, stiffness, tenderness and joint swelling. The main pathologic feature of OA is degradation of articular cartilage, but other joint tissues, such as synovial fluid and membrane and subchondral bone are involved (2).

Therapeutic strategies for OA include systemic administration of non-steroideal inflammatory drugs (NSAIDs), glucosamine and/or chondroitin-sulfate, intrarticular injections of hyaluronic acid or growth factors as platelet rich plasma (PRP) (3). To date, all the described therapies have demonstrated to partially relieve symptoms, minimize disability and temporary increase joint function. None of them are able to regenerate joint tissue, with a real impact on the progression of the condition. A new possible regenerative approach has been hypothesized since the identification of stem/progenitor cells. Mesenchymal stem cells (MSCs) have been isolated in several tissues such as bone marrow, adipose tissue, synovia, skeletal muscle, placenta, amniotic fluid (4).

In the last few decades, MSCs have gained interest as a possible strategy for OA treatment, in particular in early-moderate stages (5). *In vitro* and preclinical studies demonstrated that the use of MSCs shows benefits both in terms of cartilage

Key words: osteoarthritis, stem cells, bone marrow concentrate, stromal vascular fraction

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0393-974X (2016)

restoration and local inflammation control (6, 7). MSCs can directly participate in articular reparative process differentiating in cartilage to replace the damaged one. A paracrine effect has been identified resulting in a coordination and support function (trophic effect). Chemotactic properties mediated by the secretion of cytokines and growth factors have also been shown to enhance the intra-articular microenvironment resulting in a local recruitment of further resident stem cells. Moreover, the MSCs ability to identify and deactivate macrophages in the synovial tissue has been recently highlighted (4).

Administration of MSCs can be performed in a one-day session right after harvest and concentration (one-step) or after isolation and expansion in culture (two-step). The two procedures significantly differ in terms of both number and purity of cells. The expanding procedure is characterized by enormous costs and regulatory burden, making this procedure far from clinical translation in daily use. Therefore, researchers are pursuing efforts to optimize therapies with the use of either bone marrow concentrate (BMC) or the stromal vascular fraction (SVF) isolation from adipose tissue without recurring to expansion.

BMC can be obtained by centrifugation after bone marrow aspiration form the iliac crest or other bone marrow donor sites (8). SVF can be obtained from adipose tissue immediately after lipoaspiration by enzymatic digestion and centrifugation or micro-factorization and filtration (9).

Recently, clinical studies have been carried out worldwide using either BMC or SVF cells for the treatment of OA. However, which is the most suitable cell source and the most reliable technique is still controversial.

The aim of this article is to provide a systematic review on the available clinical data on the intra-articular injection of isolated MSCs using one-step technologies from both BMC and SVF for OA treatment.

MATERIALS AND METHODS

The systematic review was performed on 1st September 2016. Two independent reviewers (GV and LLV) separately conducted the research. All journals were considered and all relevant articles were analysed. Only articles published in a peer-reviewed journal were included. All articles were initially screened for relevance by title and abstract, excluding articles without an abstract, and obtaining full-text articles if the abstract did not allow the authors to assess the defined inclusion and exclusion criteria. Authors reviewed the abstract of each publication and performed a close reading of all papers and extracted data, to minimize selection bias and errors. Trials fulfilling the review inclusion criteria were assessed for risk of bias by the two reviewers, independently, using the Newcastle-Ottawa score (NOS). The score assigns a study a maximum of 8 points, with higher scores indicating a lower risk of bias.

A cross-reference research of the selected articles was also performed to obtain other relevant articles for the study. The following databases were screened: PubMed (Medline), EMBASE and Cochrane Central Registry of Controlled Trials. All articles reporting outcomes on BMC and SVF infiltration strategies, performed without other surgical procedures, for OA management have been included. Articles in English were included. According to the Oxford centre of EBM, level I, II, III, IV articles were found in the literature and included in our study. Literature reviews, studies on cadavers or *in vitro*, biomechanical reports, technical notes, letters to editors and instructional course, were excluded. Clinical reports with adoption of MSCs for osteochondral defects were not included. Publications reporting the use of cultured MSC were excluded.

Finally, to avoid bias, the selected articles, the relative list of references and the articles excluded from the study were reviewed, assessed and discussed by all the authors and if there was disagreement among authors regarding inclusion and exclusion criteria the senior authors (VD and MAR) made the final decision.

RESULTS

The literature search and cross-referencing resulted in 221 references. In particular, 139 articles were excluded because the abstract was out of the topic and/or inclusion criteria were not respected. Of the 82 abstracts selected as potentially relevant for review of the full text, 70 papers were excluded for

not meeting the inclusion criteria, because regarding only *in vitro* and animal studies or the use of cultured MSCs, with an agreement by κ coefficient of 0.92. Therefore, 12 papers were considered for data collection and evaluated by two reviewers, with 100% agreement for quality assessment. They were classified according to EBM guidelines as one of level II, four level III, six level IV and one level V. Among the twelve clinical trials identified focusing on OA, none were randomized, four were comparative, six were case series, and one was a case report; six concerned the use of SVF (1332 patients, Table I) and five the use of BMC (963 patients, Table II) with preliminary interesting findings in the OA treatment.

BMC one-step injections

The first experiences in the field of one-step stem cells therapy applied to the treatment of OA were conducted with BMC, which was the most widely investigated donor site. In 2006, Centeno et al. published the first clinical case reporting the use of stem cells for articular disorders (10). These authors described the use of a one-step technique in a 64 years-old male patient affected by a severely degenerated hip. At last follow up patient reported both clinical (pain and function) and radiological improvements.

Two authors published data on BMC injections in OA of the knee. Kim et al. reported data of 41 patients (75 knees) treated with injection of BMC combined with adipose tissue harvested from abdominal fat (11). BMC injection significantly improved both knee pain (mean VAS values from 7.0 preoperatively to 3.3 at 12 months of follow up) and patient-reported survey of patient health (mean SF-36 values from 31.5 to 47.7 at last follow up). Knee functional scales were also improved at last follow-up. Improvement of VAS score and functional scales were significantly poorer in patients with severe OA [Kellgren-Lawrence (K-L) grade 4] than those affected by early-moderate OA

Table I. *Adipose-derived stromal vascular fraction cells used as one-step stem cell based therapy for the treatment of OA.*

Study	Level of evidence	Patients number	Mean Age (yrs)	Follow-up (months)	Patient population	MSC origin	Administration details	Safety	Efficacy at last follow up	Features
Pak et al. 2011	Level IV: case series	2	74,5	3	Patients over 70 affected by OA	Abdominal fat	8.5 cm3 of ASCs, PRP, dexamethasone and hyaluronic acid, followed by four additional PRP injections with calcium chloride every week over a period of a month	N.A.	VAS reduction (90% improvement); functional rating index (from 36 to 12); ROM (from 110^0 to 130^o flexion; from 3^0 to 5^0 extension); MRI	The MRI data for all the patients in this series showed cartilage regeneration in the patients with OA. Physical therapy outcomes, subjective pain, and functional status all improved.
Koh et al. 2012	Level III; case control study	25	54	16	Patients over 70 years, with OA, K-L IV, previous cortisone injection	Infrapatellar fat pad	3-4 hours post arthroscopy; 1.89 x 10^6 (range 1.2-2.3 x 10^6) MSC + 3.0 mL PRP in a single intrarticular injection, followed by PRP on day 7 and 14	No major adverse events. One patient with marked swelling and pain post-injection, resolved sponaneously after 2 weeks	Mean Lysholm score: from 41 to 68; mean Tegner activity scale: from 1.5 to 2.8; mean VAS from 4.9 to 2.8	Patients with ICRS III OA on scope showed greater improvement than those with OA IV; -patients <55 years had improved Tegner activity scale and VAS outocmes
Koh et al. 2013	Level IV: retrospective case series	18	54.6	24.3	Patients over 40 years, affected by knee OA (K-L III-IV)	Infrapatellar fat pad	3-4 hours post arthroscopy; 1.18 x 10^6(range, 0.3-2.7 x 10^6) MSC + 3.0 mL PRP in a single intrarticular injection, followed by PRP on day 7 and 14	No major adverse events. One patient with marked swelling and pain post-injection, resolved sponaneously after 2 weeks.	CLINICAL: mean WOMAC score from 49.9 to 30.3; Mean Lysholm score: from 40.1 to 73.4; mean VAS from 4.8 to 2.0 IMAGING: whole-organ MRI score (WORMS) from 60 to 48.3 points	Patients with ICRS III OA showed greater improvement than those with OA IV; Improvements in WORMS correlated with the number of MSC injected (r=0.54; p=0.002)
Y S. Kim et al. 2014	Level III; retrospective comparative study	54	57.5	28.6	Patients with full-thickness cartilage lesion in OA knees (K-L I-II)	Buttock fat tissue	4.23 x 10^7 SVF cells, which contained an average of 3.93x10^6 cells alone, including 39 knees (group 1), or with addiction of fibrin glue, including 17 knees (group 2)	N.A.	Clinical results: group 1, from 38.1to 62.0 (IKDC) and from 2.5 to 6 00.8 (Tegner); group 2, from 36.1 6 6.2 to 64.4 6 11.5 (IKDC) and from 2.2 6 0.8 to 3.8 6 0.8 (Tegner) Histological results: According to ICRS cartilage repair grades, 23% in group 1 and 58% in group 2 achieved a grade of I or II	Clinical and arthroscopic outcomes encouraging for OA knees in both groups, without significant differences in outcome scores between groups (better ICRS grades in group 2)
Koh et al. 2014	Level IV: case series	56	56.6	26.7	Patients with full-thickness cartilage lesion in OA knees (K-L I-II)	Buttock fat tissue	Arthroscopic assisted cartilage defect filling after debridement with 3.83 x10^6 ASC	N.A.	Clinical results: IKDC from 38.06 to 61.0; Tegner activity scale score from 2.5 to 3.6. Histological result at second-look Arthroscopy (ICRS repair grades): 5% grade I, 19% grade II, 54% were grade III, 22% grade IV.	High BMI and large lesion size affect outcomes. Second-look arthroscopic findings revealed that 76% had the repair rated as abnormal or severely abnormal according to ICRS standards
Kim et al. 2015	Level IV: case series	49	58.1	26.7	Patients with full-thickness cartilage lesion in OA knees (K-L I-II)	Buttock fat tissue	Arthroscopic assisted cartilage defect filling after debridement with 4.3 X 10^6 ASC added with fibrin glue as scaffold	N.A.	Clinical results: IKDC from 37.7 to 67.3; Tegner activity score from 2.2 to 3.8. Twenty-four patients reported their overall satisfaction with the surgery as excellent (43.6%), 17 as good (30.9%), 11 as fair (20.0%), and 3 as poor (5.5%).	Patient age (>60y) and lesion size (larger than 6.0 cm^2) are factors that affect clinical outcomes
Michalek et al. 2015	Level III: case control study	1128	62	17.2	Patients affected by OA		Single dose of SVF cells isolated from lipoaspirate by a patent pending kit (Cellthera)	N.A.	Patients gradually improved 3-12 months after the treatment. At least 75% Score improvement was noticed in 63% of patients and at least 50% Score improvement was documented in 91% of patients 12 months after SVF cell therapy.	Obesity and higher grade of OA were associated with slower healing.

Table II. *Bone morrow cells concentrated used as one-step stem cell based therapy for the treatment of OA.*

Study	Level of evidence	Patients number	Mean Age (yrs)	Follow-up (months)	Patient population	MSC origin	Administration details	Safety and Adverse Events (AE)	Efficacy at last follow up	Features
Centeno et al. 2006	Level V: case report	1	63	3	Patient affected by hip OA	Bone marrow	Two MSCs injections combined with hyaluronic acid and thrombin activated platelet rich plasma scaffold, one month aparth	-	VAS and Functional rating index improvement, ROM improvement (15°), MRI showed neocortex formation compared to pre-op MRI	Partial articular surface regeneration at last follow-up
Kim et al. 2014	Level III: retrospective comparative study	41	60.7	8.7	Patients affected by knee OA: Kellgren-Lawrence I n=12(16%); II n=24 (32%);III n=33 (44%);IV n=4 (8%)	Bone marrow	BMC with adipose tissue injection n= 63 (84%);BMC injection with arthroscopic debridement n=6 (8.0%); BMC injection and arthroscopic microfracture in n=5 (6.7 %); BMC and high tibial osteotomy in 1 (1.3 %)	Joint swelling (92.0 %) occurred 2 weeks on average (1.5 through4 weeks) after the procedure and pain (41.3 %) treated with injection of PRP or hyaluronic acid.	CLINICAL: mean VAS score from 7.0 to 3,3 at last f-up (12 months); mean IKDC from 37.7 to 69.3; SF-36 Health score from 31.5 preoperatively to 47.7. Mean KOOS score from 43.1 to 70.6; Lysholm Knee Questionnaire from 37.3 to 71.0	Significative pain and function improvement. More effective in early to moderate OA phases
Centeno et al. 2014	Level III: retrospective comparative study	Group A (without fat graft) 518; group B (with fat graft) 163	54.3	10.4	Group A: 223 K-L I; 146 K-L II; 102 K-L III-IV; Group B: 69 K-L I; 58 K-L II; 39 K-L III-IV	Bone marrow	BMC alone n. 616; BMC with adipose tissue injection n. 224	57 reported AEs, 6% group A; 8.9% group B	Mean LEFS score by 7.9 (group A) and 9.8(group B). Mean NPS score from 4 to 2.6(group A) and from 4.3 to 3(group B). Adverse events rates: 6%(group A) and 8.9%(group B).	Significative pain and function improvement, low rate of adverse events. No statistical differences adopting adipose graft
Centeno et al., 2015	Level II: prospective comparative study	34 Group A (shoulder OA) patients; group B (rotator cuff tear) 81 patients	52,1	24	-	Bone marrow	Time 0: preinjection of 3,5-5 ml 12,5% Hypertonyc dextrose solution; Time1: 3-5 days BMC injection(3.85×10^8cells) +PRP and platelet lisate (OA group)	4,9% reported AEs, 3 post-procedural local pain	DASH from 36.1 to 17.1; NPS from 4.3 to 2.4; improvement rating score 48.8%	No differences between outcomes among the shoulders with OA vs rotator cuff tears; subjective functional improvement
Sampson et al., 2016	Level IV: case series	125	57	5	Patients affected by OA (6 ankle; 27 bilateral knees; 46 unilateral knee; 5 cervical spine; 14 hip; 18 shoulder; 9 other)	Bone marrow	Time O: BMC intraritcular injection; Time1: 8 weeks PRP intrarticular injection	No AE	Pain reduction of 71.4% compared to pretreatment values. Patient satisfaction: 9 out of 10	BMC intrarticular injiection followed by PRP injiection can provide short-term benefic in moderate-to-severe OA

(K-L grade 1-3). Centeno et al. investigated the use of BMC for treatment of knee OA in a large study including 840 procedures, 616 without (group A) and 224 with adipose graft (group B) (12). Pre and post-treatment clinical assessment was performed with the lower extremity functional scale (LEFS), the numerical pain scale (NPS) and a subjective percentage improvement rating.

Outcomes at the last follow up (10 months) were encouraging with pain reduction and function improvement. The mean LEFS score increased by 7.9 and 9.8 in the 2 groups (out of 80), respectively, and the mean NPS score decreased from 4 to 2.6 and from 4.3 to 3 in the two groups, respectively. A low rate of adverse events (<10%) in both groups was also noticed. Addition of adipose graft to the BMC did not provide statistical differences over BMC alone. The author found that independently to the treatment (with or without adipose graft) used, patients with moderate OA (K-L grade 2) were significantly more likely (2.2 times) to report ≥50% improvement on the reported outcome scale in comparison with severe OA group (K-L grade 3-4).

Autologous BMC injections were also tested in a prospective multi-site registry study for symptomatic OA at the gleno-humeral joint. At last follow-up encouraging results were found, with the average DASH score significantly improved from 36.1 to 17.1, the NPS value decreased from 4.3 to 2.4 associated with an average subjective improvement of 48.8% (13).

Sampson *et al.* (14) evaluated the effect of a single BMC intrarticular injection followed by PRP injection at 8 weeks on 125 patients affected by OA. They recorded a median absolute pain reduction of 71.4 % compared to pre-treatment values and median patient satisfaction 9/10.

SVF one-step injections

History of the clinical application of stem cells from adipose tissue is relatively recent. It must be kept in mind that the identification of a stem population contained within the fat occurred about thirty years later than the bone marrow (2001). Pak et al. published the first clinical study reporting 2 cases of knee OA treated with intra-articular injections of SVF, obtained from adipose tissue of abdominal origin by digesting lipoaspirate with

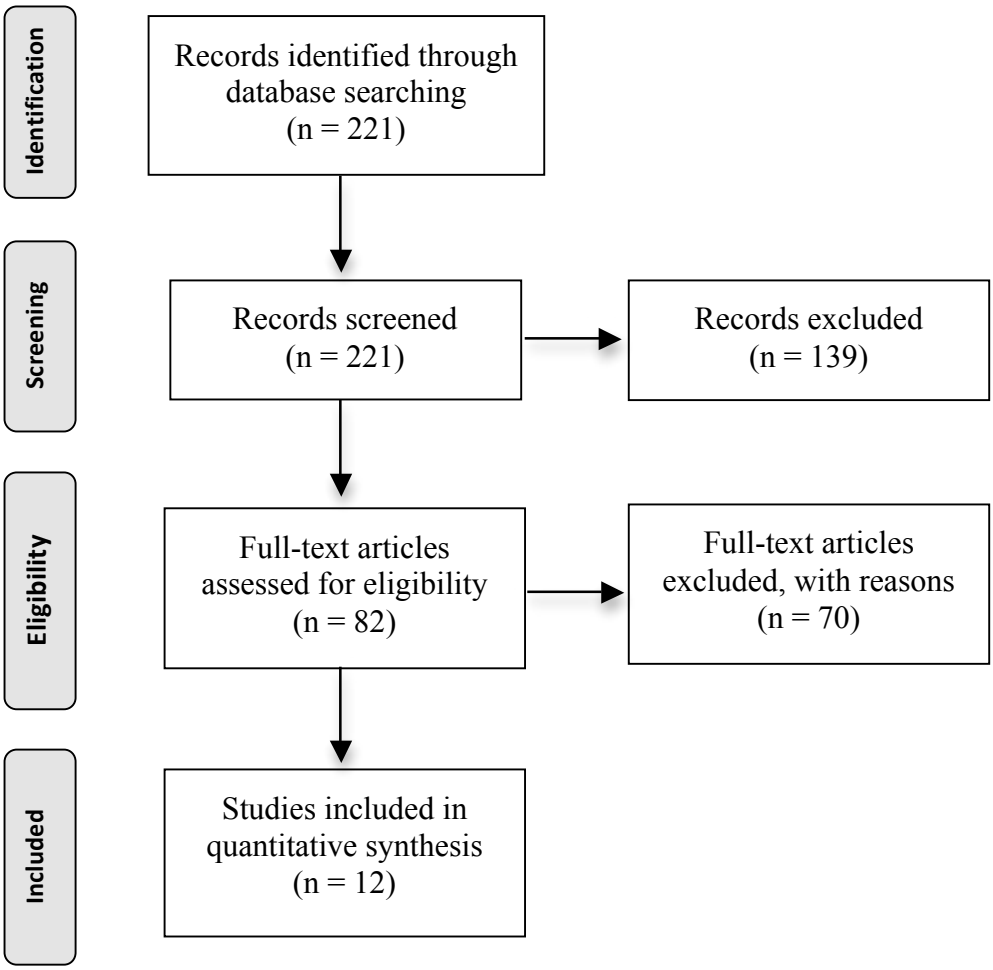

Fig. 1. *PRISMA 2009 Flow diagram.*

collagenase. Patients received a single intra-articular injection of SVF in combination with hyaluronic acid, PRP, calcium chloride and a nanogram dose of dexamethasone was performed, followed by four additional PRP injections with calcium chloride every week over a period of a month. At short term follow up (3 months), the measured physical therapy outcomes, subjective pain, and functional status, all improved and MRI analyses demonstrate increased meniscus cartilage volume (15).

First clinical study with SVF isolated from infra-patellar fat pad was published in 2012 by Koh et al. (16). Twenty-five SVF injections combined with arthroscopic debridement were administered to patients with knee OA. SVF counted a mean of 1.89×10^6 cells suspended in approximately 3.0 mL of PRP and then injected intra-articularly in the selected knees. The mean Lysholm score, Tegner activity scale and VAS scores of patients in the study group improved significantly at short term follow up (16 months) and no major adverse events related to the injections were observed during the treatment. The same research group re-evaluated the clinical and imaging results of the same group of patients (18/25) that received intra-articular injections of SVF for the treatment of knee OA. Treatment outcome appeared to be positively related to the number of cells injected, and results after 2 years were better than short-term results (17).

Kim et al. (18) retrospectively evaluated 54 patients (56 knees) who were examined with second-

look arthroscopy after one-step SVF implantation for cartilage lesions in OA knees. Patients were divided into 2 groups: 37 patients (39 knees) were treated with SVF injected directly into the knee joint, and 17 patients (17 knees) underwent implantation of SVF loaded in fibrin glue as hydrogel directly on the lesion site. At final follow-up (28 months), the mean IKDC score and Tegner activity scale in each group significantly improved. Although there were no clinical significant differences between the two groups, the cartilage repairs, assessed with the International Cartilage Repair Society (ICRS) grade, were better in fibrin glue-added group.

A study of the same research group investigated the clinical and second-look arthroscopic outcomes of SVF implantation evaluating the prognostic factors associated with this treatment. Second-look arthroscopic findings at a mean of 12.7 months postoperatively showed that 24% of the lesions had achieved a normal or near-normal state (ICRS grade I or II) but that 76% of lesions remained in the abnormal state (ICRS grade III or IV) and most ICRS grade III or IV lesions showed incomplete integration with the adjacent cartilage. However, clinical outcomes at mid-term follow up were encouraging, reporting improvement of both IKDC and Tegner score. High BMI (>27.5 kg/m2) and large lesion size (>5.4 cm2) were found to be significant predictors of poor clinical and arthroscopic outcomes (19). A subsequent study including 49 patients (55 knees) treated with SVF associated with fibrin glue as hydrogel, demonstrated that high BMI and large lesion size are important negative prognostic factors. Authors identified the cut-off points for the risk of clinical failure in patients with a lesion size larger than 6.0 cm^2 and those older than 60 years (20).

Michalek et al. (21) followed and evaluated 1114 patients (median age 62, range 19-94 years; 52.8% male) treated with a single dose of SVF cells isolated from lipoaspirate. Patients were followed for a median of 17.2 months. Their evaluations were based on pain, non-steroid analgesic usage, limping, extent of joint movement and stiffness before treatment and at 3, 6 and 12 months. At last follow-up, at least a 75% score improvement was noticed in 63% of the patients and at least a 50% score improvement was documented in 9% of the patients after 12 months.

DISCUSSION

Nowadays, clinical applications of stem cell therapy seem to be very promising, particularly in cardiac, neurologic and orthopaedic fields. Articular cartilage is a potential target of stem cell therapy. Most of the studies in literature report attempts to restore cartilage using *ex-vivo* expanded MSCs. However, expanded MSCs are classified in Europe as Advanced Therapy Medical Products (ATMP) and the regulation beyond their clinical use requires strict controls upon expansion and implantation limiting their large-scale diffusion and increasing the costs. A cellular fraction harvested from bone marrow or adipose tissue followed by centrifugation, enzymatic digestion or micro-fracturing include MSCs that might be directly used on the patient bypassing the expansion process. Therefore, these biologic products are not considered ATMPs allowing a more wide diffusion of this technology with lower costs. There is no agreement whether adipose or bone marrow is a better source for musculoskeletal tissues regeneration in humans. Recent animal studies showed that bone marrow MSCs demonstrated a better chondrogenic potential than adipose derived MSCs (22-24) with production of more hyaline-like matrix and improved glycosaminoglycan (25). However, chondrogenesis can be achieved with adipose derived MSCs at comparable amount than bone marrow MSCs by using greater doses of currently known chondrogenic growth factors (24).

Although MSCs have been isolated in several tissues, their potential applicability in clinical practice as one-step technology for OA treatment has been reported using the MSC isolated from the bone marrow (BMC) and adipose tissue (SVF). Bone marrow is the most studied donor site, as the first to be identified containing a resident stem cell population. BMC injections for OA treatment have been tested for the first time in 2006, reporting

encouraging results both for pain control and for functionality increase. Their application has been described in different joints such as hip, knee and shoulder. Several authors have tested the possibility of combining the infiltration of BMC with fat tissue. However, significant differences were not observed in comparison with the treatment of BMC alone. The best clinical results were registered in patients affected by early stages of OA.

SVF injections have been studied for the treatment of OA since 2011. Different donor sites of adipose tissues graft were tested, including the buttock, the subcutaneus abdominal panicles and the infrapatellar fat pad. Clinical studies showed promising results in terms of pain control and increased functionality. Only the knee joint was treated with injections of ASC. Four studies investigated the use of ASC one-step injections after arthroscopic debridement of the chondral lesions associated with OA. Addition of fibrin glue to ASCs was not associated with clinical improvement.

Regardless of the type of MSCs, the best results were obtained in patients affected by early OA (K-L grade I-II) compared to advanced stages.

CONCLUSIONS

MSCs intraarticular injection using one-step technologies is a promising cell-based strategy for the treatment of OA. Until now, a restricted number of reports on the clinical use of MSCs in OA have been published. Clinical data available from observational studies and case series on the use of MSCs one-step infiltration for OA treatment suggest that this biological treatment is clinically safe showing a positive influence on OA symptoms. BMC and SVF have shown to be more effective in the early stages of OA. However, further investigations with higher levels of evidence, including large randomized trials should be conducted to help answer questions about dosing, timing of intervention, type of MSCs, methods and route of delivery of MSCs.

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THE CYTOKINOME IN OSTEOARTHRITIS, A NEW PARADIGM IN DIAGNOSIS AND PROGNOSIS OF CARTILAGE DISEASE

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At present, diagnosis and progression monitoring of osteoarthritis (OA) is made through radiological and clinical assessment. Several studies investigated the role of synovial fluid analysis, to find out whether joint disease could be characterized by the pattern of cytokines, which acts during the pathogenic process or in specific stages of it. Online PubMed-Medline search was performed in order to retrieve evidence concerning synovial fluid analysis of cytokines involved in OA degenerative process. Concerning pro-inflammatory cytokines, it has been shown that interleukin (IL)-6, TNF-α and IL-17 are mainly over-expressed in the synovial fluid of OA joints, as well as anti-inflammatory cytokine IL-10. Variations of cytokines levels occur with radiological and clinical progression. It was also reported that metalloproteinases are involved. Synovial fluid analysis may be helpful in defining stage and type of OA, but more research is needed, especially focusing on the variation of sets of cytokines during OA stages and correlating these patterns with clinical features.

The term "cytokinome" was first used in 2010 to define the array of immunological molecules which play a role in an inflammatory process of a given tissue. Costantini et al. (1) utilized this term in the perspective of "omics" definition system, to refer to the whole of molecules, their action and their complex interaction network, which act in an organism.

Actually, the term can be applied to every tissue where an inflammatory pathogenesis occurs and cytokines are mainly involved. The exact pattern of molecules implied in a given process is useful in defining the process itself and to determine it as unique. This statement derives from the observation that cytokines always act in concert with other ones, in order to create a defined system of cell-cell signalling (2). Furthermore, given the molecular changes which occur during the disease progression, multiple stages can also be found which analyse the alternation of molecular patterns. Osteoarthritis (OA) is a degenerative disease of cartilage which affects joints of the human body, yielding also an inflammatory process which involves cartilage and synovia (3, 4). It has been well documented that cytokines, including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), cover a main role in degenerative process, along with metalloproteinases (MMP), in degradation of matrix which the chondrocytes are surrounded by (5).

It has been well established that chondrocytes are the main target of cytokines during arthritic process, although exact timing and action of these molecules is far from being clarified. Furthermore, the source of production of cytokines in the joint is not well known (6). Synovia, the tissue which surrounds cartilage and other articular structures and which provide them with nutrition, is commonly involved in OA inflammatory process. In fact, synovitis can be retrieved in many OA models in human and animals. It is commonly accepted that fibroblast-like and macrophage-like cells of the synovia are

Key-words: osteoarthritis, cytokines, Synovial fluid, cytokinome, molecular signalling, cartilage disease

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0393-974X (2016)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

the main sources for cytokines during OA and the study of cytokines array in synovial fluid of arthritic joint may lead to a huge step in diagnosing OA and defining the disease stage. This observation has led to formulating the hypothesis that anti-cytokine therapy may be helpful in the treatment of OA, limiting its progression and restoring a healthy environment, free from pro-inflammatory molecules.

The present work has the main objective of collecting evidence concerning the cytokine ensemble which is present during the various stages of OA in the synovial fluid of joints. Synovial fluid has been taken as a main focus since harvesting and analysis of this tissue is easy and application of experimental procedures in clinical practice is likely to be introduced.

MATERIAL AND METHODS

For article online research, PubMed database was used (http://www.ncbi.nlm.nih.gov/pubmed) in October 2016. Combinations of key-words were used, including: "cytokinome AND osteoarthritis", "cytokine AND osteoarthritis", "inflammation AND osteoarthritis". We searched for any type of studies concerning the role of cytokine and other pro-inflammatory molecules which are found in synovial fluid of joints affected by OA. We excluded studies in which rheumatoid arthritis patients were evaluated, studies on animal models or cadaver studies. No time interval for year of publication was considered. Of each of the articles retrieved, the whole bibliography was screened, in order to enrich the burden of potentially relevant studies. Studies were retrieved in full text, accurately read and main data were extrapolated ad put into tables. A summary of the included studies is reported in Table I.

RESULTS

Cytokines

Several interleukins and similar proteins have been investigated in recent studies, although attention has been particularly focused on IL-6 (6-9), IL-10 (9), TNF-α (8-10) and IL-17 (11, 12). Doß and colleagues (6) showed that IL-6 can be normally expressed or hyper-expressed in synovial fluid of arthritic knees, showing different immunological patterns of OA. Among 49 subjects affected by OA, they found eight of them showing IL-6 concentrations of up to 4500 pg/mL, with SF/serum ratio up to 300 (6). In a sample of 47 patients with knee OA, TNF-α and IL-6 were detectable in SF, whereas nerve growth factor (NGF) was not. When OA stage was evaluated and related to cytokines expression, TNF-α was not correlated with the Kellgren-Lawrence (K-L) grade, whereas IL-6 had negative correlation (8). Moreover, concentrations of those pro-inflammatory cytokines were positively correlated with WOMAC scores in patients with knee OA (8).

In a similar fashion, IL-17 levels were found significantly higher in SF of OA affected knees when compared to healthy controls. Levels also increased with K-L grade and positively correlated with Lequesne index (r=0.6232) (11). Comparable results were found by Liu et al. (13), showing that IL-17 levels in SF were significantly higher in OA patients compared with controls ($P<0.01$), were negatively correlated with OA severity, but positively with WOMAC scale for pain ($r=0.279$, $p<0.05$) (13). Another study by Teunis et al. (9) showed that IL-6, IL-10 and interferon levels were increased in OA of the wrist compared to healthy controls and pre-OA subjects. Conversely, IL-13 and osteoprotegerin (anti-inflammatory) and IL-1β (pro-inflammatory) were lower in arthritic knees when compared to healthy controls (14). Furthermore, antinflammatory cytokine IL-10 showed an increase in OA knees of women compared to sedentary subjects so an exercise protocol was assigned (15). In addition, a study by Sequiera et al. (16) showed that IL-6, along with IL-8 SF levels were over-expressed in the end-stage OA, when compared with SF of patients which had knee injury ($p=0.04$ and $p=0.006$, respectively). It has been therefore hypothesized that IL-6 and IL-8 may be a potential inflammatory role in long-term arthritic inflammation (16). Similarly, IL-18 was found to be increased in knees affected by OA. Its role in inducing the production of PGE2, which in turn can cause cartilage degradation, may reflect a major role of IL-18 in osteoarthritis degenerative process (17). Other studies correlated cytokines expression and OA progression. Among these, Vangsness and colleagues

Table II. *Details of the included studies.*

Study	Year	Study design	Biomarkers evaluated	Significant biomarkers observed into SF	N.er of patients
Mabey et al.	2014	Nine angiogenetic cytokines in plasma and synovial fluid were measured using a multiplex immunoassay.	angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB, PECAM-1, VEGF	angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PECAM-1, VEGF	31 knee OA patients and 15 healthy controls
Chen et al.	2014	Serum and synovial fluid were collected from patients with primary knee osteoarthritis. Cytokine IL-17 was quantified by ELISA.	IL-17	IL-17	98 knee OA patients and 50 healthy controls
Orita et al.	2011	Synovial fluid was harvested from the knees and the levels were measured using ELISA.	TNFa, IL-6, NGF	TNFa, IL-6	47 knee OA patients
Siqueira et al.	2016	SF samples were collected from knees undergoing arthroscopic surgeries due to ligamentous or meniscal knee injuries and primary arthroplasty due to OA. Multiplex cytokine immunoassay were measured in a plate-based format using electrochemiluminescent detection.	IL-6, IL-8, TNF-α, GM-CSF, IFN-γ, IL-1β, IL-12p70, IL-2, IL-10	IL-6, IL-8, TNF-α, GM-CSF, IFN-γ, IL-1β, IL-12p70, IL-2, IL-10	16 knee PTOA 14 End-Stage OA Excluded: 2
Liu et al.	2015	Correlation of cytokine Level in Synovia and Severity of Knee Osteoarthritis. ELISA was used for detection.	IL-17	IL-17	226 knee OA patients and 106 controls
Vangsness et al.	2011	Synovial biopsies were taken in four zones at the time of surgery for histological analysis.The SF was assayed for 21 cytokines using a LINCOplex™ Immunoassay with Luminex® 100 flow cytometry instrumentation.	TNF-α, IL-1α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15 IL-17, INF-γ, GCSF, GM-CSF, Eotaxin, MCP-1, MIP-1a, IP-10	IL-2, IL-5, MCP-1, MIP-1, IL-1β, IL-6, IL-8, IL-12p70, IFNγ	12 knee PTOA patients
Adams et al.	2014	PTAA ankle joint synovial fluid analyzed using ELISA.	IFN-γ, TNF-α, MIP-1β, MCP-1, IL-1β, IL-1Ra, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15	IL-1Ra, IL-6, IL-8, IL-10, IL-15, MCP-1	20 PTAA
Rübenhagen et al.	2012	Valuation of the roles of cytokines and metalloenzymes in knee OA in relation to OA grading, age, and BMI. Multiplex ELISA-based immunoassay was used to measure biochemical mediators in the synovial fluid (SF) of patients undergoing knee surgery.	IL-1Ra, IL-6, IL-7, IL-18, CCL2 (MCP-1), CCL3 (MIP-1 α), CXCL8 (IL-8), HGF, VEGF, MMP-1, MMP-2, MMP-9, MMP-13	IL-7, VEGF, MMP-1	82 OA patients
Mabey et al.	2016	A multiplex immunoassay was utilized for 10 cytokines in plasma and synovial fluid.	IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN-γ, TNF-α	IL-1b, IL-2, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNF-α	32 knee OA patients and 14 healthy controls
Schmal et al.	2013	Lavage fluids of ankle osteoarthritis were analysed by ELISA.	BMP-2/7, IGF-1/R, bFGF, CD105, MMP-13, IL-1b	bFGF, BMP-7, CD105	49 ankle OA
Futani et al.	2002	Synovial fluid was harvested and cytokines concentration were measured by ELISA	IL-18, IL-1β, TNF- α, IL-6, IL-8, PGE2	IL-18, IL-6, IL-8, PGE2	30 knee OA patients
Beekhuizen et al.	2013	SF was collected from control donors and end-stage knee OA patients and were measured by ELISA.	EGF, Eotaxin, FGF-2, Flt-3, G-CSF, GRO, IFNa2, IFNg, IL-10, IL-12 (p40), IL-15, IL-1a, IL-1ra, IL-1b, IL-3, IL-6, IL-8, IP-10 MCP-1, MCP-3, MDC, MIP-1a, MIP-1b, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, sIL-2ra, TGFa, HGF, Leptin, Resistin, Adiponectin, OSM	IL-6, IP-10, MDC, PDGF-AA, RANTES	18 End-Stage knee OA and 16 healthy controls
Heard et al.	2013	Multiplex technology was used to quantify expression levels for cytokines in the synovial fluid in different stage of OA.	EGF, Eotaxin, FGF-2, FIT-3Ligand, Fractalkine, G-CSF, GM-CSF, GRO, INFa2, INFg, IL-1a, IL-1b, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP10, MCP-1, MCP-3, MDC, MIP-1a, MIP-1b, PDGF-AA, PDGF-AB/BB, Rantes, CD-40L, sIL-2Ra, TGFa, TNFa, TNFb, VEGF	EGF, Eotaxin, FGF-2, FIT-3Ligand, G-CSF, GM-CSF, GRO, IL-1a, IL-1b, IL-1ra, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IP10, MCP-1, MCP-3, MDC, MIP-1a, MIP-1b, PDGF-AA, CD-40L, sIL-2Ra, TGFa, VEGF	20 severe OA 12 mild/moderate OA 34 control
Larsson et al.	2015	Evaluation of cytokines level in SF in patients with OA after meniscectomy by ELISA.	IL-6, IL-8, TNFa	IL-6, TNFa	132 knee OA after meniscectomy
Koskinen et al.	2014	Levels of cytokines in SF of OA patients who underwent to knee replacement surgery were measured by ELISA.	Resistin, IL-6, MMP-1, MMP-3	Resistin, IL-6, MMP-1, MMP-3	88 OA patients who underwent to knee replacement surgery.
Driban et al.	2010	Levels of cytokines in SF of OA patients and normal knee partecipants were measured by ELISA.	IL-1α, IL-1β, TNF- α, RANKL, IL-1, IL-1ra, IL-4, IL-10, IL-13, OPG, MMP-2, MMP-3, MMP-13, TIMPs, TIMP-1, TIMP-2; adiponectin, leptin	TIMP-2, MMP-2, MMP-3	8 subjects: 4 OA-knee participants and 4 normal-knee articipants
Teunis et al.	2014	Synovial fluid was collected from primary OA knee and posttraumatic osteoarthritic wrist joints. Mediators were measured by ELISA.	Chemokine ligand 5, INF-γ, LIF, oncostatin-M, osteoprotegerin, TNF-α, VEGF, IL-1α, IL-1β, IL-1 receptor antagonist, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-17	TNFα, IL-1α, IL-1RA, IL-6, IL-10, IL-17, oncostatin-M, INF-γ, chemokine ligand 5, LIF	20 knee OA 20 posttraumatic osteoarthritic wrist joints
Teunis et al.	2013	Radiocarpal synovial fluid samples were obtained from 3 groups of patients. Cytokines were measured by ELISA.	IL-1 β, TNF-α, oncostatin-M, INF-γ, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-1RA, osteoprotegerin	INF-γ, IL-6, IL-10	12 healthy control 16 pre-OA 20 end-stage OA
Doß et al.	2006	Serum, synovial fluid, synovial tissue, and articular cartilage samples were collected from OA patients with end-stage knee or hip OA and the cytokines were assessed though ELISA.	IL-6	IL-6	49 OA patients with end-stage knee or hip OA
Helmark et al.	2010	ELISA evaluation of IL-10 levels into intra-articular, peri-synovial fluid and SF of patient with knee OA randomized to non-exercise (NEx) or exercise (Ex) groups.	IL-10, IL-6, IL-8, COMP	IL-10, COMP	31 female subjects with OA of the knee

Table II. *Main cytokines.*

Pro-inflammatory	Anti-inflammatory	Modulatory	Pro-catabolic	Proteinases	Angiogenic
TNF-α	IL-10	IL-6	IL-17	MMP-1	IL-8
IL-1β	IL-13	IL-3	IL-8	MMP-2	PECAM-1
MIP-1		TIMP-2	IL-18	MMP-3	VEGF
MCP-1			IL-1β		HGF
IL-2					Angiopoietin-2
IL-5					
IL-1RA					

(18) found significant concentration differences in IL-2, IL-5, Monocite Chemoattractant Protein (MCP-1), and Macrophage Inflammatory Protein (MIP-1) comparing subjects with advanced OA and subjects with little or no OA on the ICRS scale ($p<0.05$) (18). Moreover, Larsson et al. (10) observed risk ratio for cytokine expression levels and radiographic progression of OA, showing that where at first examination (time of meniscectomy) high levels of IL-6 and TNF-α were observed, there was an increased risk of osteophyte formation (OR 1.05). At second examination (4 to 10 years after meniscectomy) high levels were associated with joint space narrowing and worsening of KOOS score (respectively OR 1.70 and 1.50) (10).

Several studies performed wide multiplex immunoassays, studying a whole of several cytokines and proteins in SF of joints affected by OA. In post-traumatic osteoarthritic wrist synovial fluid it was found that TNFα, IL-1α, IL-1RA, IL-6, IL-10, IL-17, oncostatin-M, interferon-γ, chemokine ligand 5 and leukemia inhibitory factor were over-expressed ($P<0.001$), while IL-1ß, IL-4, IL-7 were not present. In knee OA, TNFα was not detected, thus suggesting that post-traumatic OA has a more inflammatory pattern than primary knee OA, with faster progression (12).

Another multiplex ELISA study (19) showed that IL-6, interferon inducible protein (IP)-10, macrophage derived chemokine (MDC), platelet derived growth factor (PDGF)-AA and regulated on activation normal T cell expressed and secreted (RANTES) levels were higher in OA compared to SF of healthy controls ($P < 0.001$). Leptin, IL-13, macrophage inflammatory protein (MIP)-1b, soluble CD40 (sCD40L) levels were found to be higher, while eotaxin and granulocyte colony-stimulating factor (G-CSF) levels were lower ($P < 0.05$) (19). Post-traumatic ankle OA showed a pattern very similar to aforementioned ones, in a study by Adams et al. (20), where increased levels of IL-1Ra, IL-6, IL-8, IL-10, IL-15, and MCP-1 were found, significantly higher than levels of healthy subjects free from symptoms or radiographic signs of ankle OA (20).

Plasma levels of cytokines have been also investigated and matched with SF findings (21). IL-2, IL-4, and IL-6 levels in plasma were higher in patients affected by OA, when compared with controls ($p=0.03$, $p=0.003$, and $p<0.001$, respectively). Similarly, IL-1b, IL-5, IL-6, IL-10, IL-13, and TNF-a in OA plasma were significantly higher than in paired synovial fluid samples ($p<0.001$) (21) A summary of main cytokines involved is provided in Table II and Fig. 1.

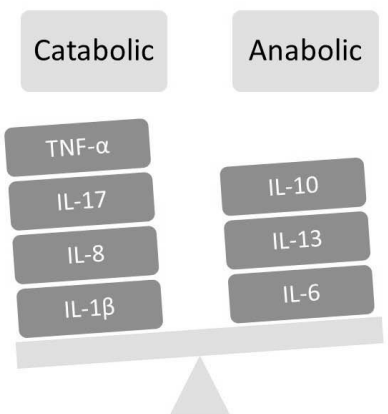

Fig. 1. *Main cytokines.*

Angiogenetic factors

Only one study reported data concerning pro-angiogenetic substances (22). Platelet endothelial cell adhesion molecule (PECAM)-1, hepatocyte growth factor (HGF), VEGF, angiopoietin-2, follistatin, granulocyte-colony stimulating factor (G-CSF), and IL-8 levels were measured in plasma of OA patients and resulted significantly higher than those in healthy subjects. Correlation with OA progression showed that angiopoietin-2 level in plasma was significantly higher in advanced than in early OA, in an o. VEGF levels in SF were found to be positively correlated with the severity of OA (r=0.367, P=0.04) (22).

Metalloproteinases

Matrix degradation enzymes were studied in more than one study (7, 14, 23), as their role is fundamental in the degenerative process of cartilage. MMP-2 and MMP-3 levels were evaluated in the SF of arthritic knees, and resulted significantly elevated when compared to controls (14). Similarly, a metalloproteinase inhibitor, TIMP-2, was significantly higher (14). Correlation studies with the grade of OA showed that there was a negative correlation between MMP-1 levels in SF and grade of OA ($p<0.001$) (23). Levels of resistin in SF were also measured and correlated with the expression of proteases, showing a positive correlation with MMP-1 (r=0.31, p=0.004) and MMP-3 (r=0.24, p=0.024) (7).

DISCUSSION

Several factors are involved in the degenerative process of osteoarthritis. Although a clear overview on the role of each molecule in the pathogenesis is not available, it is known that pro-inflammatory and anti-inflammatory cytokines are involved in the process, along with angiogenetic factors and metalloproteases. Conversely, other growth factors, including NGF (8), are not present when synovial fluid analysis is carried out. Concerning pro-inflammatory cytokines, it has been shown accordingly to several studies that IL-6 (6-8,12), TNF-α (8-10), and IL-17 (11,12) are mainly over-expressed in the synovial fluid of joints affected by OA. When levels of these molecules were correlated with radiographic grade of OA, it was shown that IL-6 expression, the main inflammatory cytokine, was negatively correlated with K-L grade progression (8,10). Conversely, discordant results were available for correlation of IL-17 levels with OA progression, since it was shown to be positively correlated with K-L grade and Lequesne index (11) but negatively in a study by Liu et al. Both studies evaluated knee OA, and both evaluated IL-17 SF levels through ELISA method. However, results showed by Liu et al. lack of statistical significance. The same cytokine, IL-17, was also thought to be responsible for pain generation (13), therefore, a correlation with knee pain has been investigated. Liu et al. showed in fact that a positive correlation is present, with the pain scale of WOMAC score.

Anti-inflammatory cytokine IL-10 was found to be increased when an exercise protocol was carried out by women affected by knee OA. Thus, a protective role of moderate exercise on arthritis-induced inflammation, can be advocate (15). Plasma levels of cytokines is also a relevant issue, since the presence of these molecules in the circulating blood reflects the systemic character of this pathology. Contradictive results were reported since SF/serum ratio was shown to be low in the study by Mabey et al. (21) and high in one other (6).

The explanation for the presence of an increased concentration of cytokines in SF should be searched in the direct production of this cytokine by the synoviocytes (6) of the joint. Conversely, permeability of synovial membrane to cytokines is limited (21) and this could explain why peripheral production of cytokines does not reflect a SF increase of cytokines levels.

Given the results of analysed studies it can be observed that OA, its post-traumatic form and its progression can be individuated and followed-up by the analysis of cytokines. The cytokines implied in the degeneration process coexist and act in concert with other cytokines of opposite action. IL-17 and TNF-α are the main catabolic cytokines involved in degradation processes and activation of proteases for matrix disruption. However several in vitro studies (24), showed a fundamental role of IL-1 as the main cytokine which is responsible of cartilage matrix degradation.

An opposite, anti-catabolic, action is provided by IL-10, IL-13 and IL-4, which contrast degeneration and stimulate anti-inflammatory patterns (2).

Recently, a downregulation role of IL-3 on metalloproteinases has been investigated on an animal model, defining this cytokine as one of the main anti-catabolic and modulatory ones (25). As already mentioned the target of cytokines are chondrocytes, while the elements the cytokines derive from are not clearly identified, though synoviocytes are thought to play a major role in synthesis. Thus, during OA processes, the joint signalling results altered and behave as an imbalanced system, where catabolic processes are overstimulated, compared to anabolic ones.

A recent investigation by Yang and collaborators (26) showed that a main role in catabolic signalling is played by CXCL-8 and CXCL-11, though the JAK-STAT, NF-kB and JNK MARK pathways, which results overstimulated in OA patients (26). The term cytokinome, in the context of OA, refers actually to this kind of complex system of interactions. Research has already clarified the main interaction which characterize this pathogenic environment, but clinical implication are not well defined yet. First of all, individuating the different patterns of interaction which intervene in each OA stage may be detrimental in diagnosis and prognosis of the disease, since a simple synovial fluid harvest could help the physician in define stage and individuate perspectives for progression. Moreover, therapy may benefit from the analysis of cytokines which may be inhibited.

In the era of biological therapy applied to inflammatory diseases and tumours, a similar action of cytokine-inhibitory drugs should be expected also in degenerative-inflammatory, non-rheumatic joint disease. The use of anti-cytokine drugs in the field of orthopaedics have been already introduced and investigated, to modulate the action of growth factor complexes, such as platelet rich plasma, in tissue regeneration (27). The use of synovial fluid harvesting may be a simple method for OA clinical framing, and should be introduced in order to evaluate not only the progression of the disease, but also the phases characterized by a more inflammatory pattern. However, more research on this topic should be carried out.

Main focus should concern the whole set of cytokines which are present during a specific stage or type of OA, and not only the time-dependent and stage-dependent behaviour of a single molecule. Successive steps could face the topic of anti-cytokine drug use in degenerative joint disease.

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THE MENISCUS VASCULARIZATION: THE DIRECT CORRELATION WITH TISSUE COMPOSITION FOR TISSUE ENGINEERING PURPOSES

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Meniscal lesions still represent an unsolved problem in clinical practice. Like the articular cartilage, meniscus has a scarce healing potential. Thus, when this tissue is damaged, the joint biomechanics is completely altered, leading to the development and progression of premature osteoarthritis. Therefore, in the last years, several tissue-engineering strategies have been developed to regenerate the meniscus with debated results. The comprehension of complex processes underlying meniscus maturation and structure is essential for a correct approach for the generation of a biomimetic meniscal substitute. In this chapter, we will first review the morphology of the meniscus during growth, focusing on the unique pattern of vascularization, and then we will discuss the most common tissue engineering strategies for meniscus repair.

The development of an engineered meniscus represents one of the most promising methods to regenerate a tissue, which, in the common clinical practice is rarely able to spontaneously repair or regenerate, with the consequent loss of functionality of the entire knee joint. The production of meniscal substitute requires a profound knowledge of the native meniscus morphology, of its biochemical composition and of cell phenotype in its different regions. Current information on meniscus is the result of several studies in human but also in animal models; for example, the swine meniscus is very similar to the human one and it is therefore useful both for the characterization of the native meniscus and for the validation of an engineered scaffold (1). Although several studies have been performed to clarify the cellular and structural heterogeneity of the meniscus, there are still some open questions regarding its structure and its insufficient repairing and regenerating capability. In particular, as the reduction of the vascularization is strongly connected to meniscus maturation (2), it is important to determine a correlation between vascular changes and the modulation of cell phenotype during the course of meniscus development. These two processes could be regulated by common molecules that are already known as vascular modulators but are not yet associated in the signalling involved for the regulation of cell phenotype. New insights about the pathways involved in vascular and phenotypical changes will guide the generation of better meniscal substitute and will enlighten the physiological development of the meniscus.

Meniscus Morphology

The meniscus is a fibrocartilaginous tissue, which is essential for proper knee function, playing an active role in the biomechanics of the knee joint.

Key words: meniscus, vascularization, tissue engineering, animal models

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0393-974X (2016)

It is mainly composed of water and some highly differentiated cells of various phenotypes, surrounded by an intricately arranged extracellular matrix (3). The cells of the meniscus are responsible for the synthesis and maintenance of the extracellular matrix. Two main cellular phenotypes have been described within the meniscus: fusiform cells, which are found along the entire superficial margin of the meniscus and fibrochondrocytes, which resemble chondrocytes but they also synthesize fibrous matrix (Collagen type I). These cells can secrete different products of the extracellular matrix and they also interact differently with other molecules present in the articular environment, particularly growth factors. Both the fusiform cells and the fibrochondrocytes have abundant endoplasmic reticulum and Golgi complexes indicating a great cellular secretion ability. Mitochondria are rarely found, suggesting that the major resource of energy production is the anaerobic glycolysis (3).

It is still unclear whether there is only one single cell type, which can modulate its gene expression and phenotype depending on its environment, or whether distinct cell types coexist in the meniscus.

The extracellular matrix of the meniscus is mainly composed of water, collagen and proteoglycan aggregates (3).

The meniscus displays great regional variation in its extracellular matrix components. Its periphery is highly fibrous, abundant in cells and collagen type I, while the inner portion of the tissue resembles hyaline cartilage with fewer cells, a higher proteoglycan content and the presence of collagen type II. In addition, the collagen fibers are differently oriented in the various meniscal regions. Kambic et al., have analyzed the spatial distribution of collagen type I and II in canine meniscus and by immunofluorescence analysis, they demonstrated that collagen type I distributes as a continuous, diffuse band in the superficial region, while in the main body of the meniscus collagen type I fibers appear as an intricate network of thick strands, stretching from the peripheral attachment to the inner tip of the tissue (4). Collagen type II is also present both in the superficial zone and inner body of meniscus, but its distribution is less uniform than collagen type I (4). Nowadays, the spatial relationship between collagen type I and II is not completely understood.

Nevertheless, it is known that the collagen fibrils within this matrix are highly structured into three layers that together allow the compressive forces to be dissipated both peripherally and tangentially into hoop stresses. This distribution creates an extremely effective mechanism of load sharing among the body and horns of the meniscus (4). On the meniscal surfaces, the collagen fibrils are randomly oriented and this disposition optimizes meniscal function in this region: here the fibers allow for a low-friction motion between the meniscal surface and the femur or the tibia as the femoral condyles slide against the menisci and the tibial plateaus during knee motion. Deep in these superficial layers, there are two structurally distinct regions with a different collagen fibers orientation. In the innermost third, the collagen bundles predominantly show a radial pattern, whereas in the outer two-thirds, the collagen bundles have a circumferential orientation that follows the C-shape curve of the tissue, from cranial to caudal attachment sites. This suggests that the inner third may play an important role in compression, and that the outer two-thirds may have a function when tensile stresses are generated. Circumferential fibers are surrounded by small radial fibers, which provide structural rigidity to compressive load.

Vascularization of the meniscus

The meniscus possesses a unique pattern of vascularization. In particular, the outer portion of the meniscus is called *red-red zone* because it is rich in blood supply; the remaining part of the meniscus is mostly avascular and it is defined as *white-white zone*. An intermediate region is also described, the *red-white zone*, localized between the outer and the inner ones, that is vascularized by only a limited number of vessels. Blood supply originates from a small reflection of the vascular layer of the synovium, i.e. the synovial fringe, present on the femoral and tibial surfaces of the meniscus. The synovial fringe creates a perimeniscal capillary plexus, originating from the medial and lateral genicular arteries (5). Only the outer portion receives vascularization as blood vessels reach only 15%-25% of the periphery of the

menisci (3) and this tissue must rely to a large degree on synovial sources of nutrition. The nutritional requirement of the menisci is indeed supplied by alternative mechanisms. During the knee motion, the tissue of the synovial cavity is compressed and the synovial fluid is pumped over menisci allowing for the diffusion of nutrients into the fibrocartilage (5). The particular vascularization of the meniscus plays an important role in decision-making in the presence of meniscal tear, as the regeneration of an avascular tissue is very limited (6).

Meniscal maturation and differentiation

Meniscus morphology and vascularization change during growth and therefore the knowledge of the different phases of meniscus maturation is essential to generate valid meniscus substitutes, which can recapitulate the physiological development of this tissue.

After birth, the meniscus continues growing and it refines its structure in response to biochemical and mechanical stimuli (2). Different load and stress distribution and growth factors can induce structural and cellular modification in the meniscus morphology. Collagen fibers assume their final orientation and the cells acquire a specific phenotype in the different regions (7).

A recent study from our group has shown that during meniscal development, the cartilaginous component increases with age in the inner zone, whereas a fibrous tissue persists on the outer region (2). These data illustrated that in young animals both medial and lateral menisci show scarce positivity for GAGs deposition, while in the adult specimens the concentration of

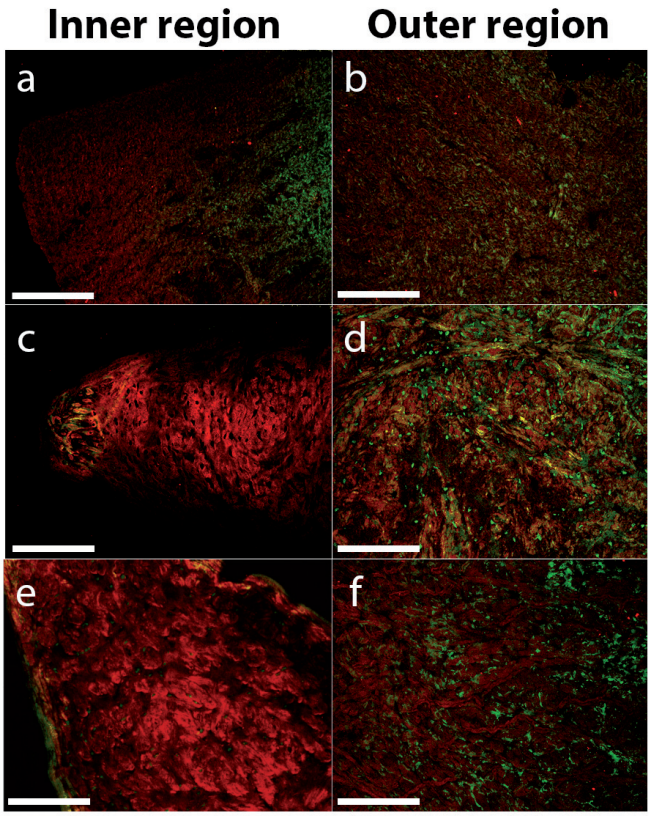

Fig. 1. *Double immunofluorescence for Collagen type II (COLII, red signal) and Vascular Endothelial Growth Factor (VEGF, green signal) in swine neonatal (**a, b**), young (**c, d**), adult meniscus (**e, f**). In the neonatal meniscus, a stronger VEGF signal is present boh in the inner and in the outer region (**a, b**); in the young and adult meniscus, a slight immunopositivity for VEGF and a much stronger signal for COLII are present in the inner zone (**c, e**); in the outer zone, slight signs of co-localization are evident in both the young and the adult tissues (**d, f**). Scale bar: 100 µm.*

GAGs in the inner area significantly increased in both menisci. The maturation of the meniscus towards a fibrocartilaginous tissue was evident along with the medial to the lateral aspect, as shown by the predominant distribution of collagen type II compared to collagen type I in the inner and intermediate zones of the horns and the body of the adult menisci. This different matrix distribution is the result of changes in the cell phenotype; these cells acquired an increased competence for the expression of cartilaginous markers and at the same time they lose the typical fibroblasts phenotype, which is characterized by the expression of collagen type I. The data obtained in the study had enforced the idea that growth of the swine knee joint is accompanied by a specific fibrochondrogenic maturation of the meniscus that occurs first posteriorly, and is then extended anteriorly, particularly in the inner and intermediate areas.

These modifications in cellular phenotype are associated with changes in vascularization. Specifically, blood vessels progressively decrease in the inner region with age. Meller et al. showed that in very young animals, blood vessel density is higher and few blood vessels can be found in the inner white-white zone; with age, the white-white zone becomes an avascular tissue (7). Our group also obtained similar results in a swine model. At the neonatal stage, we observed expression of Vascular Endothelial Growth Factor (VEGF) (a pro-angiogenic factor) in the meniscus, especially in the outer region. Conversely, in the young and adult meniscus, we demonstrated a decrease in VEGF expression in the inner region that was associated with strong expression of Collagen type II (Fig. 1). Therefore, it is likely that signals involved in the decrease in vascularization (i.e. antiangiogenic factors: Endostatin (8) may also regulate the phenotype switch of cells towards a cartilaginous tissue, which is able to survive in an avascular and hypoxic environment.

Tissue engineering strategies for meniscus repair

Tissue engineering studies first introduced the use of acellular scaffold in the clinical practice to replace a damaged meniscus. In the last years, researchers tried different materials: from synthetic (i.e. polyglycolic acid) to natural biological products (i.e. collagen), using several techniques (basic molds, electrospinning or three-dimensional printing) with promising results (9). However, the generation of a valid meniscal substitute still represents a challenge in orthopedic research. An ideal scaffold should indeed be able to mimic the complex architecture of the meniscus and several efforts have been made to meet this goal (10, 11).

Along with the scaffold structure, basic research is nowadays focused on the potentials of cell-based therapy for the healing of meniscal lesions and for the regeneration of this tissue (6). In regards to repair of the meniscal tissue, some studies have been carried out with the aim of biological repair in the avascular zone (1, 6). These studies initially used only articular chondrocytes associated to different types of scaffold. The models showed that chondrocytes are able to adhere to devitalized cartilage and produce new extracellular matrix that promotes the adhesion of these juxtaposed cartilaginous sections (12).

Morever, these cells are able to adhere to devitalized meniscal matrices used as a scaffold for the repair of meniscal lesions. We have also demonstrated that articular chondrocytes, associated with different biomaterials, are able to repair a lesion in the inner area of a devitalized meniscus (13-15). This approach has been validated in a preclinical experimental model, characterized by the use of swine autologous chondrocytes, seeded on allogeneic meniscal matrices, for the repair of bucket-handle lesions in the medial meniscus of pigs (16).

The choice of the optimal cellular population is still debated. The most logical choice is the use of meniscal fibrochondrocytes (17). However, autologous fibrochondrocytes or articular chondrocytes require a tissue explant from the meniscus itself or from the cartilage, an invasive procedure that causes a further trauma to the joint. For this reason, the use of allogeneic chondrocytes, both articular and auricular, has been investigated in one of our previous studies demonstrating the potential of these cell sources with significant difference between the treated lesions and the control groups (18, 19). Additionally, researchers have also directed their efforts towards the identification of an alternative cell population that can acquire a

fibrochondrocyte-like phenotype (20). For example, mesenchymal stem cells have been proposed as an alternative cell source as their differentiation towards a chondrocyte can be supported by the use of growth factors and by a 3D culture system, in particular by using matrices made of the typical components of the cartilaginous matrix such as collagen, hyaluronic acid or chondroitin sulphate (9). Mesenchymal stem cells have already been used in meniscus repair studies; following in vitro expansion, they were seeded onto a hyaluronic acid and collagen scaffold (21) or cultured in micropellets (22) and maintained *in vitro* with chondrogenic factors (TGF-beta1). These cells were then placed into a meniscal lesion on a scaffold or in fibrin glue and in both cases they showed a repair potential. One eligible cell source is represented by mesenchymal stem cells from adipose tissue (ASC) that can be isolated with an easy surgical procedure.

Nevertheless, the ideal cell source for meniscus repair has not yet been found. Recent studies (23, 24) suggest that the co-culture of different cell types (i.e. chondrocytes and meniscal cells, or MSC and meniscal cells) may be the solution to obtain a better meniscal substitute. Along with the cell source, researchers are also investigating the possibility to improve a potential meniscal scaffold with growth factors and promising results have been obtained by implementing the scaffold with connective growth factor (CTGF) or TGF-beta3 in a sheep model (25). Growth factors are indeed able to induce proliferation, differentiation and even migration of cells and they may therefore guide the cells seeded onto a scaffold towards a specific phenotype. Along these lines, vascular modulators are essential for meniscus maturation, and the use of anti-angiogenic factors in the inner portion of a meniscal substitute may increase the chondrogenic potential of the seeded cells leading to the formation of neo-tissue, which can closely resemble the native meniscus.

Like the articular cartilage, meniscus repair is still an unsolved problem. However, the deeper knowledge on meniscus maturation will provide new insights for tissue engineering strategies. Along these lines, we have now several available tools (scaffolds, cells, growth factors), and, by identifying the correct combination, we may be able to find the right balance that will lead to meniscus regeneration.

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ARTHROSCOPIC ALL-INSIDE TREATMENT OF POPLITEOMENISCAL FASCICLES TEARS: SURGICAL TECHNIQUE AND RESULTS FROM THE FIRST 6 CONSECUTIVE PATIENTS

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Athletes whose knees are subjected to sudden changes of direction and high jumps such as martial arts athletes, dancers, wrestlers and football players are at higher risk of injuring popliteomeniscal fascicles. Painful squatting and mechanical symptoms such as locking sensation are common. Current available treatments includes open or arthroscopic in repair. Arthroscopic repair with all-inside device can relieve symptoms and restore knee function. Six patients from two surgical centers with isolated popliteomeniscal fascicles tears were treated with arthroscopic all-inside repair. The surgical technique is thoroughly described. All patients showed consistent symptoms and MRI findings, as well as meniscal hypermobility during arthroscopic probing. Moreover, four out of six showed a chondral lesion of the lateral femoral condyle. All of them had their lateral meniscus sutured with one or more sutures. Symptoms were relieved and all but one were able to return to play at the pre-injury level. No postoperative complications were encountered. The diagnosis of the disruption of popliteomeniscal fascicles is challenging and often seen in athletes that play sports which involve repetitive twisting. However, patients' complaints are consistent. Arthroscopic repair with an all-inside device showed to be a reliable and easy technique for addressing the condition, although some issues still need to be investigated, such as how much constraint the repair should provide. Arthroscopic all-inside repair of popliteomeniscal tears prove to be safe and effective in the short-term follow-up, allowing for sport activity resumption.

The popliteomeniscal fascicle is a thin synovial mesh connecting the lateral meniscus at the popliteal hiatus (1).

Athletes whose knees are subjected to sudden changes of direction and high jumps such as martial arts athletes, dancers, wrestlers and football players are at higher risk of injuring popliteomeniscal fascicles (2-5). Current literature also reports how the tear of polpiteomeniscal fascicle often occurs in association with acute anterior cruciate ligament injuries in over 25% of cases (6). This leads to most of the cases being ignored because the ACL rupture draws all the post-injury diagnostic and therapeutic care. Isolated lesions of the popliteomeniscal fascicle are not always symptomatic, presenting pain over the posterolateral region and locking of the knee joint (6, 7).

Clinical (8) and radiological (9) diagnosis of this lesion can be very difficult. Despite a careful physical examination of the knee and a scrupulous visualization of MRI images, sometimes only the arthroscopic visualization of the structures and the surrounding area, is able to lead to the correct identification of the lesion (2). Currently, few data

Key words: popliteomeniscal fascicles, lateral meniscus, menisco-capsular lesion, all-inside repair

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0393-974X (2016)

are available on outcomes of surgical repair of popliteomeniscal fascicles and no standard treatment has been established. This study reports on treating isolated popliteomeniscal tears by means of an arthroscopic all-inside technique.

MATERIALS AND METHODS

After receiving IRB approval, data from six consecutive patients from two orthopaedics surgical centres were collected. Preoperative symptoms, diagnostic images and information on injury mechanism, time from injury to surgery and time to return to activity, have been recorded, as well as intraoperative images.

Surgical technique

The patient is placed supine on the operative table with the indexed leg hanging out from the leg holder. Tourniquet is insufflated. Surgery starts with a standard diagnostic arthroscopy through the antero-lateral portal with a 30° scope. Successively, under direct visualization, the antero-medial portal is established. After having assessed the whole joint and excluded other concomitant sources of pain, the lateral compartment is accurately examined, place patient's knee in the "figure 4" position. Using an arthroscopic probe, the lateral meniscus is then hooked and checked for its stability. If the body subluxes towards the medial joint compartment, the meniscus is unstable and the popliteal hiatus is checked for fascicles disruption (Fig. 1, 2). In the case of torn fascicles, by using a 70° scope and looking at the posterior area of the lateral femoral condyle, it is often possible to find a chondral lesion of the outer edge of the condyle, due to the chronically subluxing meniscus.

Repair of the lesion is performed by the all-inside technique (Fastfix, Smith and Nephew meniscal repair system). Usually two to three sutures are placed on either side of the popliteal hiatus (Fig. 3, 4), in a vertical fashion between the capsule and the meniscus, making sure the knots are on the capsular side. Once the meniscal stability is double-checked, the skin wounds are closed and the knee is placed on a hinged brace locked in full extension for two weeks.

Postoperative rehabilitation

Patients have to use a knee brace locked in extension for two weeks and are allowed weight bearing as tolerated using crutches (10). Passive 0-90° range of motion are allowed during the first two postoperative weeks, together with single leg isometric raise.

Successively patients are cleared from knee brace and

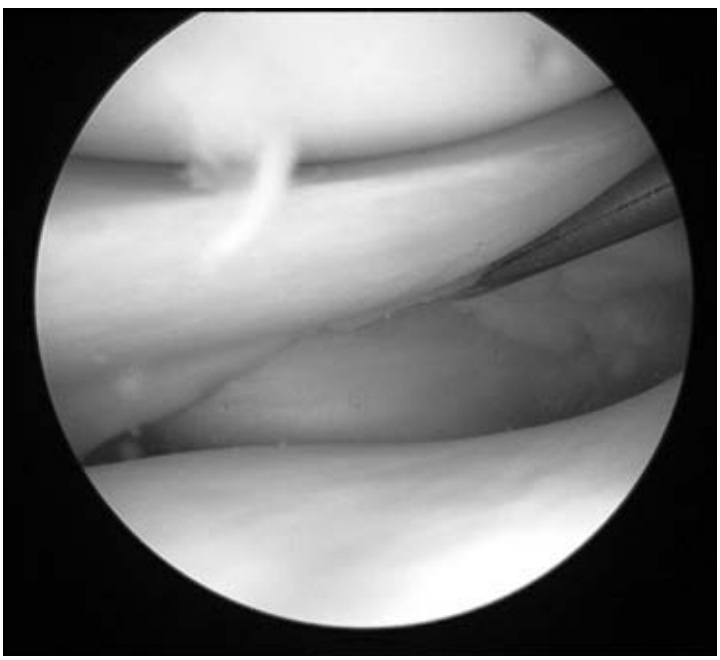

Fig. 1. *Arthroscopic view with a 30° scope, showing unstable lateral meniscus, with medial displacement.*

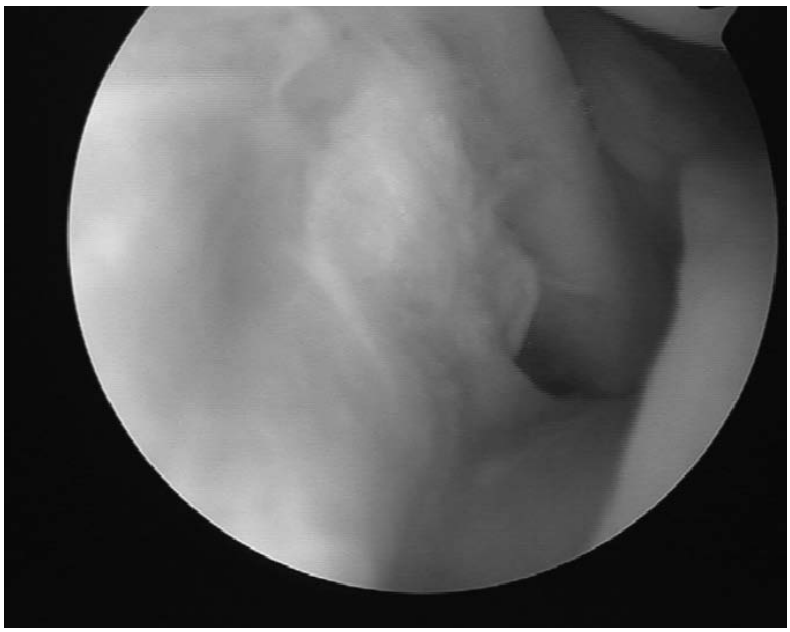

Fig. 2. *Arthroscopic view with a 30° scope, showing disruption of the popliteo-meniscal fascicles disruption.*

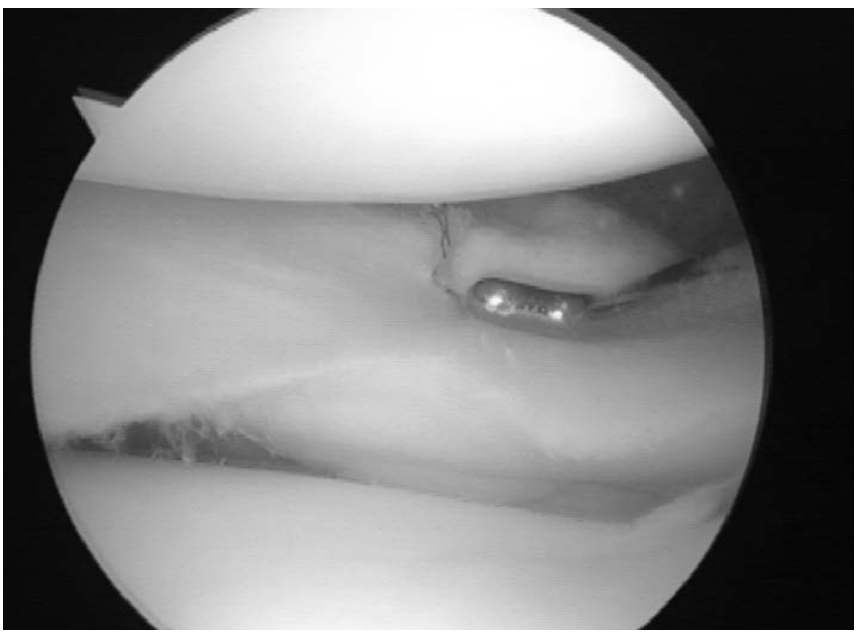

Fig. 3. *Arthroscopic view showing placement of the meniscal all inside suture.*

allowed progressive weight bearing and active 0-90° range of motion in two weeks. After the first postoperative months, patients are allowed full weight bearing, full passive range of motion and to start closed kinetic chain exercises with knee flexion limited to 90°. Running is allowed after the second postoperative month and a return to sports after the fourth postoperative month.

RESULTS

Six patients from January 2012 to January 2015 suffering from popliteomeniscal tears were identified. Five out of six were semi-professional athletes, meaning that they played in minor leagues (soccer or basketball players) or involved in national

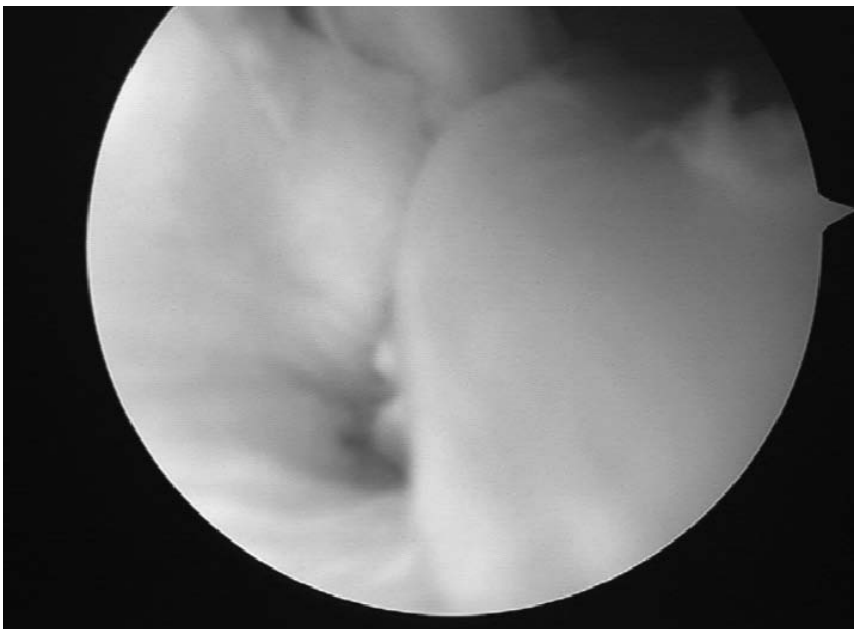

Fig. 4. *Arthroscopic view showing the result of sutured lateral meniscus.*

Table I. *Patients' demographic features and pre- and post-operative information.*

Patient #	Age, years	Gender	Activity	Duration of symptoms, months	Mechanism of injury	Symptoms	Chondral lesion lateral condyle	Resolution of symptoms	Follow-up	Time to return to sports, months
1	16	Female	Semi-pro Dancer	24	Twisting	Painful squatting; Mechanical (locking)	Yes	Yes	48	4, 7 to pre-injury level
2	17	Male	Semi-pro Soccer player	12	Tackle during soccer match	Painful squatting; Mechanical (locking)	Yes	Yes	25	4, 6 to pre-injury level
3	15	Male	Semi-pro Soccer player	6	Tackle during soccer match	Painful squatting; Mechanical (locking)	No	Yes	46	4, 6 to pre-injury level
4	16	Male	Semi-pro Basketball player	10	Twisting	Pain, giving away and mechanical (locking)	No	Yes	32	4, 6 to pre-injury level
5	17	Female	Semi-pro Dancer	>24	Twisting	Painful squatting; Mechanical (locking, snapping)	Yes	Yes	43	Retired
6	19	Male	Recreational Soccer player	12	Car crash	Painful squatting; Mechanical (locking, snapping)	Yes	Yes	39	4

competitions (dancers). One was a recreational soccer player. The mean age was 16.6 years (15-19 year old). Most of them had ongoing symptoms for more than one year. During physical examination, all of them complained of mechanical symptoms, such as locking sensation, and they were tender to palpation of the lateral compartment. The "figure 4" test was found positive in 3 patients and negative in the others. All of them had a preoperative knee MRI which showed fascicles disruption in all patients in the sagittal plane and T2 sequences. During the diagnostic arthroscopy, a chondral lesion of the outer edge of the condyle was found on 4 out of 6 patients. Four months after surgery all but one patient were

able to return to sports, however a minimum of two further months were needed to play at the pre-injury level. One patient, a semi-pro dancer, withdrew her sport activity. After an average follow-up of 3.2 years, all the patients were satisfied and with full symptoms relief, without reporting any postoperative complication (table I).

DISCUSSION

This study demonstrated that arthroscopic all-inside meniscal suture is an effective technique to treat lateral meniscus instability secondary to popliteomeniscal tear. Symptoms and knee function were restored in 6 patients, allowing 5 of them to return to sport at the pre-injury level. Few studies on the same issue are available, with comparable outcomes. Simonian et al. (15) reported satisfying outcomes on 3 patients, treated by an inside-out technique. Complete healing of the lateral meniscus was shown either by postoperative MRI or second-look arthroscopy.

La Prade and Konowalchuk (11) described an open technique to repair the lateral meniscus with the popliteomeniscal fascicles and popliteus tendon complex. They reported on the results of 6 patients, which were postoperatively asymptomatic and all returned to unrestricted activity with an average 3.8-year follow-up.

In 2012 Shin et al. (7) reported an arthroscopic all-inside technique, using a postero-lateral portal through which an arthroscopic suture hook is inserted. No outcomes data are reported. Interestingly, the authors described a triad of mechanical symptoms, hypermobility probing the meniscus during knee arthroscopy and osteochondral lesion in the posterior area of the lateral femoral condyle, which strongly suggested a popliteomeniscal tear. In our series, 4 patients showed that particular chondral lesion, and it was noticed that these patients had longer lasting symptoms comparted to the 2 other patients, which did not have the lesion.

Camarillo and Johnson (2) reported on two cases treated with inside-out repair. While one was successfully able to return to sport activity, the other one had subsequent surgeries with revision of the repair and eventually partial lateral meniscetomy.

Although anatomical studies on the popliteal hiatus have been performed for 30 years (1, 3, 6), the exact number of popliteomeniscal fascicles is still debated. Most of the studies has described two bundles: an anteroinferior fascicle and a posterosuperior fascicle (6, 9, 12-14). The presence of a third popliteomeniscal fascicle, known as the posteroinferior popliteomeniscal fascicle, is controversial (5, 8). This fascicle is reported to be located medial to the popliteal hiatus (11, 15-18).

The embryology analysis by Sussmann et al. (19) further strengthened the hypothesis that the fascicle could be an important source for the vascularization of the lateral meniscus, although it mainly acts as a restraint to lateral displacement of the meniscus throughout the whole range of movement of the knee (1, 19).

Stäubli and Birrer (6) highlighted the importance of those fascicles for the lateral meniscus biomechanics, in fact their disruption leads to increased lateral meniscus mobility and medial displacement, causing pain and locking symptoms (3).

In the case of a minimal or no meniscocapsular detachment associated to popliteomeniscal fascicles tears, it could be difficult to identify those lesions on MRI. The rest condition could not displace the lateral meniscus enough to detect the lesions (2, 7, 11). Therefore, it is always recommended to probe the lateral meniscus mobility. It has been proposed that when more than half of the meniscus body shows mobility, a tear in the popliteomeniscal fascicles should be suspected (2, 7, 11). Simonian et al. addressed lateral meniscal stability after the sequential disruption of one or two fascicles. This study assessed how mobility of the lateral meniscus significantly increased when one or both fascicles were torn (respectively +1.8mm and +2.8mm increased mobility compared to intact state), although the meniscus does not lock but spontaneously reduces to its original position (16). The same authors showed that even in the intact state, the lateral meniscus shows a certain degree of mobility (3.6 mm when a 10 newton anterior directed force is applied). Thompson et al. (20) analyzed the normal excursion of the lateral meniscus throughout the knee range of motion through an MRI model. During flexion, the posterior excursion of the medial meniscus was 5.1 mm while that of the lateral meniscus was 11.2 mm.

This question arises about how stable the lateral

meniscus should be after suture repair. The final construct should be stable enough to prevent the meniscus from displacing towards the medial compartment, however it should allow for some motion in order to preserve the native biomechanics of the knee lateral compartment.

The all-inside technique the authors used seems to provide enough stability to the lateral meniscus to restore normal knee function. However, the short follow-up and the exiguous number of patients prevent the authors from drawing a definite conclusion on the long-term effect of such surgical treatment. Moreover, no subjective or objective outcome measures were available. Other limitations include the lack of second-look arthroscopy or postoperative MRI to assess the healing status.

CONCLUSION

Although there are still many cloudy areas on the popliteomeniscal fasciculi and the lateral meniscus, as well as the entire knee lateral compartment, it is possible to conclude: a) disruption of the popliteomeniscal fasciculi occurs more often in people playing sports that involve repetitive twisting of the knee; b) consequent hypermobility of the lateral meniscus typically causes mechanical symptoms (locking) and painful squatting; c) knee arthroscopy allows for definitive diagnosis and minimally invasive treatment, with all-inside repair; d): full symptom-relief and return to sport is often possible after surgery.

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TEARS OF POPLITEOMENISCAL FASCICLES, DIAGNOSTIC AND CLINICAL IMPLICATIONS. A REVIEW OF THE EVIDENCE

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Postero-lateral corner of the knee is composed of several structures including the popliteo-meniscal fascicles (PMFs). These fibrous structures form a stable ligamentous complex around the popliteus tendon, which stabilize the lateral meniscus, increasing the strength of postero-lateral corner. Studies were retrieved through an electronic search of CINAHL, EMBASE, and Pub-Med, until May 2016. Studies in English, Italian, French, and Spanish were considered for inclusion. Randomized controlled trials, prospective and retrospective comparative studies, case series, and case reports were included. Studies eligible for inclusion concerned PMFs anatomy, biomechanics, diagnostic assessment of PMFs tears and clinical options for tears management. Thirteen studies were included in this review. There were: 7 case series, 4 case reports and 3 anatomical studies. Through anatomic dissection, two or three PMFs (antero-inferior fascicle, aiPMF; postero-superior fascicle, psPMF; postero-inferior fascicle, piPMF) can be indentified and isolated. Evaluation through MRI can be a useful diagnostic tool in detecting PMFs tears, especially using proton density (PD) sequences. The biomechanical analysis assessed that lateral meniscus (LM) motion is directly related with PMFs integrity and increased with section of one or both the fascicles. The clinical studies clearly state that a snapping syndrome, associated with lateral knee pain, can develop when one or both PMFs are torn. The three PMFs described are considered as relevant components of the popliteal hiatus, in the posterolateral aspect of the knee. MRI evaluation can detect these fibrous fascicles with good sensitivity. More studies with larger samples would be needed for a clear comprehension of PMFs function and clinical management of PMFs tears, especially with large case series and modern biomechanical testing.

The anatomy of the lateral meniscus and more broadly the postero-lateral corner (PLC) of the knee is a relevant topic with still unclear issues (1). It is known that PLC is composed of several structures, including the lateral meniscal wall, the popliteus muscle tendon and the arcuate popliteal ligament, all of them reinforced by the deep lateral collateral ligament (2). Its function has been clearly defined as the stabilization of the knee during tibial internal rotation and change of direction (3).

Several anatomical studies, described accurately the anatomy of the PLC, focusing on some small, though relevant, structures called popliteo-meniscal fasciculi (PMFs) (4). These fibrous structures form a stable ligamentous complex around the popliteus tendon, which stabilize the lateral meniscus,

Key words: menisci, knee, biomechanics, knee arthroscopy, sports medicine

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0393-974X (2016)

increasing the strength of PLC.

Some of the studies concerning PMFs report the presence of two ligaments (5-8), while some others retrieved three types of PMFs, by anatomical dissection of the knee (2, 4, 9). These ligaments are disposed around the popliteal tendon, in its way across the synovial space to the insertion at the lateral femoral condyle. The direct attachment of these fasciculi to the posterior horn of the lateral meniscus allows a better stabilization of the meniscus, that by its nature has a lower stability of the medial one (10). PLC injuries occurs in 25-80% (3) of the acute multiligamentous injuries. It was reported (11) that PMFs tears are often related and concomitant to ACL injuries, or may be reported consequently to a chronic ACL lesion (4). This may be misleading because the focus is usually drawn on bigger ligament injuries, with lower attention payed to these structures. Tears of PMFs lead to symptomatic lateral pain of the knee (9) and unstable lateral meniscus. This results in a painful locking syndrome of the knee due to biomechanical changes of the stability of the lateral compartment, defined recurrent subluxation of the lateral meniscus (RSLM) (12).

It has been hypothesized that meniscal instability (13), with internal subluxation of the meniscus, may lead to a chronic collision of the meniscal body with the lateral femoral condyle and pinching of the posterior horn during flexo-extention movements and rotational motion of the knee during gait and run.

However, a clear description of the meniscal biomechanics, and the influence that PMFs have on it, has not been given yet. In addition, questions still looking for an answer are: how many fasciculi must be torn to have a symptomatic knee, who needs surgical care, which kind of surgery should be performed. The main aim of the present piece of work is to provide a summary of the evidence which is present in literature on such a relevant topic, especially concerning anatomy, imaging assessment, clinical and biomechanical features of PMFs.

MATERIALS AND METHODS

The work for the present review was undertaken following the PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (14).

Eligibility criteria

Studies in English, Italian, French, and Spanish were considered for inclusion. Peer-reviewed randomized controlled trials (RCTs), prospective and retrospective comparative studies, case series, and case reports were included. Furthermore, studies eligible for inclusion concerned popliteomeniscal fascicles anatomy, biomechanics, diagnostic assessment of PMFs tears and clinical options for tears management. *In vitro* studies and animal model studies were not considered for inclusion.

Information sources and search

Studies research was carried out by two reviewers (G.T. and L.S.), through an electronic search of CINAHL, EMBASE, and Pub-Med, between August and October 2015. The following search strings were utilized: popliteomeniscal (All Fields) AND fascicle (All Fields) AND ("menisci, tibial" (MeSH Terms) OR ("menisci" (All Fields) AND "tibial" (All Fields) OR "tibial menisci" (All Fields) OR ("lateral" (All Fields) AND "meniscus"(All Fields)) OR "lateral meniscus"(All Fields)) and popliteomeniscal (All Fields) AND fascicle(All Fields) AND ("menisci, tibial" (MeSH Terms) OR ("menisci" (All Fields) AND "tibial" (All Fields)) OR "tibial menisci" (All Fields) OR ("lateral" (All Fields) AND "meniscus" (All Fields)) OR "lateral meniscus" (All Fields)) AND ("magnetic resonance imaging" (MeSH Terms) OR ("magnetic" (All Fields) AND "resonance" (All Fields) AND "imaging" (All Fields)) OR "magnetic resonance imaging"(All Fields) OR "mri"(All Fields)).

Study selection

Relevant articles identified by electronic search were obtained in full-text, and a search of further related articles was conducted through their bibliography. After duplicates were removed, all the articles retrieved were carefully read and evaluated by two reviewers (G.T. and R.P.). After reading the full-text papers, studies considered ineligible were excluded. The studies included were categorized by study type, according with the Oxford Centre for Evidence-Based Medicine. Moreover, the following categories were utilized in order to give a better presentation of the results: anatomic studies, imaging studies, clinical studies.

Data collection process

Data related to performed intervention, aim, patients number were analysed and summarized in tables using Microsoft Excel (2010 version, Microsoft Corporation, Redmond, WA, USA). Type of study, level of evidence, year of publication were also extracted and tabulated. Data concerning clinical features and outcomes were discussed in triplicate (R.P., J.E.M, G.T.), before they were reported in text. Where available, scores and statistical measures were inserted in text and tables.

RESULTS

Of the 13 studies included in this review, there were 7 case series (5, 6, 8, 9, 12, 15, 16), 4 case reports (3, 17-19) and 3 anatomical studies (4, 7, 20). Different types of intervention were carried out in the included studies, therefore they were divided in three classes: anatomical studies, imaging assessment studies and clinical studies. In the following paragraphs, results of each of these classes are accurately described.

Anatomy of popliteo-meniscal fascicles

Among the studies included, most reported the presence of two PMFs, defining them as antero-inferior fascicle (aiPMF) and postero-superior fascicle (psPMF). Nevertheless, a few studies (11) reported the presence of a third fascicle in the medial inferior part of the PLC, which has been

Table I. *Anatomical studies.*

Study	Year	N.er of patients	Type of intervention	Main findings	N.er of PMFs	Conclusion
Peduto et al.	2008	10 cadavers	MR arthrograpy and specimen sampling and analysis. Radiologic and anatomic correlation	iPMF and sPMF in all 10 knees, piPMF in 4 knees	3	Radiologic comparison with anatomy can be achieved
Stäubli and Birrer	1990	7 cadavers (14 knee) - 175 patients (107 control group; 40 with acute and 28 with chronic ACL injuries)	Cadavers dissection and videoarthroscopy in vivo	In the control group, lesions of the popliteus system were found in 20 patients (18.7%). There were: 5 isolated lesions of the sPMF and 9 isolated lesions of the iPMF. There where no combined lesions. In the acute ACL injuries, lesions of the popliteus system were found in 38 patients (95%) in wich 25% have an isolated lesion of iPMF. In the chronic ACL injuries, lesions of the popliteus system were found in 24 patients (85,7%) in which 7 knees (25%) had a lesion of the iPMF and 6 knees (21.4%) had lesions of both fascicles	2	PMFs tears are significantly related to ACL injuries, either in acute or chronic injuries
Munshi et al.	2003	1 cadaver dissectioned - 7 cadavers examinated through MR	Evaluation of posterolateral corner of knee using dissection, MRI and arthrogaphy	At anatomic dissection, all known ligaments were found. At MR examination, not all ligaments were showed (popliteofibular ligaments 57%; oblique popliteal ligament 100%; arcuate ligament 71% and PMFs 100%)	2	Posterolateral corner of the knee is characterized by complex and variable anatomy. Anatomy and MR imaging correletion is important for a better assessments.
Jelaso	1975	1 cadaver	Specimen was dissectioned and findings were evalueted through double contrast arthrography	Fasciculi are ligaments: two thin bands of connective tissue su by synovial membrane. Fasciculi prevent excessive centripetal movement. Moreover they connect LM to the joint capsule	2	Fasciculars impairments contribute to the disability of the posterolateral corner of the knee joint
Simonian et al.	1997	7 cadaveric knee	Biomechanical evaluation of the LM before and after sequentially sectioning PMFs through arthroscopy	When the fasciculi were intact, the average lateral meniscal motion with a 10-N load was 3.6 mm. When the anteroinferior fascicle was dis-rupted, the average lateral meniscal motion with a 10-N load was 5.4 mm. The mean increase in motion from the intact state was 1.8 mm or 50%, which was significant. When both fasciculi were disrupted, the average lateral meniscal motion with 10-N load was 6.4 mm. The mean increase in motion from the intact state was 2.8 m m or 78% and from the single fascicle disruptionstatewas 1.0mm or 18%,bothdifferences were significant	2	PMFs impairments could be diagnosed through a LM motion increased

iPMF: *anteroinferior Popliteomeniscal Fascicle;* ***sPMF:*** *poterosuperior Popliteomeniscal Fascicle;* ***piPMF:*** *posterosuperior Popliteomeniscal Fascicle;* ***ACL:*** *Anterior Cruciate Ligament;* ***LM:*** *Lateral Meniscus;* ***MR:*** *Magnetic Resonance.*

termed postero-inferior fascicle (piPMF). Among five studies concerning anatomy, one study reported the presence of 3 fascicles, while four reported only 2 fascicles. These three structures are precisely disposed around the popliteal tendon where it enters the knee joint capsule, forming the popliteal hiatus. Proceeding from the anterior to the posterior aspect of the PLC, the aiPMF originates from the lateral aspect of the meniscal wall. It directs inferiorly and posteriorly to insert at the lateral proximal tibia, just behind the proximal tibiofibular joint (4). Although its thickness varies among examined specimens (4), it is disposed to form the lateral wall and the floor of the popliteal hiatus (20), also reinforcing the popliteofibular ligament with some inferior fibres (4). The psPMF is thinner than the aiPMF and embraces the popliteal tendon superiorly, contributing to form the roof of the popliteal hiatus (7). It originates from the posterior aspect of the posterior meniscal horn, merging with the musculotendinous junction of the popliteus muscle, posteriorly and inferiorly. Since its origin is composed of some capsular fibres, it also allows the connection of the popliteus muscle to the joint capsule (20). The piPMF is placed at the medial posterior margin of the floor of the hiatus (4), originating from the musculotendinous junction and inserts at the inferior margin of the posterior meniscal horn superiorly. A study by Stäubli and Birrer (20) arthroscopically assessed whether the PMFs would be visible by introducing the 30° arthroscope into the popliteal hiatus. Giving the tibia a varus stress, an opening of the hiatus is achieved and aiPMF can be visualized in the proximal part of the hiatus. Details of the anatomical studies are summarized in Table I.

Table II. *MRI studies.*

Study	Year	N.er of subjects	Aim of the study	Type of intervention	MRI details	Main findings	Conclusion
Suganuma et al.	2012	145 (238 knees)	Find abnormal PMFs	Artroscopical assessment of RSLM, MRI evaluation of the knees with and without RSLM	Signa EXCITE 1.5 T; GE Healthcare	Abnormal PMF is found in 100% of RSLM knees	High incidence of abnormal PMF is found in RSLM affected knees
Johnson and de Smet	1999	66	Verify whether the PMFs are normally seen on MRI and whether certain imaging factors influenced their visualization	MRI evaluation of the knees (T2-weighted axial and PD- and T2-weighted sagittal scans)	1.5-T scanner with a phased-array knee coil	Both popliteomeniscal fascicles, either separately or together, could be identified in 97 % of knees	Visualization is significantly better on T2-weighted images. Fluid sensitive sequences will be needed to diagnose abnormalities. Joint effusion didn't affect visualization.
Sakai et al.	2004	Stage I: 6 knees x 11 angles = 66 images Stage II: 34 patients (right knees)	Check the percentage of knees in which MRI shows the PMFs. Stage I was to determine suitable parameters for depicting the PMFs. Stage II was to classify the "normal" image	Parameters examinated: MRI sequence, number of matrices, slice thickness, and slice angle	Signa Horizon 1.5-T MR scanner (GE Healthcare)	Stage I: PD–weighted images, 512 × 256 matrix, 4mm slice with 45° or 50° slice angles showed the best results. Stage II: 94.1% (32/34) of knees show iPMF and 88.2% (30/34) of knees show sPMF	Abnormal findings of PMF on MRI ins't sufficient to diagnose derangement of the knee. About 30% of healthy knees lack the iPMF and about 50% lack the sPMF if we consider only MRI in which PMFs are perfectly highlighted
De Smet et al.	2001	59 (30 with LM tears; 29 with healthy LM)	Verify possible correlation between abnormal sPMF or posterior pericapsular edema and LM tears. It could be an usefull indirect sign of MR diagnose of LM tear	Check paired sagittal PD- and T2-weighted MRI for abnormal sPMF and pericapsular edema	1.5-T scanner with a phased-array knee coil	Abnormal sPMF was showed in 30% (9/30) of patients with LM tear (9/9 with damage in the posterior horn). Normal sPMF is showed in 100% (29) of patients with healthy LM. 10/22 patients with a torn posterior horn had edema. 1/8 patients with a torn not about posterior horn had posterior edema	A really stong correlation between abnormal sPMF or posterior pericapsular edema and LM tears, especially in the posterior horn, is showed. Noting both of them could improve the accuracy of MR diagnose of LM tear
Blankenbaker et al.	2002	121		MR images were checked with the classic criteria of a LM tear and the new signs. These observations were correlated with the arthroscopic findings	Signa 1.5-T MR scanner with a phased-array knee coil	An improving of 5% (from 89% to 94%) in MR diagnose sensitivity of LM tear is showed using an abnormal sPMF as an additional criteria	Abnormal sPMF is highly associated with a LM tear, but it not significantly improve MR diagnose of LM tear. Posterolateral pericapsular edema was not associated with LM tears

iPMF: *anteroinferior Popliteomeniscal Fascicle;* ***sPMF:*** *poterosuperior Popliteomeniscal Fascicle;* ***piPMF:*** *posterosuperior Popliteomeniscal Fascicle;* ***RSLM:*** *Recurrent Subluxation of the Lateral Meniscus;* ***MRI, MR:*** *Magnetic Resonance Imaging;* ***LM:*** *Lateral Meniscus;* ***PD:*** *Proton Density.*

Magnetic Resonance Imaging assessment

Several studies evaluated the assessment of the PMFs and their integrity, through MRI in conventional T_1 and T_2 sequences and proton density (PD). A debate is open about sensitivity of MRI evaluation, paying attention first to the rate of knee MRI in which PMFs are visualized. With PD or T_2 sequences, a rate of 97% was reported in a study (15) for visualization of at least one PMF. Comparably, one more study (8) reported separate rates for aiPMF (94.1% 32/34 knees) and psPMF (88.2%, 30/34 knees). The same study also assessed that the best visualization can be achieved using a PD sequence, with a matrix of 512×256 points, 4mm slice width and 45° or 50° slice angle (8). Driving the focus on PMFs lesions which are considered signal abnormalities, a 100% rate was reported by Suganuma et al. (12), evaluating knees with RSLM. In the study by De Smet et al. (6), abnormalities of the psPMF were seen in 30% of the knees with tears of the posterior horn of the LM, while a 100% rate of normal psPMF was reported for knees with healthy lateral meniscus (LM). Furthermore, Blankenbaker et al. (5) demonstrated that sensitivity of MRI for LM tears improved by 5% (from 89% to 94%) when psPMF signal alteration was used as additional criteria for diagnosis (5). Details of the MRI studies are summarized in Table II.

Clinical and biomechanical studies

Studies concerning PMF clinical assessment and therapy were mostly case reports or small case series. Twenty patients were included in all the studies. Clinical assessment of PMFs pathology is not a

Table III. *Clinical studies.*

Study	Year	N.er of patients	Diagnostic technique	Treatment	Outcomes	Conclusion
Camarillo et al.	2014	2	Physical and MR examinations	Inside-out lateral meniscus repair	The patients were able to start activity without restrictions	Arthroscopic evaluation of the PMFs and mobility of the LM provides the best diagnostic study for isolated tears. Surgical stabilization of the LM must be performed for resolution of symptoms, preservation of the lateral compartment, and return to previous activity.
La Prade et al.	2005	6	Physical and MR examinations	Open repair of the PMFs of the LM	Complete resolution of their symptoms at a mean follow-up of 3.8 years postoperatively	The "figure-4" test was found to be useful in identifying the source of lateral compartment knee pain due to PMFs tears. Open repair of isolated PMFs tears was also found to be effective in resolving lateral compartment knee pain due to PMFs tears
Park et al.	2012	1	Physical and MR examinations "-". Arthroscopic view was needed to confirm diagnose sPMF tear	"Fast-Fix" was used for the direct repair of the peripheral portion of the LM and joint capsule, targeting the popliteo-meniscal junction.	Early mobilization immediately postoperatively. After 24 months, the patient was performing heavy athletic exercises	On arthroscopy, the popliteal hiatus view can be valuable for the diagnosis of sPMF tear, difficult to diagnose due to non-specific results on physical and MR examinations
Shin et al.	2012	1	Physical examinations: existence of mechanical symptoms such as pain, locking, and giving way in the lateral compartment of the knee; identification of hypermobility of the lateral meniscus through arthroscopic probing and occurrence of an osteochondral lesion in the posterior area of the lateral femoral condyle	Suture hook and polydioxanone with an all-inside repair	Full return to activity was allowed at 14 to 16 weeks	Due to its static nature, MRI may not be able to diagnose all knee injuries. Studies will be necessary for the development of more objective methods of diagnosis
Simonian et al.	1997	3	Physical and MR examinations	Arthroscopic repair of meniscal detachments	At 1 year follow up, all patients had resolution of mechanical symptoms and all had regained symmetric motion	Disruption of PMFs can result in gross instability of the meniscus producing locking of the knee.

iPMF: *anteroinferior Popliteomeniscal Fascicle;* ***sPMF:*** *poterosuperior Popliteomeniscal Fascicle;* ***piPMF:*** *posterosuperior Popliteomeniscal Fascicle;* ***LM:*** *Lateral Meniscus;* ***MR:*** *Magnetic Resonance.*

simple issue to deal with. Lateral knee pain is the main symptom of PMF tears (9), but it is not always recognized as an objective element strictly connect with PMF, especially in injured knees (9). A simple clinical test has been proposed by LaPrade and colleagues (9), in order to better evaluate the lateral compartment of the knee and PMF-related pain. The "figure-4 test" consists of placing the affected knee in flexion, varus and external rotation, causing popliteal complex tension with varus stress. The test results positive when pain occurs at the lateral joint line (9). In regards to PMF tears treatment, arthroscopic techniques are mostly preferred. Camarillo and Johnson (17) treated 2 young patients (14- and 15-year-old girls) with arthroscopic inside-out meniscal repair. Four months from the operation, after a strengthening rehabilitation program, both returned to sport training. Similarly, full return to activities was allowed at 16 weeks from all-inside repair surgery in one patient described by Shin et al. (3). An athlete treated by Park and colleagues (18) with all-inside technique for direct meniscal repair, returned to heavy sport activities within 24 months from surgery.

Arthroscopic repair was also performed on 3 patients (16), with resolution of locking symptoms within 1 year. Open repair was performed by LaPrade group in 6 patients, which were assessed with "figure-4 test" (9). A complete resolution of symptoms in those patients was achieved at a mean follow-up of 3.8 years. Biomechanical testing was carried out on cadavers by Simonian et al. (19) through sequenced radiographs. They assessed that average LM motion with a load of 10N, which almost doubled when aiPMF was lesioned (3.6 mm *vs* 5.4 mm), rising up to 6.4 mm when both fasciculi were sectioned. Details of the clinical studies are summarized in table III.

DISCUSSION

Through anatomic dissection, two or three PMFs can be indentified and isolated. These structures are disposed around the popliteus tendon, in its intra-articular way to the lateral femoral condyle. The connections of these fascicles to the lateral meniscus justify their role in meniscal stabilization, which is compromised, yielding to meniscal lesions, when the PMFs are torn. It has also been evaluated that MRI can be a useful diagnostic tool in detecting PMFs tears, especially using PD sequences. A close relationship between the popliteal tendon and lateral meniscus has been observed in anatomic studies concerning dissection of the PLC of the knee. This relationship is not only spatial, since LM and popliteus tendon stand really close to one another, but it has also been justified by an embryological point of view (23). Sussmann and his group (21) reported that in human foetuses of 23 to 36 weeks, lateral meniscus and the popliteal tendon had a similar cellularity and are connected by superior and inferior fibrous fascicles (21). In adults, this popliteo-meniscal complex develops and organizes to form the popliteal hiatus, with a clear definition of the popliteomeniscal fascicles. One of the key-points of the present topic is the proper understanding of the structure of the hiatus, especially caring about the number and the shape of the PMFs. The studies included in the present piece of work mostly report the presence of two bundles, which are the posterosuperior and anteroinferior fascicle. It is known that a third inferoposterior fascicle does exist (2, 4, 9), and is placed in the medial inferior aspect of the popliteal musculotendinous junction forming part of the floor of the hiatus (4).

None of the studies, which report the results of MRI evaluation, document the presence of this fascicle, probably because of its thinness and its position, masked by the popliteus tendon itself. Given the superficial position of the fascicles, a paper about ultrasound evaluation of the popliteal hiatus, with a special concern for these structures, would be interesting. In regards to the shape of these fascicles, iPMF has been described with a thicker, round-sectioned shape (4, 20), while sPMF has a more fibrous and wide structure and can be considered the inner part of the roof of the popliteal hiatus. It results unclear if there is an anatomical variability of PMF development, especially because of the small number of cadavers sectioned in the studies, which are present in the literature. The already mentioned hypothesis regarding meniscal instability

and incoming lesion, needs by far more study, seeing the low evidence present in worldwide literature. The only study reporting data on a biomechanical analysis (19), assessed that LM motion is directly related with PMFs integrity and increases with the sectioning of one and both the fascicles. The LM motion should also be assessed, not only in a quantitative way, but a qualitative assessment must also be carried out. Directions of meniscal displacement have to be investigated, in order to elucidate the position that LM assumes during knee movements and the effects that surrounding bony structures have on it.

Studies concerning clinical features of PMFs tears, clearly state that a snapping syndrome, associated with lateral knee pain can develop, when one or both PMFs are injured. The "figure-4 test" proposed by LaPrade and Konowalchuk (9) seems to be efficient for clinical assessment of popliteus complex related pain, although few subjects have been evaluated through this manoeuvre. Meniscal repair, either open or arthroscopic, revealed beneficial for lesions of the LM associated with torn PMFs, allowing complete healing, recover of function and return to sports activity.

Strength and limitations

To our knowledge, the present review represents the first attempt to summarize the evidence concerning popliteo-meniscal fascicles of the knee and its relationship with the lateral meniscus. A methodological guideline has been used in order to avoid methodological biases and to accurately review the included papers. However, limited literature is available on the present topic, and published studies are actually heterogeneous concerning study design and analysed feature, thus preventing the systematic approach to the evidence.

CONCLUSIONS

The popliteomeniscal fascicles described are considered as relevant structures of the popliteomeniscal hiatus, in the posterolateral aspect of the knee. MRI evaluation can detect these fibrous fascicles with good sensitivity. The functional connection of PMFs to lateral meniscus increase the value of their role in meniscal stability and integrity, although insufficient evidence is available concerning biomechanical evaluation. Clinical and therapeutic studies are of scarce quality, especially concerning the number of patients included. Furthermore, the number and position of these fascicles are controversial. More studies with larger samples would be needed for a clear comprehension of PMFs function and clinical management of PMFs tears, especially with large case series and modern biomechanical testing.

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TGF-β1 DIFFERENTIALLY MODULATES THE COLLAGEN VI α5 AND α6 CHAINS IN HUMAN TENDON CULTURES

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Collagen VI is a microfibrillar collagen with a potential regulatory role in tendon repair mechanism. We studied the expression of collagen VI α5 and α6 chains in normal human tendon fibroblast cultures, both under basal condition and in response to TGF-β1, a potent regulator of tendon healing. Under basal condition, we found that the α5 chain was expressed, although to a lesser extent with respect to the α3 chain; in contrast, the α6 chain was absent. The treatment with TGFβ1 induced an opposite effect on the expression of the α5 and α6 chains; in fact, while the α5 chain was dramatically reduced, the α6 chain was induced and released in the culture medium. These data indicate that collagen VI α5 and α6 chains are differentially involved in tendon matrix homeostasis. The α6 chain may represent a new potential biomarker for monitoring TGFβ1-related events in tendon, as healing and fibrotic scar formation.

Tendon is a dense connective tissue composed of a highly ordered extracellular matrix (ECM), mainly constituted by collagen fibrils which are hierarchically organized to withstand tensile forces transmitted from muscle to bone axis. Fibrils contain mostly type I collagen and other ECM components which contribute to tendon collagen fibrillogenesis (1).

Collagen VI, a microfibrillar collagen widely expressed in most tissues, has been identified in the matrix of both developing (2) and mature (3) tendon. It has been detected as a network of beaded filaments anchored to the cell surface of tenocytes (3). Recently, we found that collagen VI localizes in the pericellular matrix of tendon fibroblasts, by interacting with the NG2 proteoglycan (4).

In humans, five distinct collagen VI α chains have been identified (5). The best characterized and widely expressed form of collagen VI is the α1α2α3 chain containing heterotrimer that assembles intracellularly into dimers and tetramers. After secretion, tetramers associate end to end constituting typical 100 nm-spaced beaded microfilaments, which may form fibrils, by parallel alignment, or web-like structures, depending on the association with cell receptors and ECM-binding proteins (6). In humans, two novel collagen VI chains, the α5 and α6 chains, have been recently identified, which structurally resemble the α3 chain but display a more restricted and often alternative tissue specific pattern (5, 7, 8). With respect to the collagen VI α3 chain which is ubiquitous, the α5 chain is selectively detected in areas subjected to tensile stress, such as the dermo-epidermal junction (7) and the myotendinous junction (8). However,

Key words: collagen VI, transforming growth factor β1, tendon, myotendinous junctions

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0393-974X (2016)

cells responsible for its production have not been identified, as muscle cells (8) and skin fibroblasts (personal observation) do not express the α5 chain under conventional culture conditions. The α6 chain co-assembles with the α3 chain in muscle cell cultures; its localization is mainly interstitial (7, 8) and it appears to be implicated in ECM remodeling during muscle fibrosis (8).

Mutations in *COL6A1, COL6A2, and COL6A3* genes cause muscular dystrophies associated with distal joint hyperlaxity and contractures (9). *Col6a1-/-* mice, a collagen VI null model (10), and *Col6a3* deficient mice (11) develop mild myopathy; altered tendon fibrillogenesis and tendon dysfunction have been also reported in mouse models (11, 12).

Interestingly, col6a1 mutant zebrafish embryos display alterations of the myotendinous junctions (13), the primary site of force transmission from the muscle to the tendon and bony skeleton. In the tendon biopsy of a patient with a mutation in the *COL2A2* gene, we found dramatic changes of collagen fiber morphology and accelerated tendon matrix turnover, possibly due to the up-regulation of MMP2 (14), suggesting a critical role of collagen VI in tendon matrix homeostasis.

Collagen VI is also thought to be implicated in tissue remodeling and wound healing. Variations in collagen VI expression have been reported in many pathological conditions, like muscle and liver fibrosis (15) and injured tendon (16). Notably, collagen VI α3 and α6 chains, but not the α5 chain, are up-regulated in fibrotic areas of muscle biopsies of patients affected by different forms of muscular dystrophies (17), likely in response to increased levels of transforming growth factor β1 (TGF-β1). Recently, we reported that the collagen VI α3 chain plays a crucial role in tendon repair, by regulating tendon fibroblast behavior and the assembly of tendon matrix (4). It remains to be established whether the α5 and α6 chains are involved in tendon healing.

TGF-β1 is one of the most potent regulators of tissue wound healing and fibrosis (18). The responsive profile of TGF-β1 plays a critical role in tendons and ligaments healing. Notably, TGF-β1 stimulates cell growth, collagen production and matrix contraction, a process mainly supported by myofibroblasts (19). The involvement of TGF-β1 in tendon healing is supported by the presence of myofibroblasts in ruptured cuff tissue (20), tendon adhesion (21), chronic paratenonitis (22), and experimental models of tendon injury (23).

In this study, we explored the expression of the collagen VI α5 and α6 chains in normal human cultured tendon fibroblasts and in response to TGFβ1 exposure.

MATERIALS AND METHODS

Tendon cell cultures

Human quadriceps tendon fragments were harvested from four patients (17, 21, 51, and 60 years old) during standard orthopedic surgery (total knee arthroplasty and anterior cruciate ligament replacement). All patients granted informed consent.

For tendon fibroblast cultures, quadriceps tendon fragments were subjected to mechanical dissociation, and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% antibiotics plus 10% fetal bovine serum (FBS); 0.25mM L-ascorbic acid was added to the medium to allow collagen VI tetramer secretion. Tendon cells were grown onto coverslips, cultured in multiwell plates and, when indicated, treated with 10 ng/ml TGF-β1 (Sigma-Aldrich) for 10 days.

Immunofluorescence and confocal analysis

Cells grown onto coverslips were incubated with affinity-purified rabbit polyclonal antibodies against the collagen VI α3, α5, α6 chains (7), collagen I (Sigma-Aldrich), and with FITC or TRITC-conjugated secondary antibodies (DAKO). Cell nuclei were stained with 1 mg/ ml DAPI (Sigma-Aldrich). Samples were mounted with an anti-fading reagent (Molecular Probes Life Technologies Ltd, Paisley, UK) and observed with a Nikon epifluorescence microscope. The confocal imaging was performed with a A1-R confocal laser scanning microscope (Nikon, Melville, NY), equipped with a Nikon 60x, 1.4 NA objective, and with a 488 and 561 nm laser lines to excite FITC (green) and TRITC (red) fluorescence signals. Each final confocal image, of 1.024x1.024 pixels and 4.096 gray levels were obtained

by maximum intensity projection of ten optical sections passed through the central region of the cells (recorded at z-step size of 300 nm).

Western blot analysis

Cultured tendon fibroblasts were harvested by scraping. The media recovered from the different culture conditions were concentrated with Vivaspin sample concentrators (Vivaspin 2 MWCO10000, GE Healthcare) according to the manufacturer's operating procedures. Cell lysates and concentrated culture media were resolved by standard SDS-PAGE, electroblotted onto a nitrocellulose membrane and incubated with antibodies against the collagen VI α3, α5 and α6 chains, actin, β-tubulin and the collagen VI α1 chain (Santa Cruz), and with a horseradish peroxidase (HRP)-conjugated antibodies. Chemiluminescent detection of proteins was carried out with ECL detection reagent Kit (GE Healthcare Amersham, Pittsburgh, PA) according to the supplier's instructions.

RESULTS

For a comprehensive analysis of collagen VI chains in cultures, tendon fibroblasts were grown to confluence onto coverslips, and treated with

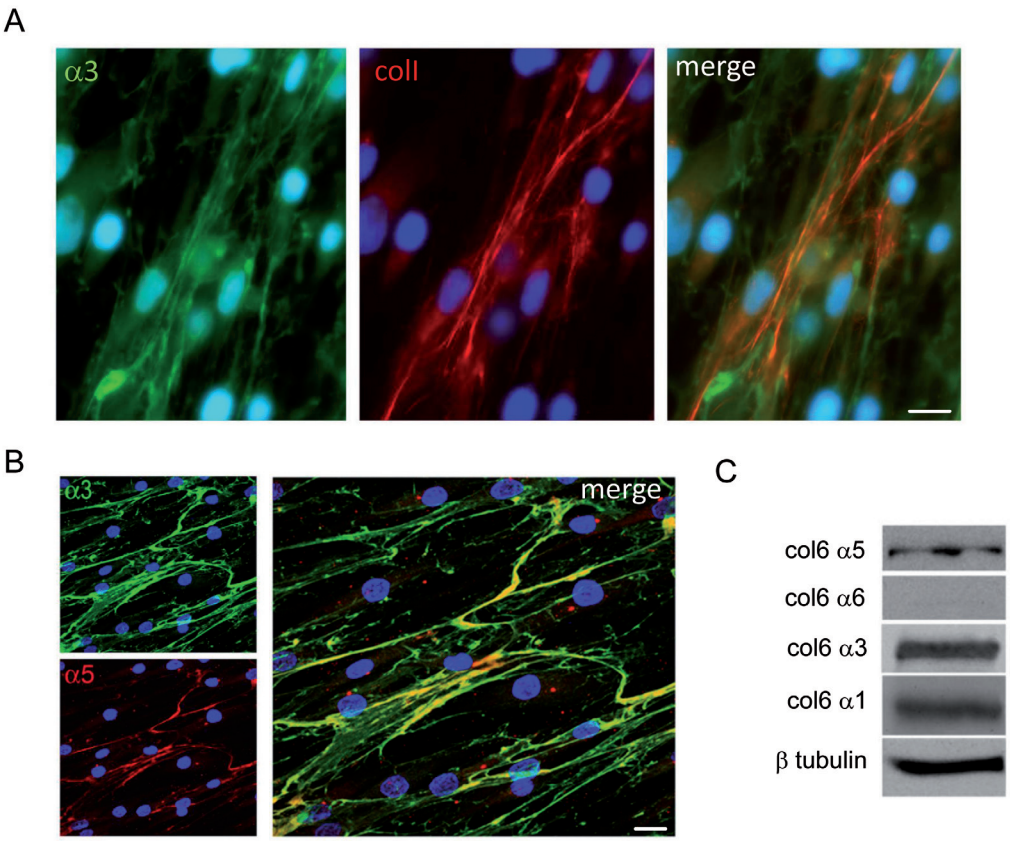

Fig. 1. ***A****): Immunofluorescence analysis with antibodies against thecollagen VI α3 chain (α3, green) and collagen I (colI, red) in post-confluent human tendon cultured fibroblasts. Typical filamentous and web-like structures containing collagen VI α3 chain are secreted and appear parallel aligned with respect to the cell axis. Collagen I fibrils co-localize with the collagen VI α3 chain, as demonstrated by merge image. Nuclear staining, DAPI. Scale bar, 10 μm;* ***B****): Confocal imaging of double labeling for collagen VI α3 (α3, green) and α5 chain (α5, red) at the same culture condition as in A, showing that the α5 chain co-assembles with the α3 chain forming filamentous and web-like structures in the extracellular matrix of human tendon fibroblasts. Nuclear staining, DAPI. Scale bar, 10 μm;* ***C****): Western blot analysis of tendon cell culture lysates. Cells were grown ten days post-confluence in absence of L-ascorbic acid. The collagen VI α1, α3, α5 and α6 chain expression was evaluated by specific antibodies. β-tubulin was used as loading control.*

ascorbic acid to induce the secretion of collagen VI tetramers. By immunofluorescence analysis, the α3 chain was abundantly secreted in the extracellular matrix and displayed a filamentous arrangement aligned to the cell axis. Double labeling for collagen I revealed a close co-localization with the collagen VI α3 chain (Fig. 1A). The collagen VI α5 chain was also expressed, although to a lesser extent than the α3 chain, and co-localized with the α3 chain containing filaments, suggesting that collagen VI α3 and α5 chains may form a network (Fig. 1B). On the other hand, the collagen VI α6 chain was not detectable (not shown). Western blot analysis confirmed the presence of the α5 chain and of the α3 chains, and the absence of the α6 chain in cell lysates (Fig. 1C).

TGFβ1 is a potent regulator of extracellular matrix production during healing process and scar fibrosis (18) by inducing trans-differentiation of tendon fibroblasts into myofibroblasts (4, 19). Tendon fibroblasts were treated with 10 ng/ml TGFβ1 for ten days and the cell phenotype was assessed by immunofluorescence analysis of the ED-A fibronectin splice isoform. Consistent with myofibroblast trans-differentiation ED-A fibronectin was increased (Fig. 2A). Western blot analysis of cell lysates in absence of ascorbic acid did not reveal significant quantitative changes of the collagen α1 and α3 chains in cells after treatment with TGFβ1 (Fig. 2B). However, the analysis of medium after induction of collagen VI secretion with ascorbic acid revealed an increased amount of both α1 and α3 chains released in the medium of treated with respect to untreated cells (Fig. 2C). These data further support the hypothesis that TGFβ1 treatment did not affect the synthesis of collagen VI α1 and α3 chains, but impairs their retention on the cell surface by means of its interaction with specific cell membrane receptors (4). Strikingly, TGFβ1 treatment induced a dramatic reduction of the collagen VI α5 chain as demonstrated by western blot analysis of cell lysates and cell culture medium (Fig. 2B, C). Conversely, the collagen VI α6 chain was induced by TGFβ1 treatment, as indicated by the presence of a band at

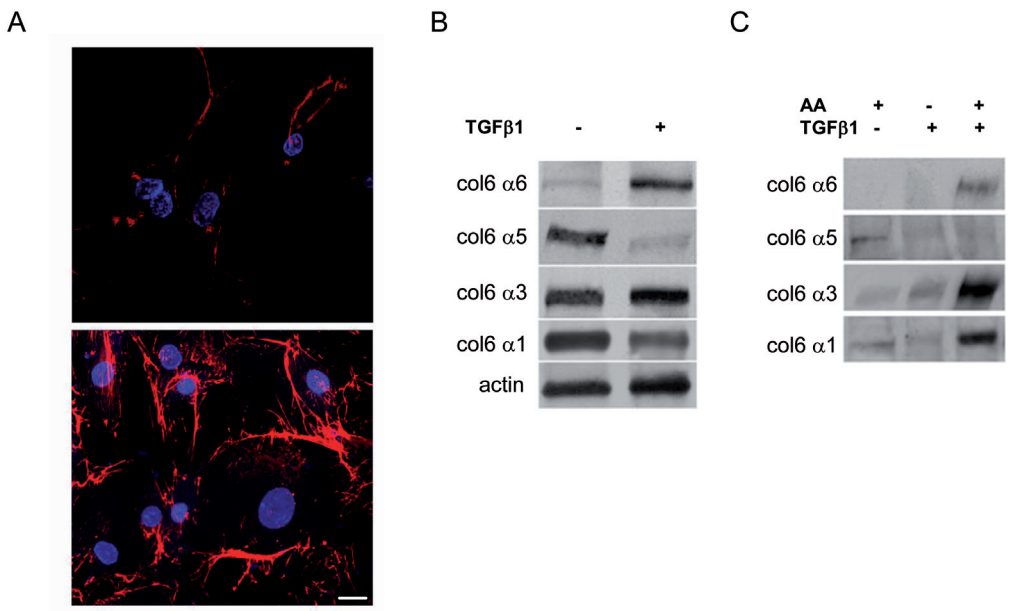

Fig. 2. ***A****): Immunofluorescence analysis of ED-fibronectin in untreated (upper panel) and TGFβ1 treated (lower panel) tendon fibroblasts. Nuclear staining, DAPI. Scale bar, 10 μm;* ***B****): Western blot analysis of cell lysates derived from tendon cultured fibroblasts under basal condition and after treatment with 10 ng/ml TGFβ1. Cells were grown ten days post-confluence in absence of L-ascorbic acid. Collagen VI α1, α3, α5 and α6 chains were evaluated by specific antibodies. Actin was used as loading control;* ***C****): Western blot analysis of conditioned medium of tendon fibroblasts. The addition of L-ascorbic acid (A.A.) and TGFβ1 are indicated.*

the expected molecular weight in cell lysate (Fig. 2B), and in the medium (Fig. 2C). However, the α6 chain was still undetectable by immunohistochemical analysis (not shown).

DISCUSSION

In this paper, we provide new insights in the expression of collagen VI α5 and α6 chains in human tendon cultures, both under basal condition and after treatment with TGFβ1, a key regulator factor of tendon healing.

We previously reported that the collagen VI α5 chain is a specific component of the myotendinous junction, while it is absent in the muscle extra-junctional matrix of adult human tissue. It remains to be established which cells produce and deposit the α5 chain at the myotendinous junction, as muscle cells (both myoblasts and fibroblasts) are not able to produce it *in vitro* (8). Here we show that tendon fibroblast produce and secrete the collagen VI α5 chain in cell culture, suggesting that tendon fibroblasts could represent potential α5 chain-producing cells *in vivo*. In addition, we found that the α5 chain co-localized with the α3 chain-containing microfibrils, suggesting that α3 and α5 chains may co-assemble and form a common network. This data is consistent with recent evidence that, like the α3 chain, the novel chains assemble to homotetramers that are incorporated into mixed microfibrils (24).

Differently from the collagen VI α5 chain, the α6 chain was not detected in tendon fibroblast cultures, confirming that the α6 chain is not essential for tendon matrix organization and function under normal/basal conditions (8). However, the treatment of tendon fibroblast cultures with TGFβ1 induced the expression of the α6 chain, while the α5 was dramatically down-regulated. Though the role of the α6 chain in the extracellular matrix remains unclear, it is evident that it may have a specific role during tissue remodeling and repair. We previously demonstrated that the α6 chain is increased during muscle fibrosis in muscular dystrophies (8, 17), possibly as result of TGFβ1 over-expression (8). The increased expression of the α6 chain in response to TGFβ1 treatment may correlate with changes of biomechanical properties of the extracellular matrix related to myofibroblast differentiation induced by TGFβ1. We previously demonstrated that myofibroblasts produce the collagen VI α6 chain in muscle cell cultures. Myofibroblasts are mechanically active cells that promote tissue contraction by establishing cell-cell and cell-matrix contacts. The mechanism of contraction implicates the development of specialized cell-matrix contacts for the transduction of mechanical forces between cells and the surrounding ECM. Thus, the integration of the collagen VI α6 chain into the extrcellular matrix produced by myofibroblasts may be functional for matrix contraction during specific phases of tissue repair.

It has been suggested that the restricted tissue distribution of the collagen VI α5 and α6 chains with respect to the ubiquitous collagen α3 chain, could reflect their different ability to integrate into the extracellular matrix. It has been suggested that the N-terminal globule of the α3 chain projects out from the rest of the molecule, potentially providing interaction surfaces for cell receptor or matrix molecules (25). This feature may explain the presence of the α3 chain both at the cell surface and matrix of tendon fibroblasts both *in vitro* (4) and *in vivo* (16). It has been suggested that the α5 and α6 chains may alter the mechanical properties of the microfibrils, particularly as they contain significantly more cysteine residues than the α3 chain which may affect the microfibril flexibility by formation of additional disulfide bonds (24).

Our data indicate that collagen VI α5 and α6 chains are differentially involved in tendon matrix homeostasis; the α6 chain may represent a potential biomarker for monitoring TGFβ1-related events in tendon, as healing and fibrotic scar formation.

ACKNOWLEDGEMENTS

This work was supported by the “5 per mille” 2013 in favor of the Laboratory of Musculoskeletal Cell Biology of the Rizzoli Orthopedic Institute,

"Conto Capitale 2013" Ministero della Salute and by the Deutsche Forschungsgemeinschaft (SFB829/B2).

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HEAT SHOCK INDUCES THE EXPRESSION OF PRO-INFLAMMATORY CYTOKINES IN HUMAN ACHILLES TENDON TENOCYTES

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The aim of our study is to investigate the behaviour of healthy and tendinopathic human tenocytes after a heat shock. After we harvested tendinopathic and healthy human tendon samples, we split tenocytes into 4 groups: 3 groups were submitted to heat shock, followed by different periods of post-heating (2, 4 and 20 h). The other group represents our negative control. The target genes were analysed using Real Time PCR. IL-1β and IL-6 expression were significantly increased in tendinopathic samples after heat shock. COL1 and COL3 expression were increased in non-stimulated tendinopathic tenocytes, but their levels significantly decreased after heat shock ($p<0.01$). COL3 levels increase in healthy samples after 20 h post-heating ($p<0.01$). COL1 and COL3 decreased after heat shock as a sign of the failure of repair mechanisms in tendinopathic tendons. Heat shock in *in vitro* models was insufficient to trigger pro-inflammatory cytokines in healthy human tenocytes.

Tendinopathy is the best generic descriptive term for the clinical conditions in and around tendons arising from overuse (1, 2). The term "tendinopathy" defines the clinical syndrome characterised by a combination of pain, swelling and impaired performance (3). Tendon injuries produce considerable morbidity, and the disability that they cause may last for several months despite what appropriate management. The aetiology of tendinopathy remains unclear and many causes have been hypothesised (4). Hypoxia, ischaemic damage, oxidative stress, hyperthermia, impaired apoptosis, inflammatory mediators, fluoroquinolones, and matrix metalloproteinase imbalance have all been implicated as mechanism of tendinopathies (4). Biomechanical factors, functional alterations, repetitive mechanical loading, aging and metabolic disorders may predispose to tendinopathy, with high risk of re-injury (1, 2, 5).

The process of tendinopathy involves both the collagen matrix and the tenocytes (6, 7, 8). Overloading of tendons after intensive exercise and training induces micro-rupture of tendon (9), with damage expression associated molecular patterns (DAMPs) in an attempt to produce tissue healing (10). Several models have recently implicated pro-inflammatory cytokines to initiate the catabolic process, such as Tumor Necrosis Factor-α (TNF-α),

Key words: Stress-related changes, pro-inflammatory cytokines, heat shock, in vitro, human tenocytes

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0393-974X (2016)

Interleukin-1 β (IL-1β) and Interleukin-6 (IL-6), Growth Factors such as Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), TGF-β, EGF, IGF-1 (1) and transcriptional factors such as Nuclear Factor-kappa B (NF-κB), especially in the earlier phases of the process (11).

Mechanical loads play a key role in the maintenance of tissue homeostasis (12). Sedentary individuals present high levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and VEGF and low levels of COL1, which improve the state of inflammation and prelude to an increased activity of MMPs (MMP-2, -9, -13) with a degenerative response and an higher risk of tendon rupture (13). Without initial inflammation, the healing process and the subsequent changes that characterize chronic tendinopathies (>12 weeks) cannot take place (14).

The present study evaluated the molecular mechanisms and the behaviour that characterise human tenocytes during stress condition. A thermal stress (heat shock) was performed on healthy and tendinopathic tendon cells, to analyse the inflammatory mediators and matrix genes involved and to evaluate the response to heat stress.

MATERIALS AND METHODS

We enrolled 15 patients in the present study (12 men and 3 women; average age 34.4 years; range 15-57 years) with failure of conservative management of chronic tendinopathy of the main body of the Achilles tendon. All patients had received at least 6 months of appropriate conservative management, but no local injections. The average time between onset of symptoms and surgery was 9.3 months (range 7 to 14 months). The Local Ethics Committee approved all procedures and all patients gave written informed consent to participate, according to the Declaration of Helsinki (2008). Tendon tissues that would otherwise be discarded were obtained from healthy and injured/diseased tendon areas during surgery procedures performed in accordance to clinical indications. After exposure of the lesion, we obtained two specimens from each patient, one from the injured/diseased area of tendon and the other approximately 5 cm proximal to it, in the visually healthy area of the tendon. The injured/diseased tendon samples were placed in 20 mL of normal saline in a sterile container for transportation to the laboratory. The healthy tendon samples were transferred in a sterile container containing culture medium (D-MEM) (7).

Primary tenocytes explants cultures

Preparation of culture medium: Culture medium consisted of Dulbecco's modified Eagle's medium (D-MEM, Sigma-Aldrich, St.Louis, MO, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin solution. In preparation, 50 ml of fetal bovine serum and 15 ml of penicillin/streptomycin solution were then added to D-MEM. *Preparation of Collagenase Solution*: To prepare the collagenase solution, 50 ml of D-MEM was added to 100 mg of Clostridopeptidase A (crude bacterial collagenase type 1, Sigma-Aldrich, St.Louis, MO, USA). This solution was then sterilised by filtrating through a 0.2-micron pore-sized filter. The solution was stored in 2-ml aliquots at -20°C.

Establishment of Primary Cultures of Tenocytes: The tendon samples were washed with sterile PBS. Any visible fat, muscle, or paratenon was removed from the tendon. The samples were cut into small pieces, rinsed again with PBS and placed in a sterile container. Two millilitres of a prepared 10 mg/ml collagenase solution was added to each container and diluted to a concentration of 2 mg/ml using D-MEM. The container was placed in a shaking incubator at 37°C, where small samples of injured/diseased tendon were digested for 2 to 3 h, and larger samples were digested for 18 to 20 h. After collagenase digestion, the samples were centrifuged for 10 min at 1200 rpm, and the supernatant was transferred to another container. The cell pellet produced by centrifugation was broken up with the tip of a sterile glass Pasteur pipette, and the cells were suspended in 2 ml of culture medium. The 2 ml of cell suspension were transferred to a sterile 25 cm^2 ventilated cell culture flask. The supernatant was re-centrifuged for 10 min at 1200 rpm to remove as many cells as possible. The supernatant was then aspirated, and the cell pellet was put in culture. Two millilitres of culture medium were added to the container, and the cells present were suspended. The 2 ml of cell suspension was then added to the 25-cm^2 flask. The flasks were incubated at 37°C in 5% carbon dioxide. The medium was changed every 3 to 4 days until confluent growth was achieved. The cells were then ready to be sub cultured. Samples of

healthy tendons were rinsed and cut up in the same way as the samples of injured/diseased tendons. The samples were left to digest in the collagenase solution for 18 to 20 h at 37°C. Once the collagenase digestion was complete, the samples were centrifuged as described previously. The cell suspension from each container was transferred to a different 25-cm^2 flask.

Once the tenocytes reached confluence, they were sub cultured in 75 cm^2 ventilated flasks. The tenocytes used for the experiment, from which the mRNA was extracted after the heat shock, were taken between the first and the third passage (24-48 h).

Heat shock

The heat shock was performed for six samples (three healthy and three tendinopathic), immersing the Eppendorf (1.5 mL) which contained suspended tenocytes, in a bath (Julabo Labortechnik, USA), reaching the temperature of 42°C for one h. The heat shock was followed by post-heating, in which the three pairs of samples (one healthy and one tendinopathic sample) were incubated at 37°C for 2, 4 and 20 h, respectively. Another pair of samples were not submitted to heat shock and represented our negative controls.

RNA isolation and Real-Time PCR

After post-heating incubation, total RNA was extracted from human tenocytes, by using TRIZOL reagent according to manufacturer's instruction (Invitrogen, Carlsbad, California, USA). Real-time RT-PCR was carried out with cDNAs reverse-transcribed from total RNA by using Go Taq qPCR Master Mix (Promega, Madison, WI, USA) and Light Cycler 480 Real Time PCR (Roche, Basel, Swiss). The primers used were:

GAPDH:	FW:	5'-AAATTCCATGGCACCGTCAAGGCT-3'
	RV:	5'-CTCATGGTTCACACCCATGACGAA-3'
COL1:	FW:	5'-GGTTACTACTGGATTGACC-3'
	RV:	5'-TTGCCAGTCTCCTCATCC-3'
COL3:	FW:	5'-GCTGGCATCAAAGGACATCG-3'
	RV:	5'-TGTTACCTCGAGGCCCTGGT-3'
MMP-2:	FW:	5'-CGTACGATGGAGGCGCTAAT-3'
	RV:	5'-TCAGGTATTGCACTGCCAACT-3'
IL-6:	FW:	5'-CTCATTCTGCCCTCGAGCC-3'
	RV:	5'-GACCGAAGGCGCTTGTGGA-3'
IL-1β:	FW:	5'-CAGGCTGCTCTGGGATTCTC-3'
	RV:	5'-GTCCTGGAAGGAGCACTTCAT-3'
IL-10:	FW:	5'-AAGACCCAGACATCAAGGCG-3'
	RV:	5'-AATCGATGACAGCGCCGTAG-3'
TNF-α:	FW:	5'-ACGAAACATCCAACCTTCCCA-3'
	RV:	5'-CCCAATTCTCTTTTTGAGCCA-3'

The concentration of mRNA samples was estimated using a spectrophotometer NanoDrop. The integrity of the extract of mRNA was checked using a run in 1.2% agarose gel for RNA. The absorbance ratio A260:A280 was 1.93±0.05, with an average concentration of 376.3 ng/ml. The results of RT-PCR were analysed by calculating the ratio between the target gene and the gene GAPDH, used as normalizer.

Statistics

A *t-student* test to compare two means was performed using Graph Pad Software (California, USA) to test for statistically significant differences in mRNA levels. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Pro-inflammatory cytokines levels increase in tendinopathic human tenocytes after heat shock

Real Time-PCR shows a marked increase of IL-1β levels in heat shocked tendinopathic samples, reaching a peak at 4 h post-heating in comparison with healthy samples. In non-shocked samples, an increased expression of IL-1β was observed in the healthy samples compared to the tendinopathic (Fig. 1a).

IL-6 levels, similarly, significantly increased in tendinopathic samples after heat shock, with a peak after 4 post-heating compared to shocked healthy samples, while in non-shocked samples, an increased expression of IL-6 was seen in healthy samples in comparison with the tendinopathic samples (Fig. 1b).

The expression of IL-10 shows a statistically significant increase in the healthy samples after heat shock at 20 h post-heating in comparison with the tendinopathic samples ($p<0.01$). IL-10 levels are significantly increased in the tendinopathic samples after heat shock at 4 hours post-heating compared to the healthy samples ($p<0.01$) (Fig. 1c).

COL1 levels are increased in not stimulated tendinopathic tenocytes

COL1 levels increased in the non-stimulated tendinopathic samples compared with the healthy non-stimulated samples ($p<0.01$). COL1 levels

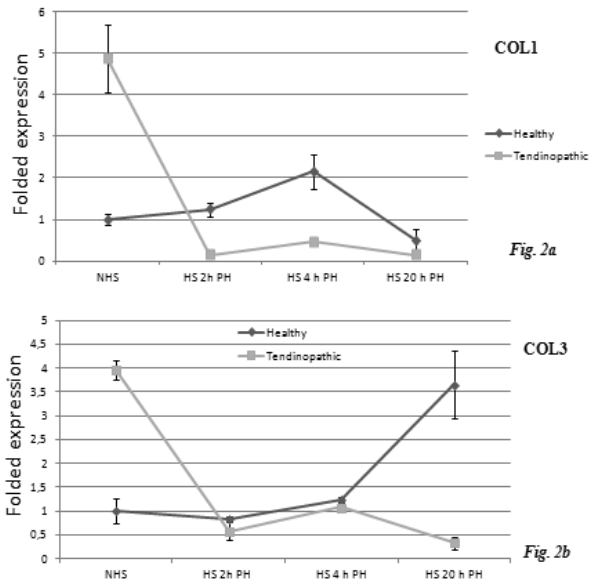

Fig. 1. *Cytokines levels increase after heat shock in tendinopathic samples.*
***NHS**: not stimulated tenocytes; **HS 2h PH**: shocked cells after 2 h post-heating; **HS 4h PH**: shocked cells after 4 h post-heating; **HS 20h PH**: shocked cells after 20 h post-heating. IL-1β (**Fig. 1b**) and IL-6 (**Fig.1a**) levels were increased in tendinopathic samples after heat shock compared to healthy samples, showing a similar peak at 4 h post-heating ($p<0.01$). On the contrary, IL-6 and IL-1β levels are higher in the non-shocked healthy samples compared to non-shocked tendinopathic samples ($p<0.01$). IL-10 (**Fig. 1c**) levels showed a peak at 4 h after heat shock in the tendinopathic samples compared to the shocked healthy samples ($p<0.01$), while their levels were increased in the shocked healthy samples at 20 h post-heating compared to the healthy non-shocked samples ($p<0.05$).*

decreased in the tendinopathic samples after heat shock at 2 and 4 h post-heating compared to the healthy samples ($p<0.01$), while after 20 h post-heating no differences were evident (Fig. 2a).

COL3 levels are increased in healthy tenocytes after heat shock

COL3 levels similarly increased in the non-stimulated tendinopathic samples compared to healthy non-stimulated samples ($p<0.01$).COL3 increases at 20 h post-heating in healthy samples in comparison with the tendinopathic samples ($p<0.01$). No differences were found after heat shock at 2 and 4 h post-heating (Fig. 2b).

DISCUSSION

In our study, we analysed IL-1β, IL-6 and TNF-α as pro-inflammatory cytokines, IL-10 as anti-inflammatory cytokine and MMP-2, which belongs to the family of metalloproteinases, has a double function, enhancing degradation and remodeling of the ECM at the same time, COL1, the main collagen in tendons and COL3, a reparative collagen, whose variations are considered crucial to outline the state of health of a tendon (7). The results of MMP-2 and TNF-α still need to be clarified and further studies are necessary to better investigate their role in stress condition. TNF-α and IL-1β are typical pro-inflammatory cytokines, which induce and mediate the expression of other actors in the inflammatory response in tendons, as MMPs (MMP-1, 13) (9, 15), PGE_2 (14), as well as other inflammatory cytokines such as IL-8, -15, -21(16, 17), and IL-6, which has both a pro-inflammatory function and the induction of the resolution of the state of inflammation. IL-10,

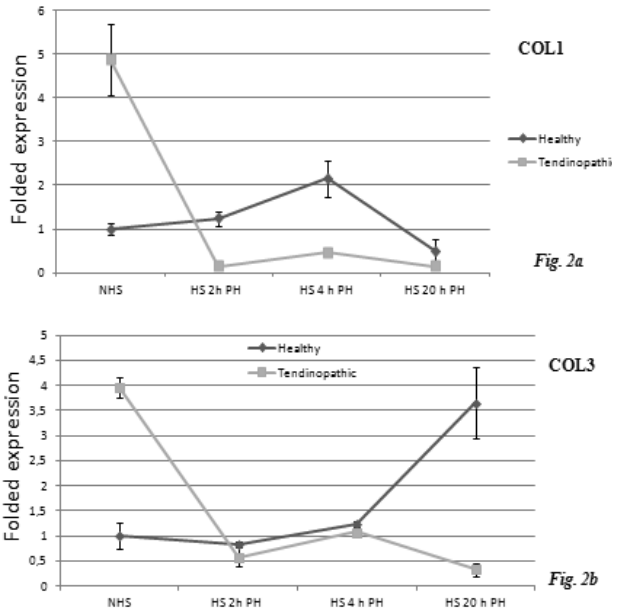

Fig. 2. *COL3/COL1 ratio increases in healthy samples after heat shock.*
***NHS**: non-stimulated tenocytes; **HS 2h PH**: shocked cells after 2 h post-heating; **HS 4h PH**: shocked cells after 4 hpost-heating; **HS 20h PH**: shocked cells after 20 h post-heating. COL3 levels were increased in the healthy samples after 20 h post-heating compared to the tendinopathic samples ($p<0.01$). No significant differences were found in samples after heat shock at 2 and 4 h post-heating. COL1 and COL3 levels were increased in the non-stimulated tendinopathic samples in comparison with the tendinopathic samples after heat shock ($p<0.01$). COL1 levels decreased drastically after heat shock in tendinopathic cells, while there was an increase of COL1 levels in healthy samples after 4 h post-heating in comparison with tendinopathic samples ($p<0.01$). After 20 h post-heating, no differences were found between healthy and tendinopathic samples.*

in contrast to previous cytokines, has mainly anti-inflammatory features and it is responsible, along with IL-6 and IL-8, of the resolution of inflammation in tenocytes (14, 18). We chose to analyse MMP-2, which belongs to the family of metalloproteinases, enzymes capable of reshaping the matrix in normal conditions, to induce damage in response to a pathogenic noxa and to stimulate the repair processes of the tendon. MMP-2 was chosen for its dual function, inducing the degradation and subsequently, the healing of the ECM, and for its action to induce VEGF (16, 17, 19).

The exact aetiology of tendon disorders has not been fully clarified yet and the essential pathological lesion is a failed healing response (21, 22, 23). Although histopathological signs of inflammation are largely not evident in operative specimen, lately its role has been re-evaluated and in the last decade, its implication in the pathogenesis of tendinopathy have been studied extensively (21, 22). Our *in vitro* model allows to analyse what happens in healthy tenocytes during a stress condition and compare them with pathological samples from tendinopathic areas of tendons submitted to the same stimulus. For this purpose, we applied a thermal stress (heat shock), a harmful stressor for tenocytes (20). Our protocol involves exposure to 42°C for 1 h, followed by incubation at 37°C for 2, 4 or 20 h. In this way, the tenocytes are subjected to thermal stress for a prolonged period, to allow them to develop a response to the stimulus, avoiding reaching temperatures too high to sustain.

The present study showed that, in healthy tenocytes, the expression of COL1 significantly increased after heat shock at 4 h post-heating compared to COL3, followed by a significant

decrease 20 h post-heating. COL3, instead, increased in healthy samples after 20 h post-heating, when the expression of COL1 decreased. In tendinopathic samples, COL1 and COL3 peaked in the non-stimulated samples.

The expression of COL1 and COL3 is altered in tendinopathy (7, 21): COL1 is the main constituent of tendons and COL3, whose expression increases in tendinopathy, gradually replaces the predominant collagen, and determines the formation of a mechanical structure weaker than the native one (7). Most likely, the tenocytes from the tendinopathic areas had exhausted their repair capability and were thus unable to produce collagen I. On the other hand, in the non-stimulated tendinopathic samples, the expression of COL1 was high, as these tenocytes were chronically stimulated and when subjected to heat shock, they are not able to react, with a decrease in COL1 level.

The expression of IL-1β and IL-6 is similar. Tendinopathic cells increased the expression of these genes, with minimum levels in non-shocked tendinopathic cells because of their exhausted healing potential. These levels then increased gradually, starting at 2 h post-heating, peaking at 4 h post-heating, followed by a gradual decrease 20 h post-heating. IL-10 shows a similar trend, with a peak in the tendinopathic samples at 4 h post-heating and low levels for the other non-shocked tendinopathic samples, at 2 and 20 h post-heating. Tenocytes from healthy tendon samples after heat shock show an increase of this cytokine, in particular 20 h post-heating, compared to healthy non-shocked samples.

It is necessary to compare the changes in expression of pro-inflammatory genes, such as IL-1β and IL6, with those of the genes involved in healing, such as COL1 and COL3. The reason why pro-inflammatory genes do not increase in response to heat shock in healthy tenocytes is controversial and this requires further reflection. The likely explanation may lie in the different types of responses that a tenocyte, depending on their condition, is capable of expressing. Healthy tenocytes respond to heat shock not with inflammation, as one would expect, but with an increase in the expression of genes involved in healing, as observed from COL3 levels, compared to those of tendinopathic cells. In healthy tenocytes which have not been subjected to a failed healing response, their defense mechanisms to the damage, such as the expression of HSPs 27 and 70 (19, 23), are still intact. For this reason, to be most effective, stressor stimulus must necessarily exceed a threshold value that can be overcome only with a more prolonged and more intense stimulus, as *in vivo*. On the contrary, tendinopathic tenocytes have already compromised the defence and healing mechanisms: they respond to thermal stress mainly inducing the expression of inflammatory genes, which activate other genes, such as collagenases, elastases and MMPs, thereby undermining the healing ability of the tendon and amplifying the pre-existing damage. This may be regarded as a memory of the inflammation to which tendinopathic cells may have been subjected. The rise of this anti-inflammatory cytokine could imply that a stress of this entity and duration is not able to induce the expression of pro-inflammatory mediators such as IL-6, TNF-α and IL-1β, in healthy tenocytes subjected to heat shock.

Legerlotz et al. demonstrated an increased expression of IL-6 in tendinopathic human cells (24), while Sun Hui et al. showed an increase of IL-1β in damaged tendons of rats subjected to mechanic overload (25). In contrast, in healthy tenocytes, this increase does not take place, as one would expect. The expression of these genes decreases in shocked samples, compared to healthy controls. We still do not know the reasons for this: probably, the simultaneous effects of other anti-inflammatory genes involved initially in response to injury, as in the case of IL-10, causes these genes not to increase. It is also possible that a far more intense and prolonged stressor would be necessary to induce a change in expression of these genes.

The present study evaluated the response to heat shock by healthy and tendinopathic tenocytes. In healthy tenocytes, reparative processes prevail, without relevant induction of an inflammatory response, which is instead evident in tendinopathic tenocytes, in which the healing potential is likely exhausted.

The present study is the first to investigate the role

of inflammation and repair genes in human samples using a novel heat-shock protocol, comparing the expression of these genes in healthy and tendinopathic samples, analysing what may happen during the first stages of tendinopathy.

A most important limitation of the studies on human samples is that tendon biopsies are usually obtained from patients with end stage tendinopathy (10). Therefore, it is difficult to understand what happens before these phases. Since the tests were carried out *in vitro*, using only heat shock, further studies are needed to try to understand the possible pathogenic mechanisms underlying the early tendinopathy. In our view, more intense stimuli administered close together, combining mechanical and heat stressors for longer duration, could induce a greater rise in the expression of pro-inflammatory genes in healthy tenocytes, reproducing the *in vivo* conditions.

ACKNOWLEDGMENTS

Dr. A.D. for the organization and collection of samples and data and their interpretation. Prof. N.M. and the entire department of Musculoskeletal diseases of the University of Salerno, for his essential scientific and surgical contribution and to have critically reviewed the manuscript. Prof. S.F., Dr. D.S. and all the department of Molecular Biology and Pathology of Federico II University of Naples, for the interpretation of data and their suggestions about biological approaches and laboratory techniques. Prof. D.R. and Dr. S.L.D. for their contribution to the writing and for the review of this article.

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RISK FACTORS FOR POST-OPERATIVE SHOULDER STIFFNESS: ARE THERE NEW CANDIDATES?

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The aim of this study was to document the incidence of postoperative shoulder stiffness (SS) after arthroscopic rotator cuff repair and evaluate the role of risk factors for its development. Seventy-five consecutive patients that underwent arthroscopic rotator cuff repair were included. The incidence of post-operative SS was prospectively investigated and the presence of 20 potential risk factors was documented retrospectively. The incidence of post-operative SS was 10.4%. All patients were women, and sex was significantly associated to pathology development (p=0.0067). The presence of gastroesophageal diseases was found to be significantly associated with post-operative SS development (p=0.0046). A significant association between the occurrence of post-operative SS and the presence of gastroesophageal diseases was identified. This finding, not yet reported in literature, deserves further investigation. The incidence of post-operative SS fell among previously reported ranges, with females significantly more affected than men.

Rotator cuff (RC) tears are a common contributing factor to shoulder pain and occupational disability, and RC surgery evolved rapidly from a minor niche to a fully recognized subspecialty in orthopaedic surgery. Nowadays, arthroscopic RC repair is accepted as gold standard in surgical treatment of RC tears. This technique has proven to be effective, safe and has high clinical success rate that is durable in time (1). There are however some possible complications described in literature. Restriction in glenohumeral joint range of motion (ROM) has been reported with variable incidence in literature and has been defined as post-operative shoulder stiffness (SS) (2, 3). The term SS has been recommended to replace the sometimes misleading previously used "frozen shoulder" and "adhesive capsulitis" (2).

SS may have various aetiologies, including immobilization, trauma or surgical interventions, and numerous risk factors for primary and post-operative SS have been described. This article evaluates the role of pre-operative conditions as risk factors for SS in the development of a specific type of post-operative condition, namely post-operative SS after arthroscopic RC repair.

MATERIALS AND METHODS

After institutional review board approval (55/INT/2016),

Key words: shoulder stiffness, rotator cuff repair, post-operative complication

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0393-974X (2016)

all patients between 18 and 75 years of age who underwent arthroscopic RC repair for degenerative posterosuperior RC tears between 01/10/2014 and 30/09/2015 were assessed for eligibility. Exclusion criteria were previous history of trauma, isolated subscapularis tears, massive RC tears (C4 in Snyder's classification) and presence of unequivocally diagnosed concomitant disorders of the shoulder, including glenohumeral arthritis, fracture, osteonecrosis, instability or infection.

Pre-operatively, all patients underwent a clinical evaluation and magnetic resonance imaging to diagnose any tear of the RC.

Arthroscopy was performed in a lateral decubitus position using an interscalene brachial plexus block associated with a deep sedation.

Diagnostic glenohumeral arthroscopy was completed in a standard manner using a 30° arthroscope. The long head of the biceps was evaluated for degeneration and stability and RC tears were classified according to Snyder and Lafosse. All pre-operative and intra-operative evaluations were performed by a single examiner (P.R.) with extensive experience in shoulder surgery.

After creation of an anterolateral and lateral portals, RC repair was performed with double or triple loaded metal suture anchors (Corkscrew, Arthrex Inc., Naples, FL, USA; Super Revo and Threvo, Conmed, Largo, FL, USA) in a single-row fashion. The choice of the implant was based on type of lesion and experience of the surgeon. In cases of tendinopathy of the long head of the biceps, a tenotomy was performed.

Table I. *Potential risk factors for SS evaluated in study population.*

1. Smoking habits
2. Diabetes mellitus and antidiabetic therapy
3. Hyperthyroidism
4. Hypothyroidism
5. Chronic obstructive pulmonary disease
6. Gastritis and gastroesophageal reflux disease
7. Autoimmune diseases
8. Parkinson's disease
9. Dupuytren's disease
10. Lipid metabolism disorders
11. Hypertension
12. Psychological disorder (anxiety, depression)
13. Gynaecological anamnesis (menarche, pregnancies, menopause)
14. Treatment with oestrogen or progestin drugs
15. History of cardiovascular surgery, neck or breast surgery
16. History of oncologic diseases
17. Dimension of rotator cuff lesion as measured at arthroscopy
18. Pre-operative diagnosis of calcific tendinosis on the operated shoulder
19. Pre-operative diagnosis of SS on the operated shoulder
20. History of SS in 1st degree relatives

Bursoscopy and bursectomy were performed routinely. Acromioplasty with Sampson's technique was carried out in patients with type 2 or 3 acromial morphology according to Bigliani's classification.

Patients were discharged the day after the operation wearing a sling (Ultrasling II; Don Joy, Carlsbad, CA, USA) and instructed to wear the sling continuously for 28 days. Passive assisted exercises began at post-operative day 29, active for a minimum of 2 months post-operatively. According to Brislin et al., SS was diagnosed when one of the following was present: total passive external rotation with the arm at the side of less than 10°, total passive external rotation with the arm in 90° abduction of less than 30°, or total passive forward flexion of less than 100°. The diagnosis of stiffness was made only when these motion deficits persisted for at least 90 days post-operatively (4).

After Medline literature review, 20 elements with certain or supposed correlation with SS occurrence were selected and defined as potential risk factors for SS (Table I). Clinical records of all patients were retrospectively evaluated for the presence of these potential risk factors for SS and all patients were prospectively interviewed at least four months after surgery to double-check the recorded findings, to confirm the occurrence of SS episodes and to note any post-operative complication.

Statistical analysis

Statistical analysis (A.M.) was performed using GraphPad Prism v 6.0 software (GraphPad Software Inc.). Continuous variables were expressed as the mean ± standard deviation (SD) or medians and first and third quartiles (Q1 - Q3) as appropriate, while the dichotomous variables are expressed in numbers of cases and frequencies. The Shapiro-Wilk normality test was used to evaluate the normal distribution of the sample and if the null hypothesis of this test could not be rejected, the non-parametric Mann-Whitney test (U test) was applied for the analysis of the samples. Variables with a Gaussian distribution were analysed with Student's *t*-test. The differences for categorical variables were tested using with the *chi*-square test or Fisher's exact test. For all analyses, the significance level was set at *p*-value lower than 0.05.

RESULTS

Seventy-five patients were available to complete all

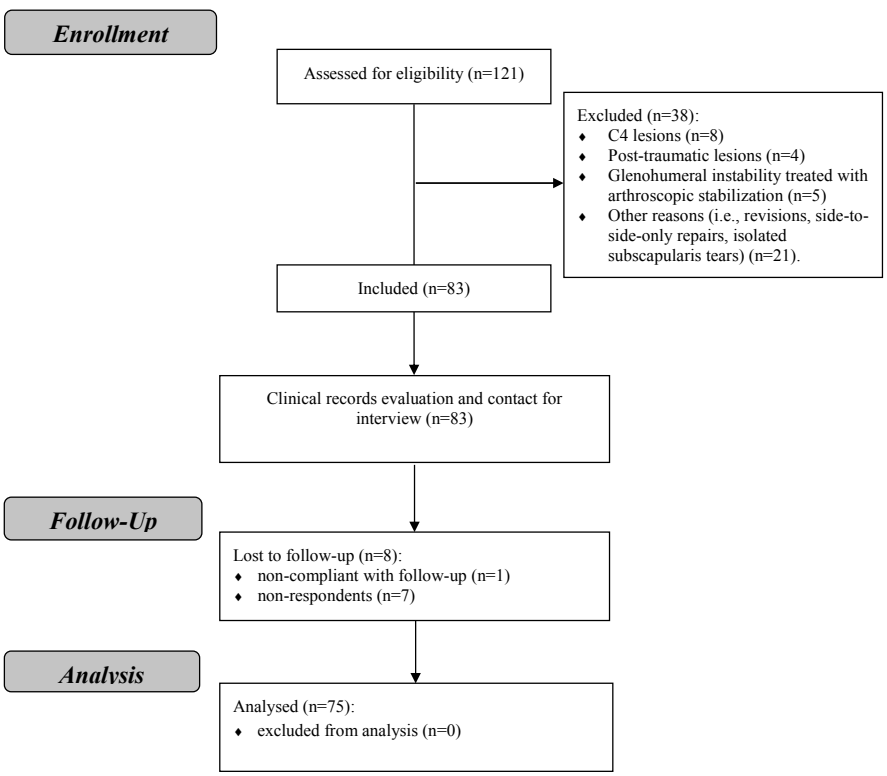

Fig. 1. *Flow diagram of the study.*

planned investigations. A flow diagram illustrates the grouping and flow of patients in our study (Fig. 1) while Patients' demographics are reported in Table II. The incidence of post-operative SS in our population was 10.4% and all patients diagnosed with post-operative SS (SS^+) were women. BMI and age were significantly lower in the population with post-operative SS (SS^+).

A significant association was found between the presence of gastroesophageal diseases and occurrence of SS (p=0.0046) and between female sex and occurrence of SS (p=0.0067). No statistically significant associations were found in the presence of any of the other examined potential risk factors and the occurrence of post-operative SS.

DISCUSSION

This study demonstrates that women and patients affected by gastroesophageal diseases are at higher risk of developing post-operative SS after arthroscopic RC repair. Moreover, these data add information to the incidence of post-operative SS.

The term "frozen shoulder" was coined by Codman to describe "many conditions which cause spasm of the short rotators or adhesions about the joint or bursae" (5). A recent consensus paper of the Upper Extremity Committee of ISAKOS discouraged the generic use of the terms "frozen shoulder" and "adhesive capsulitis", recommending the use of aetiology-based definitions: primary idiopathic SS, or frozen shoulder, which develops without any trauma or specific shoulder disease; secondary SS if a known cause is recognised (2). Post-operative SS is considered a specific subgroup of secondary SS. Numerous different definitions of post-operative SS have been reported (3) and for our study we chose the one by Brislin et al. (4).

The prevalence of post-operative SS after arthroscopic RC repair varies widely in literature. However, the lack of common criteria to define SS and the paucity of studies reporting on post-operative complications after arthroscopic RC repair make a valid comparison of the incidence of post-operative SS among published reports almost impossible (3).

A recent literature review found SS to be a commonly reported complication after arthroscopic RC repair, with rates ranging from 1.5 % to 11.1 % (6). The largest consecutive series, published by Huberty et al., found that the overall rate of post-operative

Table II. *Patients' demographics.*

Group	Overall	Post-operative SS^+	Post-operative SS^-	p-value
Age (years)	61.12 (± 8.78)	55.00 (± 6.93)	61.85 (± 8.74)	0.0361
BMI (kg/m²)	26.18 (± 3.72)	22.77 (± 4.99)	26.50 (± 3.46)	0.0177
F/M ratio	0.56/0.44	1/0	0.51/0.49	0.0067
Operated side L/R ratio	0.35/0.65	0.50/0.50	0.33/0.67	0.4367 (n.s.)
Dominant side L/R ratio	0.08/0.92	0.12/0.88	0.07/0.93	0.5044 (n.s.)

***SS**: shoulder stiffness; **SS⁺**: patients with post-operative SS; **SS⁻**: patients without post-operative SS; **BMI**: body mass index; **F/M**: female/male; **L/R**: left/right; **n.s.**: not significant. Continuous variables are reported as mean (± **SD**).*

stiffness (defined as "patients' dissatisfaction with their range of motion") was 4.9 % (7). On this basis, we expected to find a similar rate of 5 % in our population.

We encountered a higher incidence of post-operative SS than in the study by Huberty et al. (7); this is perhaps related to our choice to use an objective definition of SS, which also included patients satisfied with a limited ROM. In any case, the incidence of SS in our population falls in the range previously reported in literature (6).

The relationship between several risk factors and the occurrence of primary and secondary SS has been analysed by numerous authors, but only a minority of papers report on risk factors for post-operative SS after shoulder surgery also (8–11). In particular, no reports exist on relationships between gastroesophageal diseases and SS.

Changes in proteins related to inflammation and tissue homeostasis have been identified in patients affected by gastroesophageal reflux disease, gastritis and chronic obstructive pulmonary disease (12–17); tobacco smoke itself is known to affect the equilibrium of the extracellular matrix, altering the levels of matrix metalloproteinases and their inhibitors (18, 19). For this reason, we decided to include gastroesophageal diseases, chronic obstructive pulmonary disease and smoking habits among the possible risk factors for development of post-operative SS, although no reports relate occurrence of SS patients affected by these disorders or in smokers. The diagnosis of gastroesophageal reflux disease or gastritis had been previously confirmed either by proton pump inhibitor test or by esophagogastroduodenoscopy in all of our patients affected. Interestingly, we found a significant association between the occurrence of post-operative SS and the presence of gastroesophageal diseases. This association, not previously reported in literature, deserves investigations with further studies.

Women are more frequently affected by SS than men (20-22) as confirmed in our series. However, the attempt to identify aspects of the gynecological anamnesis or the use of estrogen or progestin drugs as predictors for developement of post-operative SS was not successful. To our knowlegde, the biological rationale underneath this preference for the female sex remains unexplained, and we advise that further, adequately powered studies to investigate this relationship both from the epidemiological and the biochemical point of view.

We observed that the population affected by post-operative SS was younger than that not affected; this is in accordance with the data reported by Huberty et al. (7), but in contrast with other reports (10, 11).

Patients with established diabetes mellitus (DM) have a greater likelihood of developing primary SS than the normal population (2); DM was also indicated as a risk factor for moderate post-operative stiffness (8). In our population, 12.5 % of the patients had a diagnosis of DM prior to shoulder arthroscopy and none of them developed post-operative SS.

No significant difference has been found in our series between occurrence of SS in patients with and without thyroid diseases.

Some neurologic conditions have been associated with SS, like surgical treatment for sub-arachnoid haemorrhage and Parkinson's disease. Different malignant diseases may also be associated to musculoskeletal manifestations. In our cohort, no patients were diagnosed with neurologic conditions and no significant difference could be found between occurrence of SS in patients with or without history of malignancy. Dupuytren's disease was found more frequently in patients with SS than in general population. In our study only one patient was affected by Dupuytren's disease and did not develop SS.

Genetics may contribute in developing SS. Having a first degree relative with history of adhesive capsulitis has been reported as a risk factor for developing idiopathic adhesive capsulitis and heritability was demonstrated in a twin study. Predilection for Caucasians and for patients having birthplace or relatives in the British Isles has also been described. At the molecular level, two single nuclear polymorphisms on the IL-6 and MMP-3 genes have been found to be significantly associated with increased susceptibility and severity of post-operative SS (9). We investigated both familiar history and presence of any autoimmune disease among our patients, but data were not enough to confirm any significant association.

Anamnestic evaluation of lipid metabolism disorders

in our cohort could not reveal significant differences between patients developing SS and symptom-free patients. To date, the role of lipid metabolism in SS has been scarcely investigated and remains questionable. However, recent research suggested intriguing hypotheses on the role of hypercholesterolemia and inflammatory lipoproteinemia in SS (22).

Treatment with barbiturates and highly active anti-retroviral therapy has been associated with development of connective tissue disorders, including SS and case reports related SS to administration of vaccines and ACTH deficiency. No patients in our group were treated with any of these medications or referred ACTH deficiency.

Although psychological factors may play a role in development of SS (23), we didn't collect enough data to confirm associations between any psychological disorder and occurrence of post-operative SS.

Secondary SS can also occur after neck, breast or cardio-vascular procedures (2). In our series, no significant associations were identified between anamnesis of neck, cardiac or breast surgery and development of SS.

Limited pre-operative ROM and surgery-related variables, such as tears less than 3 cm in diameter, partial articular-sided tears, high grade fatty infiltration, pre-operative SS, calcific tendinosis, concomitant labral repair, single tendon repair and open surgery have been associated to development of SS after RC repair (2, 4, 7, 10, 11, 24), however, no associations of this kind could be identified in our cohort.

Our study has some limitations. Firstly, it is a retrospective case series from a single surgeon on a single procedure, without any control group. However, all patients underwent a standardised operative and peri-operative protocol and timely evaluation was conducted to limit confounding variables. Moreover, the retrospective nature of this study did not account for an adequate power analysis for each single risk factor, therefore, highly powered studies are awaited to confirm the association between gastroesophageal diseases and post-operative SS.

This study did not investigate the role of rehabilitation in development of post-operative SS (2, 24, 25), nevertheless, the importance of implementing early post-operative motion is still debated, with some authors suggesting that too rapid advancing motion protocols could also lead to an inflammatory response and increased risk of re-tear (24, 25).

Finally, SS diagnosis was purely clinical, as widely accepted in literature. However, recent literature suggests that different imaging modalities may help to confirm the diagnosis and overcome the lack of common criteria to define SS which nowadays make comparison between outcomes form different studies difficult (3).

In conclusion, a significant association between the occurrence of post-operative SS and the presence of gastroesophageal diseases ($p=0.0046$) has been identified. This finding, not yet reported in literature, deserves further investigations with future studies. The incidence of post-operative SS encountered in this study (10.4%) falls within previously reported ranges, with females being significantly more affected than men ($p=0.0067$).

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SURGICAL TREATMENT OF INSERTIONAL ACHILLES TENDINOPATHY: A SYSTEMATIC REVIEW

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Insertional Achilles tendinopathy is a frequent cause of pain and performance impairment of the ankle. It is more common in runners, but may also affect general population. Conservative treatment is the gold standard in the early phases but 10% to 30% of patients require surgery. The aim of this study is to review the current literature in order to evaluate current surgical strategies for Insertional Achilles tendinopathy and to analyze the effectiveness of the available techniques. We performed a systematic review of the literature, to identify studies reporting clinical outcome after surgical treatment for Insertional Achilles tendinopathy in any population group with at least 6 months follow-up. The quality of the articles included was evaluated by the Coleman Methodology Score and correlated with the reported outcome and year of publication. We identified 16 studies reporting on 465 surgically treated Insertional Achilles tendinopathy with a mean follow-up of 29.8 months. Average age at the time of surgery was 53 years. Two different categories of surgical treatment were distinct: debridement alone or debridement with augmentation in case of excessive tendon loss. Results were excellent or good in 89.6% of cases and fair or poor in 10.4%. Average complications rate was 18.3%, with 15.7% of minor and 2.6% of major complications with no difference in the two groups. Negative correlation was found between Coleman Methodology Score and the reported outcome and positive correlation was found between Coleman Methodology Score and year of publication. Good or excellent outcome can be expected after surgical treatment for Insertional Achilles tendinopathy whatever the adopted procedure, but there is no specific evidence regarding which surgical technique provides a better outcome or a lower rate of complications. Research with higher levels of evidence and methodology that is more rigorous are needed in order to evaluate the optimal surgical strategy for patients with IAT.

Achilles tendon problems are very common in active people but they can also affect general population. According to anatomical location two different pathologic entities can be identified, the non-insertional or midportion Achilles tendinopathy and the less common insertional tendinopathy (1). Achilles tendon problems are reported to be non-insertional in 66% of the cases and insertional in 23%, rarer are the mixed forms.

Insertional Achilles tendinopathy (IAT) is more common in runners, especially if occasional athletes with an incidence of 9%, nevertheless up to 5% of professional athletes during their careers suffer for IAT (2). Non-athletes, typically older than 40 years, are the second most common category in whom symptoms appear. Older people are more commonly

Key words: Achilles tendon, tendinopathy, tendonitis, tendinosis, tendinitis, tendon transfer

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0393-974X (2016)

affected probably due to the decreased vascularity and the poor quality of tissues (2, 3).

IAT is clinically characterized by a combination of pain and stiffness at the insertion site onto the calcaneus with different grade of performance impairment. Tendon insertion is painful, swelling can be visible and bony spurs can be palpable. It can be frequently associated with retrocalcanear bursitis. In the early phases, patients are often unable to practice sports or work activities due to acute pain that increases during exercise. With chronic disease, patients experience pain even at rest (4).

Several risk factors have been identified; most relevant intrinsic factors are old age, male gender, prominent calcaneal tuberosity (Haglund's disease), lower extremity malalignment, leg length discrepancy and muscular weakness. Extrinsic risk factors include overuse or misusage, local or systemic steroid use and use of fluoroquinolone antibiotics. Nevertheless, cavus foot, hyperpronation and marked forefoot varus are the most frequently associated conditions (5, 6).

Due to its function, Achilles tendon undergoes repetitive strain from running or jumping that can create a greater susceptibility to degenerative changes or rupture. Reactive tendinopathy may be the first to be caused by overload. This results in a non-inflammatory response that thickens the tendon and increases its stiffness. If overload persists, it leads to inflammation, tissue disorganization, cellular disorders, microscopic tearing and finally to degenerative tendinopathy (4, 7, 8).

Treatment strategies for Achilles tendinopathy try to alter the biology at the diseased site inducing biological changes from degeneration to healing. Initial treatment is conservative and good clinical results have been demonstrated with NSAIDs (non steroidal anti-inflammatory drugs), immobilization, rest, ice, heel lifts, modification of sport activities and eccentric training (7-9). In 10% to 30% of patients, the conservative treatment is not effective and surgery must be take into account.

The purpose of surgery is to restore normal Achilles tendon function with long-term pain relief. Many surgical techniques have been used for IAT and they can be divided into 1) debridement of degenerative tendon, and excision of spurs and retrocalcaneal bursa; 2) when more than 50% of tendon must be removed during debridement, suture anchor repair, tendon transfer or augmentation techniques (with flexor digitorum longus, flexor halluces longus, or peroneus brevis). Multiple surgical approaches have been described including longitudinal midline incision over Achilles tendon (AT), lateral longitudinal, medial longitudinal and the two incisions approach with both medial and lateral incisions (7).

However no definitive surgical recommendations and no definitive conclusion on what could be the most effective surgical procedure are available in literature. The aim of this study is to review the current literature in order to evaluate the current surgical strategies in the treatment of insertional Achilles tendinopathy and to analyze the effectiveness of the available techniques.

MATERIALS AND METHODS

We performed a search using the keywords "Achilles" and "insertional" or "insertion" or "enthesis" or "attachment" in combination with "tendinopathy" or "tendinitis", "tendinosis", "tendinoses", "tendonitis" and "tenosynovitis". English journal articles on these items were searched via Pubmed (http://www.ncbi.nlm.nih.gov/pubmed); Ovid (http://www.ovid.com); Cochrane Database (http://www.cochrane.org); and Google Scholar on July 2, 2016. All journals were considered. The search included all case reports, letters, communications, prospective, and retrospective studies. Literature references of the selected papers were also checked in order to find further relevant publications.

All clinical studies investigating outcomes after surgical treatment for IAT in any population groups with at least 6 months follow-up were included in the review.

Exclusion criteria were: studies on midportion Achilles tendinopathy; combined, unclear or unspecified forms of tendinopathy; conservative treatment; studies without clinical scoring of patients satisfaction; studies with less than 10 patients; narrative reviews, letters to the Editor, personal opinions and technical notes.

These searches yielded 287 articles. Two authors read the abstracts, which were used to judge the relevance

and excluded the title of the papers. In case of doubt regarding inclusion of an article, the senior author made the final decision. Based on title, abstract and study design, 243 article were excluded. Exclusion was based on study design in 79 cases, the topic of the study was not related to insertional Achilles tendinopathy in 102 cases and the study did not present a surgical technique in 62 cases. The full text version of the remaining 44 articles was obtained, and the reference lists were screened, finding a further 5 articles that related to the topic. The contents of these 49

Table I. *Coleman Methodology Score Criteria.*

Section	Number or factor	Score
Part A – only one score could be given for each of the seven section		
1. Study size – number of tendons (N) (if multiple follow-up, multiply N by number of times subjects followed up)	• > 60 • 41-60 • 20-40 • < 20, not stated	10 7 4 0
2. Mean follow-up (month)	• >24 • 12-24 • < 12, not stated, unclear	5 2 0
3. Number of different surgical procedures included in each reported outcome. More than one surgical technique may be assessed but separate outcomes should be reported.	• One surgical procedure only • More than one surgical procedure, but >90% of subjects undergoing the one procedure • Not stated, unclear, or <90% of subjects undergoing the one procedure	10 7 0
4. Type of study	• Randomized controlled trial • Prospective cohort study • Retrospective cohort study	15 10 0
5. Diagnostic certainty (use of preoperative ultrasound, MRI, or postoperative histopathology to confirm diagnosis)	• In all • In >80% • In <80%, no, not stated, unclear	5 3 0
6. Description of surgical procedure given	• Adequate (technique stated and necessary details of that type of procedure given) • Fair (technique only stated without elaboration) • Inadequate, not stated, unclear	5 3 0
7. Description of postoperative reabilitation	• Well described with >80% of patients complying • Well described with 60%-80% of patients complying • Protocol not reported or <60%-80% of patients complying	10 5 0
Part B – scores may be given for each option in each of the three sections if applicable		
1. Outcome criteria (if outcome criteria is vague and does not specify subject's sporting capacity, score is automatically 0 for this section)	• Outcome measures clearly defined • Timing of outcome assessment clearly stated (e.g., at best outcome after surgery or at follow-up) • Use of outcome criteria that has been reported good reliability • Use of outcome with good sensitivity	2 2 3 3
2. Procedure for assessing outcomes	• Subjects recruited (results not taken from surgeon's files) • Investigator indipendent of surgeon • Written assessment • Completion of assessment by subjects themselves with minimal investigator assistance	5 4 3 3
3. Description of subject selection process	• Selection criteria reported and unbiased • Recruitment rate reported: >80% or <80% • Eligible subjects not included in the study satisfactorily accounted for or 100% recruitment	5 5 5

Table II. *Functional classification of outcome according to Nelen.*

Rating	Result
Excellent	No residual symptoms, sports performance unlimited
Good	Full return to the same sport as preoperatively; some stiffness after strenous activities
Fair	Improvement with regard to the preoperative situation; still stiffness and aching relating to sports
Poor	No improvement at all

articles were discussed by all the co-authors and 16 out of all the studies reviewed were considered eligible for this review.

The studies included were evaluated according to the Coleman methodology score (CMS) (10) that was used to assess the quality of studies investigating the surgical outcome for Achilles tendinopathies. CMS assesses the quality of a study assigning a score from a minimum of 0 to a maximum of 100, that represent a perfect study design without influences of chance, biases and confounding factors (Table I) (10).

Clinical outcomes were evaluated using the functional classification described by Nelen et al. (11). Results were considered satisfactory when excellent or good and unsatisfactory when fair or poor (Table II).

RESULTS

Characteristics

Through the reported search strategy, we identified 16 eligible studies published between 2002 and 2015. Of the 16 studies, 12 were retrospective case series (12-23) and 4 were prospective comparative studies (24-27). Two studies (16, 25) evaluated two different groups of patients each, with different surgical procedure performed and compared. Average Coleman Methodology Score was 62.3±12.4 (range 46-86).

Patients' data

The total number of patients included in the reported studies was 451 of 465 surgically treated IAT. Data about sex were deduced from 11 of the 16 studies for 265 patients of which 161 male (60.8%) and 104 female (39.2%). Average age at the time of surgery was 53±8 years (range 33-76 years). The mean follow-up was 29.8±13 months (range 6-48 months) (12-22, 24-26).

Intraoperative features

All articles included patients in whom previous conservative treatment had failed. The preferred operating position was prone, used in 15 studies (12-18, 20-27). The supine position was used only in one study and it was preferred because the surgical procedure was performed endoscopically (19). In the open procedures numerous surgical approaches are described with the longitudinal midline incision more frequently practiced (9 studies) (12-15, 18, 20-22, 25), followed by the medial J (3 studies) (16, 24, 26), the lateral incision (2 studies) (17, 23) and the transverse semi-circular incision (1 study) (27). All papers examined agree on the importance of an extensive debridement in order to obtain healthy tendon tissue and complete removal on any spurs and calcification. Partial or complete Achilles tendon detachment was always performed and followed by reattachment of the tendon to the bone with bone anchors suture or trans-osseous sutures. An Achilles tendon excision greater than 50% of its width was required in 4 studies for a total of 83 procedures and an augmentation with FHL or free flap transfer of sural triceps aponeurosis was done respectively in 74 and 9 patients (12, 24-26). In the other 12 studies involving 382 procedures, an augmentation technique was not performed (13, 15-23, 25, 27).

Complications

Average complication rate was 18.3%, with 15.7%

Table III. *Patient satisfaction.*

Author (year)	Study size (n tendons)	Mean FU (months)	Patient satisfaction		CMS	Preoperative score	Postoperative score	Approach
			Excellent/Good	Fair/Poor				
McGarvey et al (2002)	22	33	17	5	46	/	/	Longitudinal midline incision through AT
Yodlowski et al (2002)	41	39	41	0	57	4,7 (0-6)	1,5 (0-6)	Lateral longitudinal incision between AT and supero lateral crest of calcaneus
Maffulli et al (2004)	21	48,8	16	5	67	63,8 (VISA-A)	86,4 (VISA-A)	Longitudinal midline incision up to 1/3 of AT
Johnson et al (2006)	22	34	/	/	48	53,0 (AOFAS)	89,0 (AOFAS)	Longitudinal midline incision through AT
Wagner et al (2006)	26	47	24	2	52	/	/	Medial J incision
Wagner et al (2006)	39	33	36	3	52	/	/	Medial J incision
Elias et al. (2009)	40	27	38	2	65	56,3 (AOFAS)	96,2 (AOFAS)	Longitudinal midline incision over AT
Maffulli et al (2011)	30	36	26	4	71	62,0 (VISA-A)	88,0 (VISA-A)	Transverse semi-circular incision
Nunley et al (2011)	24	48	23	1	56	/	96,4 (AOFAS)	Longitudinal midline incision through AT
Oshri et al (2012)	21	6	13	8	54	52,0 (AOFAS)	83,1 (AOFAS)	Lateral longitudinal incision
Rigby et al (2013)	43	24	42	1	53	/	90,0 (AOFAS)	Longitudinal midline incision over AT
Ettinger et al (2015)	40	15,6	34	6	69	59,4 (AOFAS)	86,5 (AOFAS)	Longitudinal midline incision through AT
El-Tentawy et al (2015)	13	24,5	13	0	84	57,5 (AOFAS)	98,3 (AOFAS)	Medial J incision and medial foot incision (FHL harvest)
Tallerico et al (2015)	11	13,8	10	1	61	52,0 (AOFAS)	94,8 (AOFAS)	Endoscopic (Medial and lateral portal) or Open (Longitudinal midline incision)
Ahn et al (2015)	15	42	15	0	52	62,1 (AOFAS)	92,5 (AOFAS)	Longitudinal midline incision through AT
Hunt et al (2015)	18	12	18	3	82	56,7 (AOFAS)	91,5 (AOFAS)	Longitudinal midline incision over AT
Hunt et al (2015)	21	12	16	2	86	61,4 (AOFAS)	92,3 (AOFAS)	Longitudinal midline incision over AT
Rousseau et al (2015)	18	24	15	3	67	58,5 (AOFAS)	93,7 (AOFAS)	Medial incision

(range 0-41.5%) of minor and 2.6% (range 0-7.7%) of major complications. Major complications included deep venous thrombosis, major wound problems such as deep infections, nerve injury, persisting neuralgia, tendon rupture and reoperation. Minor complications included minor wound problems, superficial infection, pathological scars and discomfort. No differences in complication rate were found between those who had been subjected to debridement alone and those who had the augmentation.

Clinical outcomes

Clinical outcomes (patient satisfaction inferred with the score of Nelen et al.) were excellent or good in 89.6% of patients (range 76-100%) and fair or poor in the remaining 10.4% (range 0-24%).

In patients who were subjected to augmentations, technique satisfaction was excellent or good in 89.1% (range 76-100%) (12, 24-26). All patients treated with an augmentation technique were also evaluated using the AOFAS score: mean preoperative value was 56±5.2 points; mean postoperative value was 95.5±2.5 points, with a score increment of 39.5 points.

In the 382 patients (12 studies) in which an augmentation technique was not performed excellent or good results were achieved in 89.7% of cases (range 62-100%) (13, 15-23, 25, 27). Of these 12 studies, 4 did not evaluate outcome with a reliable score (13, 16, 20, 22). In the remaining, the AOFAS score was used in 6 studies (14, 18, 19, 21, 23, 25) and the VISA-A score in 2 (15, 27). For those who used the AOFAS score the average preoperative value was 55.9±4.2 points, and

the average postoperative value was 89.6±4.3 points, with a score increment of 33.7 points.

Methodological quality

The Coleman Methodology Score was blindly assessed twice with a high reproducibility (r=0.98, P <0.05). The CMS were correlated with the year of publication and the reported success rates found that, for the 16 studies considered, the CMS negatively correlated with the reported outcome and positively correlated with the year of publication.

CONCLUSIONS

The aim of this study was to review the current literature in order to evaluate the surgical strategies for insertional Achilles tendinopathy treatment and to analyze the effectiveness of the available techniques.

The most important findings of the present study are the overall good outcome after surgical treatment of IAT whatever surgical procedure was adopted when considering patient satisfaction as principal outcome, with satisfaction rates ranging from 82% to 97% and with improvement both in general population and in professional athletes (12, 22, 28). However, when considering the AOFAS score, we unexpectedly found a higher score in those patients who were subjected to debridement and augmentation compared to those who were subjected to debridement alone. Postoperative AOFAS score values were 89.6±4.3 with the debridement technique alone and 95.5±2.5 with the debridement and augmentation technique. This could be due to an inadequate intraoperative evaluation of the Achilles tendon impairment in those who were subjected to debridement alone, that when properly estimated would have needed a more invasive treatment.

The majority of studies examined in this review present a longitudinal midline incision approach. Although the central Achilles tendon approach seems to be the one which provides the best exposure to access the IAT and allow a good debridement of all the pathologic tissue, in the literature there is a lack of studies that compare the different approaches proposed.

Complications of surgery include nerve injury, tendon rupture and persistent or recurrence pain, while wound healing complications such as delayed healing or superficial wound infection are the most commonly reported. Nevertheless, in the majority of cases these complications are easily resolved with dressing change and only rarely require further surgery.

The main limitation of the present study is the low level of evidence of studies included with only one randomized controlled trial (level I). The majority of studies were retrospective or prospective in their design. In literature, some randomized control trials on Achilles tendon tendinopathy are present but the inaccuracy of definition of Achilles tendon, led us to exclude most of them.

The analysis by CMS of the 16 studies considered in this review showed a negative correlation with the reported outcome and positive correlation with the year of publication.

The higher the CMS the lower the outcome and the latest the year of publication. This result suggests that the high success rates achieved in the past were probably due to a poor methodology quality of the studies and that over the years the study methodology increased with a consequent reduction of the reported outcome.

Three previous reviews were performed regarding IAT and its treatment options (28-30). Kearney et al included 11 articles and investigated on current management but no outcome data were provided (29). Wiegerinck et al later systematically analysed available literature on IAT, examining 14 trials with 452 procedures, but they referred to both conservative and surgical treatment (30). Finally Khan et al recently investigated on outcome of surgery for chronic Achilles tendinopathy over the last 50 years, but their work deals with the entire Achilles tendon problem (28).

To our knowledge, this is the first systematic review available in literature considering all the available papers dealing with the outcome after surgical treatment for only insertional Achilles tendinopathy.

Studies on outcome of surgical treatment for insertional Achilles tendinopathy varies widely. Methods, surgical procedures and outcome

evaluation are often different and even if one or more of these topics are comparable, studies are, in some aspect different and/or incomplete. Furthermore, the majority of the studies available in literature are often retrospective and only few prospective studies are available and the majority reporting clinical outcomes after surgical treatment are retrospective and inconclusive. For these reasons, only limited conclusions can be made on surgical treatment for insertional chronic Achilles tendinopathy.

In conclusion, from the studies analysed in this review, good or excellent outcomes can be expected after surgical treatment for insertional Achilles tendinopathy whatever the adopted procedure, but there is no evidence that a surgical technique provides better outcomes or a lower rate of complications. Further studies with higher level of evidence are required to establish the better surgical strategy for patients with IAT.

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EXTRACORPOREAL SHOCK WAVES INDUCE OSTEOGENIC DIFFERENTIATION OF HUMAN BONE-MARROW STROMAL CELLS

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The effects of treatment with shock waves (SW) on osteoblastic cells have already been described. Furthermore, the effects of treatment with SW are also determined by the contextual stimulation of other cell lines, in particular of mesenchymal cells. This is the first experimental study of stimulation of a human mesenchymal stem cell line, taken from bone marrow, using SW (electromagnetic device), with two energy levels. The results showed a significant increase in expression of the main osteoblastic differentiation genes: BMP2, alkaline phosphatase, osteocalcin, COL1A1, RUNX2. The monitoring within 96 hours demonstrated a progressive increase of cell adhesion and an intense cell proliferation at 48 h. The differentiation response and proliferation of stem cells after treatment with SW shows that this therapy is an effective method of regenerative medicine.

In the last 10 years, Extracorporeal Shockwave Therapy (ESWT) has been used in the treatment of pseudoarthrosis and non-union, with a success rate of 70% (1). The Shock Waves (SW) is an acoustic wave characterized by a rapid increase in blood pressure and a following decrease that guarantees the focalization in tissue depth. The clinical effects are justified by the neo-angiogenesis actions, proliferation of osteoblasts and differentiation of stem cells into osteoblastic. This latter effect of SW has been reported only in animal models (2, 3).

The bone healing of a fracture follows the sequence of osteogenesis and bone remodelling that ensures the skeletal reconstruction integrity (4, 5). In the healing of a fracture, the mesenchymal cells are attracted and induced to differentiate into osteoblasts in order to activate bone tissue regeneration (6). When there is non-union, this means there has been a failure of the fracture healing process. In this case, the role of existing osteoblasts may have been inefficient. Moreover, the role of the differentiation of mesenchymal cells into osteoblasts is also very important (7-9). The important role assumed by stem cells in the consolidation of a fracture is already recognized and adopted in the therapeutic field. Recently, innovation in biotechnology has allowed the use, in fractures, of different substances, such as growth factors and bone morphogenetic proteins (BMP), which are able to stimulate the differentiation of progenitor cells present in the site (10). With these biological mediators, it is possible to speed up and optimize the response of fracture healing (11).

The aim of our study was to verify the effect of the shock waves on human pre-osteoblast mesenchymal cells, particularly as regards the expression of osteoblast differentiation genes and the adherence and proliferation of cells.

Key words: *shock waves, stem cell, differentiation, bone*

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MATERIALS AND METHODS

The pre-osteoblasts were purified from bone fragments obtained by surgical treatment of fractures. The samples were pre-treated with collagenase 100 micrograms/ml and incubated in flasks with culture medium αMEM, containing 10% fetal calf serum (FCS), penicillin/ streptomycin and glutamine. After about 7 days, the adherent cells present in a single layer in the flasks were recovered and cultivated. The local ethics committee has given consent to the research.

The ESWT electromagnetic generator system (Minilith SL1, Storz Medical) was used in this study. This ESWT device is a focused one, and the application was possible by using an ultrasound-guided system. Between the generator and the cells, we used an ultrasound compressed gel in order to guarantee the absence of air. The test-vial was placed in a special cylindrical support on the shockwave generator, which enabled the focal depth probe to be directed correctly to the vial containing the cells.

Cells were subdivided into 6 populations: 4 specimens underwent ESW treatment at two different parameters of energy levels and energy flux density (EDF=0.055 mJ/mm2, defined as E2.5; EDF=0.17 mJ/mm2, defined as E5) for 500 pulses, while two untreated vials was

Table I. *SW stimulation.*

REAL TIME			P value	P value
	MEAN	SD	Compared to control cells	Compared to cell with the addition of
BMP2				
Control cells	1	0	-	-
Cells with the addition of DF	8.3	9.2	0.2	-
Cells stimulated at E2.5	0.8	0.2	0.03	0.1
Cells stimulated at E5	1.1	0.2	0.3	0.2
Cells stimulated at E2.5 and with the addition of DF	1.2	0.6	0.5	0.2
Cells stimulated at E5 and with the addition of DF	3.3	1.9	0.05	0.3
COL1A1				
Control cells	1	0	-	-
Cells with the addition of DF	0.4	0.1	0.0001	-
Cells stimulated at E2.5	1.7	0.2	0.0002	0.0001
Cells stimulated at E5	2	0.7	0.02	0.003
Cells stimulated at E2.5 and with the addition of DF	0.4	0.3	0.005	0.8
Cells stimulated at E5 and with the addition of DF	0.7	0.4	0.2	0.2
alkaline phosphatase				
Control cells	1	0	-	-
Cells with the addition of DF	3	0.8	0.003	-
Cells stimulated at E2.5	0.8	0.1	0.002	0.002
Cells stimulated at E5	1.4	0.6	0.2	0.02
Cells stimulated at E2.5 and with the addition of DF	4.1	0.1	0.0006	0.1

Cells stimulated at E5 and with the addition of DF	6.3	0.7	0.0001	0.0008
OSTEOCALCIN				
Control cells	1	0	-	-
Cells with the addition of DF	2.75	0.960902	0.01	-
Cells stimulated at E2.5	4.15	1.126943	0.001	0.1
Cells stimulated at E5	6.875	0.826136	0.0001	0.0006
Cells stimulated at E2.5 and with the addition of DF	2.55	0.793725	0.007	0.7
Cells stimulated at E5 and with the addition of DF	2.9	1.029563	0.01	0.8
RUNX2				
Control cells	1	0	-	-
Cells with the addition of DF	1.825	0.6	0.03	-
Cells stimulated at E2.5	2.3	0.7	0.008	0.3
Cells stimulated at E5	3	1.2	0.01	0.12767
Cells stimulated at E2.5 and with the addition of DF	2.8	0.9	0.005	0.09
Cells stimulated at E5 and with the addition of DF	2.6	0.9	0.01	0.2

Cells were subdivided into 6 populations: 4 specimens underwent ESW treatment at two different parameters of energy levels and energy flux density (EDF=0,055 mJ/mm2, defined as E2.5; EDF=0.17 mJ/mm2, defined as E5) for 500 pulses, while two untreated vials was kept as control samples (control and with the addition of differentiation factors-DF). Results of Real Time PCR for the expression of osteoblastic differentiation genes such as: bone morphogenetic protein 2 (BMP-2), collagen type-I-alpha-1 (COL1A1), alkaline phosphatase (FA), osteocalcin, runt-related transcription factor 2 (RUNX2). The data are expressed as mean +/-standard deviation and p-value.

kept as control samples (control and with the addition of differentiation factors, DF), but underwent the same laboratory processing. The control vials were also kept outside the incubator for the same time as the treated cells. The environmental conditions were 25°C and an air pressure of 101 kPa.

Each sample underwent shock waves administration and subsequently treated for the induction of differentiation into mature osteoblasts in the presence of 10 mmol/L β-glycerophosphate, 100 nmol/L dexamethasone, and 0.2 mmol/L ascorbic acid, which were the differentiation factors. Cells that were not treated with shock waves and cells differentiated using only the growth factors, represented the controls.

On the cell samples undergoing the various treatments, we carried out the MTS (tetrazolium salt test), to verify the osteoblastic proliferation and staining with crystal violet as an index of adhesion.

Finally, we purified the RNA messenger from the cells, which was processed in Real Time PCR for the expression of osteoblastic differentiation genes such as: bone morphogenetic protein 2 (BMP-2), collagen type I alpha 1 (COL1A1), alkaline phosphatase, osteocalcin and RUNX2. The differences in gene expression were evaluated according to the formula 2-ΔΔct.

Statistical evaluation

Each test was performed in triplicate and the results are expressed as mean values ± standard deviations. The statistical evaluation was performed using *t*-student test. The statistical significance was set to $p < 0.05$.

RESULTS

The SW stimulation determined a significant increase of BMP2 only for E5 energy level with

Table II. *Lower energy level of SW stimulation.*

ADHESION		
	MEAN	SD
24 HOURS		
E2.5	37.4	46.5
E5	6	13.4
48 HOURS		
E2.5	67.2	81
E5	-8.2	23.7
96 HOURS		
E2.5	82.4	92.1
E5	16.2	16.2
PROLIFERATION		
24 HOURS		
	MEAN	SD
E2.5	-8.6	35
E5	-5.2	26.4
48 HOURS		
48h		
E2.5	9.8	36.4
E5	7.6	26.6
96 HOURS		
E2.5	-5.6	46.8
E5	4.4	20.1

Adhesion and proliferation was valued only for cells stimulated with SW at 24, 48 and 96 hours. The data are expressed ad mead +/- standard deviation (number of cells).

the addition of differentiation factors (Table I). For COL1A1, we verified that stimulation with SW at E2.5 and at E5, was responsible for a significant increase compared to the control cells. The SW stimulation was responsible for a significant increase of alkaline phosphatase compared to control (at E2.5, at E5 and at E5 with the addition of DF). The SW stimulation was responsible for a significant increase of osteocalcin compared to control (for each energy of SW stimulation) and control with the addition of DF (for E5 only). SW stimulation was responsible for a significant increase of RUNX2 compared to control (for each SW stimulation). When we used the lower energy level (E2.5), the pre-osteoblast adhesion was statistically greater at 24, 48 and 96 h (Table II). The pre-osteoblastic proliferation was statistically higher at 48 h for two energy levels. Considering that in the presence of the differentiation factors there occurs inhibition of adhesion and activation of proliferation for the cells treated with SW without the addition of

DF, we verified a greater adhesion and proliferation. When we used the lower energy level (E2.5), the pre-osteoblast adhesion was statistically greater at 24, 48 and 96 h. The pre-osteoblastic proliferation was statistically higher at 48 h at the two energy levels.

DISCUSSION

Until now, there is no data in the literature about the effect of SW stimulation on mesenchymal stem cells in humans. In this study, we verified that the SW stimulation of human stem cells was responsible for osteoblastic differentiation, in consideration of the general significant increase of the main osteoblastic differentiation genes expression: BMP2, alkaline phosphatase, osteocalcin, COL1A1, RUNX2.

The SW stimulation was efficacious for both energy levels, and the addition of the DF did not have an important effect for the treatment with SW. On the other hand, we underline that for the lowest energy level we recorded a reduced response for BMP2 and FA (for the stimulated cells and with DF) and for COL1A1 (for the stimulated cells and with DF). This may assume a greater specificity of therapeutic response to higher energy levels. As regards to the cells stimulated with SW, in the absence of DF, we performed an additional experiment of adhesion and proliferation, which confirmed that the biological response was determined by SW stimulation. We have to consider that the assessment of gene expression in this study at 24 h was quite early. In fact, in a previous study after SW treatment for femoral head osteonecrosis, the authors verified a significant increase of BMP2 gene expression at 24 h, whilst the increase of the protein expression occurred one week after OU stimulation (12).

Our results confirm the data of recent literature and these allow us to improve our knowledge of the therapeutic effects in bone diseases such as Complex Regional Pain Syndrome, osteonecrosis and fracture consolidation delays. *In vivo* after treatment for consolidation delay, an increase of nitric oxide and osteogenetic growth factors in serum levels has been noted in the patients (13). It is known that bone-marrow mesenchymal stromal cells have the potential to differentiate into osteoprogenitors (14).

Following *in vitro* studies regarding the SW stimulation of the osteoblast line cells, several authors have described different effects: an increase of the VEGF expression which was responsible for neo-angiogenic effect (15); modulation of membrane permeability (16); activation of the cyclin E2/CDK2 complex, which regulates the G1-S transition and cell proliferation (2); induction of the expression of numerous genes involved in osteoblast differentiation (PTHrP, prostaglandin-E2 receptor EP3, BMP-2 inducible kinase, chordin, cartilage oligomeric matrix protein, matrillin) (17, 18); a reduction of the RANKL/OPG ratio, suggesting that stimulation of osteoblasts was able to determine osteoclastogenesis inhibition (2).

Furthermore, earlier studies hypothesized that the SW osteogenetic effects are also due to the simultaneous stimulation of mesenchymal cells. It is known that the bone-marrow mesenchymal stromal cells have the potential to differentiate into osteoprogenitors (14). Until now, the number of studies conducted on the SW effect on mesenchymal cells have been few and these works regarded only animal models (19, 20). The first results showed that the SW stimulation of mesenchymal cells is responsible for an increase of CFU-osteoprogenitors, with a significant increase of bone alkaline phosphatase and TGF-beta1 (19). In the presence of a non-union, the stimulation with SW may be able to determine a recruitment of mesenchymal stem cells with a progressive osteoblastic differentiation (20). Our preliminary results confirm that the effects in humans are similar to those described in animal models.

We can hypothesize that the SW therapeutic effect on the human bone tissue is due not only to the up-regulation of multiple genes involved in osteoblast differentiation, but also to the recruitment of mesenchymal stem cells and osteoblastic differentiation. These findings allow us to better comprehend the effects of shock waves on bone tissue, considering the SW therapy as an efficacious method of regenerative medicine. Further clinical studies may verify the application of the SW treatment in pathological conditions characterized by loss of substance.

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ASSOCIATION BETWEEN MARKERS OF BONE LOSS AND URINARY LITHOGENIC RISK FACTORS IN OSTEOPENIC POSTMENOPAUSAL WOMEN

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In this study, we explored if urinary lithogenic risk parameters could have some application for monitoring bone health status. We recruited 20 women with postmenopausal osteopenia and a negative medical history for nephrolithiasis. Markers of lithogenic risk were evaluated on 24-h urine and fasting-morning urine. Serum levels of bone turnover markers (BTM) were measured in fasting-blood samples. We found that cross-linked telopeptide of type I collagen (CTX) was significantly correlated with 24-h calcium excretion. N-terminal propeptide of type I procollagen (PINP) correlated with 24-h excretion of potassium, calcium and citrate. CTX had considerably increased in patients with pH <5.5. Low citrate levels (<3.3 mmol/24 h) were associated with lower levels of CTX and PINP. Our findings suggest that a low-grade acidosis and some lithogenic risk factors are detectable in a proportion of patients with postmenopausal osteopenia. Further studies are necessary to confirm that this evaluation could be clinically relevant.

The microarchitectural deterioration of bone tissue is a current picture in postmenopausal women, with consequent lowering of bone mass and increased susceptibility to fracture (1). The bone loss starts early and continues for many years, depending on many causes, leading to a unique epiphenomenon that is the imbalance between the bone resorption and formation (2). The pathophysiology of postmenopausal bone weakening may also be influenced by adverse metabolic conditions. Bone tissue is directly involved in essential functions, such as the regulation of acid-base balance. In order to buffer the systemic acidosis, the skeleton acts as an ion exchange column modifying the composition of the mineral portion (3). There is a linear correlation between elimination of calcium and acidosis: the higher is the acidosis, the higher will be the loss of calcium from bones. Estrogen deficiency (1), a diet high in meat protein (4) and the decreased renal function due to ageing (5) are factors favouring a high acid loading that may exceed the neutralization capacity, leading to a latent or low-grade acidosis (3). The relationship among renal function, risk of osteoporosis and hip fracture has been investigated and many studies show that bone mineral density (BMD) decreases not only in end-stage renal disease (5), but also in patients with nephrolithiasis (6). In fact, the urinary stone formation is frequently associated with calcium metabolism alterations and high bone turnover and is favoured by the increased acid production occurring in subclinical acidosis (7). Recent findings demonstrated that lithogenic risk

Key words: osteopenia, acidosis, bone turnover markers, urolithiasis

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0393-974X (2016)

factors are detectable also in patients without renal stones, who show signs of osteoporosis or osteopenia, thus leading to hypothesize that the evaluation of lithogenic risk could have significant implications for monitoring the bone health status (8). While focusing on postmenopausal osteopenia, in this study we explored if lithogenic risk parameters, related to the acid-base balance, were associated with markers commonly used in monitoring the bone loss.

MATERIALS AND METHODS

The study was approved by the local Ethical Committee. The cases were selected among the patients who attended the Radiodiagnostic Unit of Rizzoli Orthopedic Institute from September 2015 to April 2016 to perform the periodic measurements of Bone Mineral Density (BMD). Lumbar and femoral BMD were measured by dual-energy X-ray absorptiometry (DXA). The eligibility criteria were *a)* osteopenia (T-score <-1 and >-2.5 at femoral neck and/or lumbar spine) and *b)* more than 5 years post menopause. We excluded patients with high risk of fracture (FRAX: >20 major osteoporotic; >3 hip fractures) (9), with renal diseases including nephrolithiasis and gastrointestinal disorders, who used protonic pump inhibitors or drugs influencing bone metabolism.

Fasting venous blood samples were collected and analyzed for creatinine, sodium, potassium, total calcium, inorganic phosphate, parathyroid hormone (PTH) and 1.25-dihydroxyvitamin D. Main chemistries and hormones were measured by routine methods. Serum levels of BTM (Carboxy-terminal cross-linked telopeptide of type I collagen-CTX, N-terminal propeptide of type I procollagen-PINP, tartrate-resistant acid phosphatase 5b isoenzyme-TRAcP5b, and bone-specific alkaline phosphatase-BALP) were measured by enzyme immunoassays using commercially available reagents (Biomedica, Vienna, Austria).

The urine analysis was performed on 24-h urine and fasting-morning urine by using a standardized system (Lithotest, Biohealth, Torino, Italy) (□10). During the 24-h collection, urine was fractioned into two aliquots: one supplemented with HCl, to prevent calcium and phosphate precipitation and one with hibitane to prevent any contamination. The acidified fraction was used to measure calcium, magnesium, phosphate, citrate and sulfate. The other aliquot was used to measure pH, urea, creatinine, urate, ammonium, chloride, sodium, potassium. Calcium, urate, citrate (all expressed as creatinine ratios) and pH were also detected in fasting-morning urine samples.

Calculations and statistical analyses

Statistical analyses were performed using the MedCalc Software 7.5.0.0 (Mariakerke, Belgium). Data were expressed as means, standard error of the means (SEM), minimum and maximum values and median. Urinary parameters were considered normal or altered based on the reference values of the lithogenic risk validated by the laboratory that conducted the analysis (Lithocenter, Torino, Italy). When the percentage of patients with altered urinary parameters was higher than 25%, the t-test was used to highlight significant differences in quantitative measurements of bone loss markers by comparing normal-group and risk-group. The degree of association between continuous variables was calculated by measuring the correlation coefficient R and the significance level using the Fisher r-to-z test.

RESULTS

The eligibility requirements were found in 82 women; 20/82 did not exhibit exclusion criteria and were enrolled in the study after signing the written informed consent. One patient was excluded because the urine was not properly collected.

The mean age was 59.3±1.1 years (range 48-69 years; median 58 years) and the postmenopausal mean time 8.9±1 years (range 5-21 years; median 8 years). The T score at the lumbar spine was -1.54 ±0.1 (range between -2.1 and -0.6; median -1.5) and -1.70±0.13 (range between -2.4 and -0.6; median -1.8) at the femoral neck. The 10-year probability of hip fracture was 0.98±0.12 (range 0-1.9; median 4.9) and the 10-year probability of a major osteoporotic fracture was 4.82±0.25 (range 2.4-7.6; median 4.9). Table I shows laboratory results of biochemical analytes, hormones, and BTM levels which were performed on serum samples. All serum biochemical analytes were within the range of normal values; PTH levels were normal, while a deficiency of 25-OH-cholecalciferol was observed in 8/19 patients (42.1%). BTM levels were

Table I. *Serum levels of biochemical analytes, hormones and BTM.*

Serum parameter	Mean±SEM; (range min-max); median	Reference value
Creatinine (mg/dl)	0.75±0.02; (0.61-0.88); 0.77	0.5-1.2
Calcium (mg/dl)	9.6±0.1; (9.1-10); 9.6	8.6-10.4
Magnesium (mg/dl)	2.16±0.04; (1.95-2.5); 2.1	1.7-2.55
Phosphorus (mg/dl)	3.7±0.1; (2.9-4.5); 3.6	2.7-4.5
Potassium (mg/dl)	4.6±0.1; (4-5.1); 4.6	3.5-5.3
Sodium(mg/dl)	142.8±0.4; (140-147); 143	134-146
25-OH-cholecalciferol (ng/ml)	31.8±1.9 (15-45); 33	<10 (failure) 10-30 (deficiency) 30-100 (sufficiency)
PTH (pg/ml)	49.3±3.9; (29.8-86); 45.0	12-88
BALP (ng/ml)	22.7±1.2; (16-32.9); 21.2	<22.4 *†
CTX (ng/ml)	0.6±0.1; (0.35-1.1); 0.6	0.14-1.35 *†
PINP (pg/ml)	22.6± 1.7 (15,4-40.8); 20.1	16-96 †
TRACP5b (U/L)	3.23±0.14; (1.66-4.06); 3.5	3.31±0.72 *

** Reference values for post-menopausal women released by manufacturer. †: http://www.mayomedicallaboratories.com.*

consistent with the values found in postmenopausal women (11).

We found that some urinary parameters related to the acid-base homeostasis and lithogenic risk were altered in more than 25% of patients (Table II). In 24-h and fasting-morning urine, the pH values were below the threshold value in 31.6% and 52.6% of cases, respectively. In 24-h urine, a proportion of patients showed a decrease in citrate levels (52.6%), potassium (89.5%), chloride (84.2%), ammonium (42.1%) and magnesium (36.8%). In fasting-morning urine, low citrate levels were found in 26.3% of cases. All patients with low pH in 24-h urine had low citrate levels, but only one exhibited an increased calcium excretion.

We did not find a correlation among pH values and other urine parameters, either in 24-h or fasting-morning samples. In 24-h urine, citrate excretion was significantly correlated with calcium (R=0.70, p=0.0006), sodium (R=0.68, p=0.003), and potassium (R=0.56, p=0.02). Calcium level showed a significant correlation with potassium (R=0.49, p=0.03), sodium (R=0.49, p=0.03), sulphate (R=0.51, p=0.02) and chloride (R=0.48, p=0.04). Sodium correlated with chloride (R=0.86, p<0.0001), sulphate (R=0.63, p=0.004), ammonium (R=0.63, p=0.004), urate (R=0.50, p=0.03) and phosphate (R=0.47, p=0.04). Potassium correlated with magnesium (R=0.66, p=0.002). In addition, phosphate, sulphate, urate and ammonium showed significant correlation with each other (data not shown).

Regarding the relationship between lithogenic risk parameters and markers of bone loss, we found that CTX levels correlated significantly with the 24-h calcium excretion (R=0.64, p=0.003). PINP levels correlated with the 24-h excretion of potassium (R=0.50; p=0.03) and calcium (R=0.45; p=0.05), and with citrate either in 24-h or fasting-morning samples (R=0.53, p=0.02 and R=0.65, p=0.003, respectively).

We evaluated if the presence or absence of urinary alterations associated to the lithogenic risk influenced significantly bone loss markers. The

Table II. *Parameters of lithogenic risk measured in urine samples.*

Urinary parameters	Mean±SEM; (range min-max); median	Reference value*	Number of patients with altered values/ number of total patients
Diuresi (ml/24-h)	1687±157; (630-2990); 1600	≥ 1000	L: 3/19 (15.8%)
24-h pH	5.92±0.15; (4.65-7); 6.0	5.5-7	L: 6/19 (31.6%)
Fasting-morning pH	5.76±0.17; (4.59-7.0); 5.55	5.5-7	L: 10/19 (52.6%)
24-hour parameters			
Ammonium (mmol/24-h)	21.15±0.98; (20.2-13.8); 29.6	20-50	L: 8/19 (42.1%)
Calcium (mg/24-h)	123.9±13.3; (44-267); 115.0	< 200	H: 3/19 (15.8%)
Citrate (mmol/24-h)	3.34±0.34; (1.2-6.1); 3.3	> 3.3	L: 10/19 (52.6%)
Chloride (mEq/24-h)	118.5±8.2; (30-179); 124	140-240	L: 16/19 (84.2%)
Creatinine (g/24-h)	0.82±0.05; (0.37-1.21); 0.77	1.3-1.8	L: 19/19 (100%)
Magnesium (mg/24-h)	60.3±6; (20-112); 61	> 50	L: 7/19 (36.8%)
Oxalate (mmol/24-h)	0.30±0.03; (0.06-0.53); 0.32	< 0.5	H: 0/19 (0%)
Phosphate (mmol/24-h)	10.3±1.6; (2.6-28.5); 10.4	< 42	H: 0/19 (0%)
Potassium (mEq/24-h)	28.1±3.7; (4.0-55.0); 22.0	50 -100	L: 17/19 (89.5%)
Sodium (mEq/24-h)	92.3±6.8; (26-136); 94	50-100	L: 2/19 (10.5%)
Sulphate (mmol/24-h)	12.2±1; (5-19.5); 11.4	6-30	L. 2/19 (10.5%)
Urea (g/ 24-h)	16.1±1; (9.8-30.1); 15.5	10-35	L: 1/19 (5.3%)
Uric acid (mg/24-h)	418.8±27.5; (249-723); 410	< 600	H: 2/19 (10.5%)
Fasting-morning parameters			
Calcium (mg/mg creatinine)	0.18±0.05; (0.02-0.95); 0.130	0.02-0.2	H: 3/19 (15.8%)
Citrate (mol/mol creatinine)	0.49±0.05; (0.1-0.88); 0.460	> 0.3	L: 5/19 (26.3%)
Relative saturation index			
Beta Calcium Oxalate	5.53±1.05 (1.67- 18.42) 3.490	< 5	H: 1/19 (5.3%)
Beta Calcium Phosphate	0.750±0.26 (0.07±5.04) 0.34	< 2	H: 1/19 (5.3%)
Beta Uric Acid	0.85±0.23 (0.03±3.54) 0.44	< 1	H: 5/19 (26.3%)

*****: *According to the assessment of lithogen risk (10-12);* ***L****: low;* ***H****: high.*

comparison showed that CTX was considerably increased in patients with pH <5.5 (Fig. 1a). Low citrate levels (<3.3 mmol/24-h) were associated with lower levels of BTM, i.e. CTX in both urine samples (Fig. 1b, 1c) and PINP in fasting-morning urine (Fig. 1d). Women with high calcium excretion (>200 mg/24-h) showed higher CTX and PINP levels in comparison to the other subjects, and a statistically significant difference was observed (data not shown). However, we consider that this finding has to be

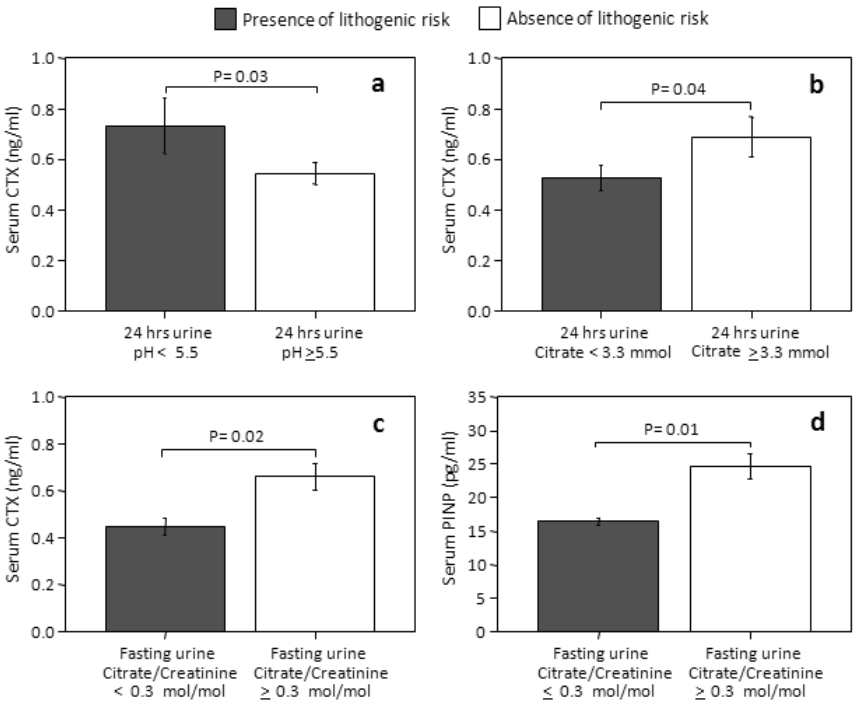

Fig. 1. *Bone loss markers and lithogenic risk parameters. The comparison has been performed when the percentage of patients with altered urine parameters was >25% (Table II) and only the significant results are shown in histograms. The bars indicate the mean±SEM of serum levels of BTM (**y-axis**) in two groups of patients classified according to reference values of lithogenic risk (**x-axis**) (10, 12). **Grey bar:** group with altered urinary parameters; **white bar:** represents the normal group. **a**: CTX levels and 24-h urine pH; **b**: CTX levels and 24-h urine citrate; **c**: CTX levels and fasting-morning urine citrate; d: PINP levels and fasting-morning urine citrate.*

further confirmed due to the low number of patients who exhibited hypercalciuria (16%).

DISCUSSION

We investigated whether parameters related to the lithogenic risk could have some application for monitoring the bone health status in postmenopausal osteopenia. All women enrolled in the pilot study had a negative medical history for nephrolithiasis, so that the alteration of urinary parameters could not be ascribed to the presence of kidney stones. In addition, they were more than 5 years post menopause, when the changes in bone turnover markers following the estrogen withdrawal are more limited (13).

Signs consistent with a low-grade metabolic acidosis were found in 24-h and fasting-morning urine (4), including low pH levels, low excretion of citrate and potassium, while high calcium levels were observed only in 3 subjects. We also found low levels of magnesium, which is usually related to the potassium and citrate excretion (14).

A recent report showed that a defective acidification process of the distal renal tubule is detectable in 23% of subjects with osteopenia or osteoporosis, without signs of systemic acidosis. This alteration is characterized by a fast morning urine pH>5.8 not modified by the acid load with NH4Cl (□15). We cannot exclude that this condition was present in some of our patients since the fasting-morning urine was pH>5.8 in more than 40% of cases. However, in distal renal tubular acidosis, also incomplete, the alkaline urine pH is associated with hypercalciuria and hypocitraturia, but these changes were not found in patients who had a pH>5.8.

According to literature data, BTM levels were consistent with the values found in postmenopausal women.(11, 16). CTX was significant increased in

patients with a low pH (Fig. 1), as well as a significant correlation was found with calcium excretion. Our findings agree with previous studies that showed a linear association between acidosis, bone resorption and calcium release (4, 15, 17). It is well known that low pH induces activation of osteoclasts, thus promoting the bone matrix degradation with the resulting increase in circulating levels of CTX (3).

The low-grade acidosis should enhance the bone turnover which physiologically occurs after menopause, thus eliciting an increase also in bone formation markers. Indeed, we found that PINP and urinary calcium were significantly correlated. Since acidosis decreases the excretion of both citrate and potassium, we also expected an inverse correlation of these parameters with bone turnover markers. On the contrary, we found a direct association, i.e. the lower excretion of citrate and potassium corresponded to the lower levels of CTX and PINP. The new knowledge on the key role of citrate in the mineralization process could explain this apparent discrepancy. Citrate is strongly bound to the collagen/apatite complex, and Costello et al. showed that it is not derived from blood plasma, but it is synthesized by osteoblasts during the osteogenic differentiation (18, 19). Since acidosis affects osteoblast differentiation and activity, the citrate production and the collagen synthesis should be hampered, and, as a consequence, the linear correlation between citrate levels and collagen turnover markers could be justified.

Therefore, it is reasonable to assume that both the low urine pH and the decreased production by osteoblast are responsible for the low citrate excretion. From a pathophysiological point of view, the latent acidosis activates osteoclasts and increases bone resorption, but the osteoblasts are not able to produce efficiently new tissue, thus sustaining the imbalance between the two processes and favouring the progression of the bone loss (2). This hypothesis is supported by literature data, but specific studies have to be conducted to highlight the clinical impact.

Taken together, our findings suggest that a low-grade acidosis may be associated with postmenopausal age also in the absence of concomitant diseases. The acidosis activates bone resorption in order to recruit phosphate groups which act to neutralize the H+ excess, increases the excretion of calcium released by the disrupted bone matrix, increases the tubular resorption of citrate due to the pH lowering, and affects the osteoclast and osteoblast function (3, 20).

The estrogen deficiency (1) and the decreased renal function due to ageing (5) could be the main reasons of the latent acidosis that we found in our case series. We cannot exclude that the diet influenced the metabolic picture that we observed. It is well recognized that some foods have more acid content than others, i.e. diets rich in salts and meat proteins (4). A retrospective nutritional assessment was not reliable, but we knew that all the enrolled subjects consumed a free and non-vegetarian diet. However, the low daily excretion of some nutritional markers (e.g. creatinine, sulphate, phosphate, urea) lead to hypothesize that in our case series, the animal protein intake was in low range.

In conclusion, the evaluation of lithogenic risk in postmenopausal osteopenia could have significant implications for monitoring the bone health status, as well as identifying the increased risk of urolithiasis. The metabolic profiling of postmenopausal women could help the clinicians to design proper treatments aimed to neutralize adverse metabolic conditions, and in turn, to counteract the osteopenia progression to prevent the development of renal stones. In this regard, the alkali citrate supplements are used to reduce kidney stone recurrence and their ability to oppose the acid overload may have beneficial effects in preventing the bone loss progression or in supporting conventional therapy for osteopenia or osteoporosis (21).

Finally, even though lithogenic risk factors and bone loss markers seem to be associated, further studies are necessary to confirm that this evaluation could be a clinically relevant tool for monitoring the bone health status.

ACKNOWLEDGMENTS

This work was supported by the “Fondazione del Monte di Bologna e Ravenna” and “5 per mille 2013”. We wish to express our gratitude to Dr. Massimo De Filippi and Donatella Tosco (Biohealth-Lithocenter) for their support in the management of the urine analyses.

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PREGNANCY-ASSOCIATED OSTEOPOROSIS (PAO) WITH MULTIPLE VERTEBRAL FRAGILITY FRACTURES: DIAGNOSIS AND TREATMENT IN A YOUNG PRIMIGRAVID WOMAN

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PAO is an uncommon condition affecting pregnant women during last trimester or early post-delivery period; it is often asymptomatic or presents with pain related to some acute fragility fractures. The diagnosis is often delayed or missed, the etiology remains unknown and no guidelines about treatment have been published. We present one case of PAO in a 33-year-old primigravid woman presenting acute worsening back pain. Our patient was treated with a TLSO brace, oral 25 (OH)-vitamin D supplementation and Teriparatide for 6 months. A short review of the literature has been included and useful advice about how to suspect and diagnose this uncommon disease were given in order to recognize and treat such a debilitating and severe condition for young mothers as best as possible, based on the available scientific

Pregnancy- and lactation-associated osteoporosis (PAO) was first described by Nordin and Roper in 1955 as an idiopathic form of acute osteoporosis occurring in late pregnancy or early post-partum period (1). Literature on PAO is scarce and mostly focused on description of isolated cases. Prevalence in general population of PAO is unknown and aetiology of this condition remains unclear. It has been suggested that genetic predisposition or hormone factors may play a role in development of PAO (2), but it is also possible that mechanical factors associated with pregnancy, (e.g. decreased level of activity, increased weight) may be involved. The most common clinical presentation of PAO is in a young, primigravid woman, with severe low back pain occurring in the last trimester or in the early post-delivery period (3, 4). Thoracic and lumbar vertebral compression fractures are the most frequently occurring fractures, although hip, ribs, and pubic rami fractures can also occur.

Due to the non-specific clinical presentation of PAO, the correct diagnosis is often missed or delayed. Chronic low back pain is a common occurrence in pregnant women. Furthermore, concerns over the use of conventional radiological investigations (e.g. x-ray or bone densitometry) during pregnancy may delay diagnosis. Therefore, clinical suspicion should be formulated when a young primigravid woman complains of severe back pain during pregnancy or lactation, without any remission of symptoms with common analgesia. When symptoms become continuous and quality of life gets worse (immobilization, inability to take care of the newborn, etc.) a gynecologist or a pediatrician should be consulted in order to decide when to safely perform X-ray examination and bone densitometry scan (DEXA). Furthermore, serum levels of calcium, phosphorus and alkaline phosphatase, thyroid and

Key words: pregnancy associated osteoporosis, vertebral fragility fractures, teriparatide

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0393-974X (2016)

parathyroid hormones, liver and kidney function, urinary excretion of calcium and phosphorus should be performed. The diagnosis of PAO is confirmed by evidence of femoral and lumbar osteopenia/osteoporosis detected by bone densitometry scan (T-score values less than -1 or -2.5 respectively), sometimes alterations of bone metabolism markers (especially vitamin D and bone alkaline phosphatase) and vertebral fragility fractures documented by spine X-ray and MRI. Common forms of treatment include functional rest, immediate cessation of lactation, calcium and vitamin D supplementation, bisphosphonates or teriparatide. Concerns have been raised with regard to the use of bisphosphonates in young women because of their long-term effects on subsequent pregnancies. Newer anabolic drugs (i.e. teriparatide) have been used with success in treatment of PAO. However, no guidelines have been published.

The aim of this report is to describe one case of PAO presenting with multiple vertebral fractures and review the relevant literature on this uncommon disorder. Diagnostic challenges, socio-economic impact and the role of orthopaedic and more recent medical treatments will be also discussed. We hope our report may help raise awareness among orthopaedic surgeons and general practitioners of this uncommon but potentially severe complication of pregnancy.

Case Report

A 33-year-old Caucasian woman attended our outpatient clinic 3 months after giving birth to a healthy boy. She started to complain of low back pain during the last two months of her pregnancy; the pain became significantly worse during the first week after delivery and by the time she came to our attention she was barely able to walk because of thoracic and lumbar pain. She denied any peripheral symptoms in the lower limbs. The pregnancy was uneventful, she gained 12.5 kg before delivery and she had breast-fed her baby after birth. She had no past medical or surgical history, no recent history of trauma and she did not exercise on a regular basis. She did not smoke or drink alcohol during pregnancy and had no family history of osteoporosis. She did not receive any corticosteroid or heparin therapy during the pregnancy and at the time of our clinical assessment, she was only on a regular prescription of Paracetamol for pain control.

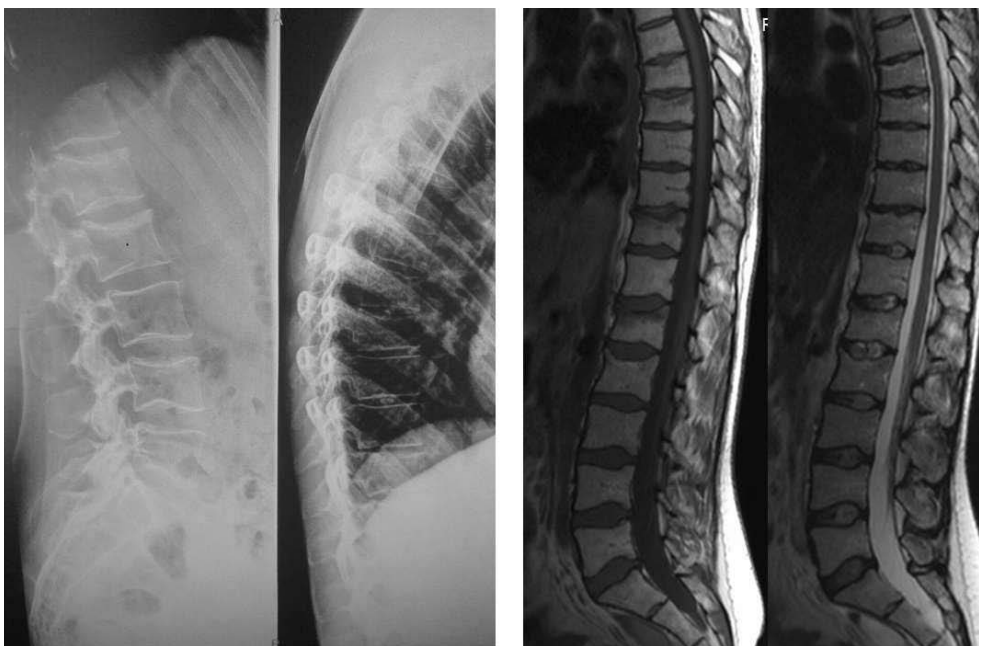

Fig. 1. *X-ray and T1- and T2-weighted MRI at diagnosis showing wedge compression fractures at T7, T8, T11, T12, L1, L2, L4, and L5 vertebral levels.*

Her height and weight were 167 cm and 60 kg respectively (BMI 21.51 kg/m^2). On examination, range of movement of the lumbar spine was severely restricted. There was severe tenderness on palpation and tapping of spinous processes of the lumbar and thoracic spine. Neurological examination was unremarkable. Standing antero-posterior and lateral X-ray of the whole spine showed wedge compression fractures at T7, T8, T11, T12, L1, L2, L4, and L5 vertebral levels (Fig. 1). MRI confirmed the presence of bone marrow edema at the fracture levels while excluding infection, tumor, or any abnormality to the spinal cord (Fig. 1). Bone mineral density (DEXA) showed femoral osteopenia (T-Score -2.3), and severe vertebral osteoporosis (T-Score -3.6). Serum calcium levels, phosphorus and alkaline phosphatase, thyroid and parathyroid hormones, liver and kidney function, and urinary excretion of calcium and phosphorus were normal. Serum 25-(OH) vitamin D was found to be slightly decreased (27 ng/ml, ref. range >30 ng/ml normal).

The patient was prescribed a thoraco-lumbar-sacral (TLSO) brace and it was recommended to discontinue breastfeeding and maintain a balanced diet. The patient expressed her wish to have more pregnancies in the future; therefore, a decision was made to avoid treatment with bisphosphonates. The patient was prescribed Teriparatide 20 μg SC daily for 6 months and Cholecalciferol 25.000 IU PO for 2 weeks. The TLSO brace was discontinued after 8 weeks. At 3 months, VAS score had decreased to 5/10 (from 9/10 at presentation); 25(OH)-vitamin D levels increased to 33 ng/ml, BMD had already increased with lumbar T-Score -2.6 and femoral T-Score -2.1 (Table I). At 6 months, lumbar and thoracic back pain had disappeared completely (VAS 0/10), serum 25(OH)-vitamin D was 35.2 ng/mL, lumbar T-Score -2.0 and femoral T-Score -1.7 (Table I). There were no side effects related to the therapy and no recurrent fractures or worsening of the deformity as shown at control X-ray and MRI (Fig. 2).

It has now been 1 year since the onset of her symptoms and no new fractures have been noted while her lumbar and femoral BMD levels have increased to normal values with normal femoral T-Score and lumbar T-Score indicative of slight osteopenia (Table I).

DISCUSSION

PAO is an uncommon condition affecting pregnant women during last trimester or early post-delivery period (3, 4). PAO is asymptomatic unless it presents with pain related to some acute fragility

Table I. *Bone Densitometry and serum 25-(OH) vitamin D results.*

	At diagnosis	3 months	6 months
BMD (g/cm^2)			
Femoral	.542	.746 (+37.64%)	.792 (+46.12%)
Lumbar	.771	.914 (+18.55%)	.995 (+29.05%)
T-Score			
Femoral	-2.3	-2.1 (+0.2)	-1.7 (+0.6)
Lumbar	-3.6	-2.6 (+1.0)	-2.0 (+1.6)
Z-Score			
Femoral	-2.6	-2.0 (+0.6)	-1.5 (+0.9)
Lumbar	-3.4	-2.1 (+1.3)	-1.8 (+1.6)
25-(OH) vitamin D (ng/ml)	27.07	33.40 (+6.33)	35.20 (+8.13)

Bone Mineral Density, T-score, Z-score and serum 25-(OH) vitamin D values at diagnosis and after 3 and 6 months of treatment. Data show the improvement in all DEXA Scan findings and the increase of serum vitamin D concentration due to therapy with Teriparatide and vitamin D supplementation.

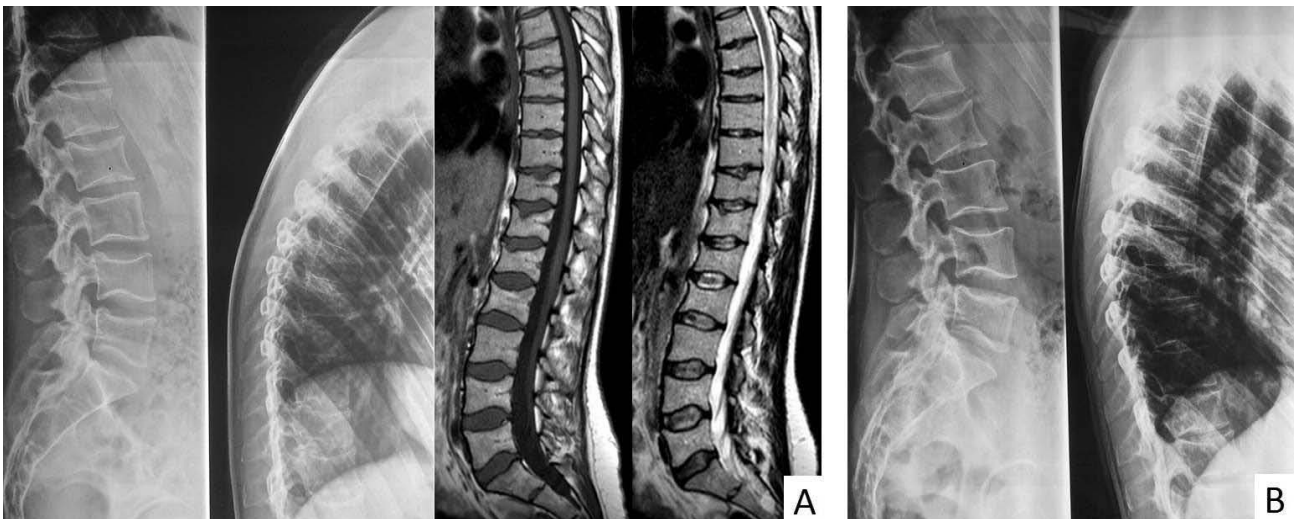

Fig. 2. *Radiological findings after 3 and 6 months of treatment. (A) X-ray and T1- and T2- weighted MRI after 3 months of treatment showing good consolidation of fractures. (B) X-ray after 6 months of treatment showing complete healing and no new fractures occurrence.*

fracture. The thoracolumbar or lumbar spine is most commonly affected site (5). Due to the very high prevalence of back pain in late pregnancy, diagnosis is often delayed or missed. Other causes of pathological fractures must be excluded (i.e. infection, tumor), and MRI imaging is usually useful in excluding secondary lesions. If needed, it is possible to perform genetic tests in order to verify the presence of primitive skeleton diseases (for example osteogenesis imperfecta), but the presence of these kind of diseases is considered unlikely in the absence of other previous signs or symptoms (6). A bone densitometry scan (DEXA) is mandatory to document decreased bone density. Finally, serum markers of bone metabolism are usually within normal limits or slightly altered (a raised title of bone alkaline phosphatase due to the acute fractures and a vitamin D deficiency can be found). Chronic administration of corticosteroid or heparin for prevention of deep vein thrombosis during pregnancy can also cause secondary osteoporosis during pregnancy (7).

Although several hypotheses have been proposed, aetiology of PAO remains unknown. As clinical syndrome, PAO may represent the "tip of the iceberg" of a more widespread and common phenomenon of osteopenia /osteoporosis during pregnancy. It has been estimated that a total of ~30 g of calcium is required for the successful mineralization of fetal skeleton during pregnancy. Eighty percent of this is transferred to the fetus during the third trimester, a fact that represents a significant stress to the maternal bone metabolism (8). Furthermore, ~300-400 mg of calcium is required daily during breastfeeding (9). A few studies have tried to assess the impact of pregnancy on maternal BMD; a ~3-5% decrease in lumbar spine BMD has been reported during physiological pregnancy. Similarly, a prospective study from Olausson et al. has confirmed a 1-5% decrease in whole body, spine, and hipbone mineral content (BMC) and BMD after pregnancy (10). Osteoporosis with clinical fractures may occur if an underlying or pre-existent condition of baseline osteopenia is present before pregnancy (11, 12). Jang et al have shown a 36.3% prevalence of osteopenia in a cross-sectional study on 1561 Korean puerperal women (13). Several hormones regulate bone metabolism during pregnancy. Concentration of vitamin D in maternal blood increases several folds during pregnancy; vitamin D is responsible for increased intestinal absorption of calcium. Several studies have shown a high prevalence of vitamin D deficiency in pregnant women (14). PTH related peptide (PTHrp) is responsible for bone reabsorption in maternal skeleton, and clinical studies have shown that PTHrp increases during late pregnancy (14). Other factors involved in controlling bone

metabolism during pregnancy are the placental growth hormone, the RANKL and OPG system, sclerostin and FGF-23 and prolactin. None of these factors has definitively confirmed to be involved in PAO and some authors have also highlighted some familiar clustering of this condition suggesting a genetic origin (2).

There is no standardized treatment for PAO. Symptomatic treatment, including rest and analgesia, is suggested. In cases that present after delivery, an early weaning of the baby is recommended to decrease the burden on maternal skeleton (15).

Case series have shown that bone density recovery happens spontaneously in the long term in some of the cases. Nevertheless, a common aim of treatment is to restore a normal or near normal BMD as soon as possible, in order to prevent other fractures, to enhance fracture healing and to allow the mother to take care of the baby and return to her usual activities.

Vitamin D and calcium supplementation is the mainstay of treatment (9). Bisphosphonates has been used for treatment of PAO by some authors (16-18). O'Sullivan et al have shown an increase of 23% in spinal BMD after 2 years of treatment with bisphosphonates compared to 11% increase in untreated women (16). A potential drawback of therapy with bisphosphonates is related to long-time retention in the skeleton of these compounds, which raises concerns with regard to fetal exposure during subsequent pregnancies. Furthermore, animal studies have shown that bisphosphonates can cross the placental membrane and form deposits in fetal bone. Teriparatide (human recombinant 1-34 fragment of PTH hormone) is the first anabolic agent for treatment of osteoporosis. A few case reports have shown its potential use for treatment of PAO (5, 9, 17, 19). Teriparatide has a very short half-life and it is administered by subcutaneous injection. Teriparatide should be administered in order to improve bone quality in a short time as demonstrated by Z-score and T-score. Bisphosphonates could have similar effects in the long term and it would translate in a worse outcome. Although still little is known about aetiology, PAO may represent the high end of a spectrum of conditions of deranged bone metabolism during pregnancy or pre-existent baseline osteopenia which is undiagnosed in young women because of lack of appropriate screening tools.

CONCLUSIONS

PAO is a rare condition that should be taken into consideration as differential diagnosis for nonresponsive and severe back or hip pain during the late pregnancy and early postpartum period. Thoracolumbar and lumbar spine are the two most common site of fragility fractures in PAO. An extensive workup is needed to rule out any secondary cause of osteoporosis, including administration of corticosteroid and heparin during pregnancy. BMD measurement (DEXA scan) is mandatory to establish a diagnosis of PAO. MRI is useful in detecting subtle fragility fractures, as well as establishing the diagnosis in pregnant women. Treatment is symptomatic with rest, analgesia and calcium and vitamin-D supplementation. The long-term aim of treatment is to restore normal BMD. Bisphosphonates have been used by several authors, but anabolic therapy with Teriparatide can potentially restore higher BMD levels by virtue of its anabolic activity.

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TREATMENT OPTIONS OF SIMPLE BONE CYSTS: THE ROLE OF BONE SUBSTITUTES, GROWTH FACTORS AND LITERATURE REVIEW

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The solitary bone cyst is a typical tumor-like lesion of the immature skeleton, whose etio-pathogenesis is still unclear. The purpose of this work is to perform a review of the literature about the different surgical approaches focusing on the role of bone substitutes and growth factors. Literature analysis shows injection techniques of substances such as methylprednisolone, autologous bone marrow, demineralized bone matrix, calcium sulphate and surgical techniques that involve the resection and curettage associated with bone graft and/or intramedullary nailing. Although there are good results currently associated to these techniques and the different ways of treatment, the only evidence-based treatment is given by injections of steroids. However, given the high rate of failure, autologous bone marrow and platelet gel represent a viable therapeutic option.

The solitary bone cyst (SBC) is a typical tumor-like lesion of the immature skeleton (between 3 and 14 years), characterized by the presence of an intramedullary cavity full of liquid frequently localized in the proximal metaphyseal and diaphyseal area of the humerus and femur, that has a tendency to grow, weakening the bone (1).

Multiple etio-pathogenethyc theories have been proposed, ranging from the presence of dysplastic area in the lesions that develop in response to trauma, to venous occlusion in medullary spaces, inflammation and congenital remains of intraosseous synovial tissue, while the origin in correspondence to the growth plate, supports the hypothesis that it represents more of a growth disorder rather than a neoplastic process (2).

Most lesions are asymptomatic and the diagnosis is often incidental or associated to simple or moderately displaced pathological fractures after a low-energy trauma (1).

The localization of the SBC near or across the physis in young children is indicative of an active cyst, while the localization in diaphyseal region and an older age is indicative of an inactive cyst. It tends to regress or heal spontaneously once they reach skeletal maturity in the majority of cases but not always (1, 2).

The aim of SBC treatment is to prevent pathological fractures, reduce pain and prolonged physical restraint, but despite the high frequency, the treatment lacks guidelines and consensus regarding timing and the type of surgical technique. It is possible that the treatment of an active cyst may not be successful while the treatment of an inactive cyst could be, but at the same time may not be necessary. However, although there are no guidelines, it is

Key words: simple bone cyst, unicameral bone cysts, tumor-like lesions, bone tumor

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0393-974X (2016)

reasonable to treat the SBC if it is active or associated to pathological fracture, if it is inactive or is of large dimension and localized in the sub-trocantheric region or in stress areas. In others cases, it is best to keep it under observation (1-3).

Analysis in literature proposes many therapeutic methods in treating the SBC. The purpose of this study is to review the efficacy of the different surgical techniques used, focusing on the role that bone substitutes and growth factors could play in healing lesions.

MATERIALS AND METHODS

A literature review using Pubmed/Medline database was performed in order to identify scientific publications relevant to the treatment of SBC. The MESH terms, simple bone cyst, unicameral bone cyst, tumor-like lesion and bone tumor were used in our search in order to retrieve the relevant publications.

RESULTS

The therapeutic methods proposed over the years and still valid today range from simple injection techniques to open surgical techniques, demonstrating how far we are from understanding the true pathogenesis of this disease (3).

Injection technique

This approach consists of a fluid aspiration, washing with a saline solution, decompression, placing the medullary canal in contact with the cavity and injecting different substances (methylprednisolone, saline solution at high speed, autologous bone marrow, demineralized bone matrix, calcium sulphate) under fluoroscopy guide (1-3) as follows:

Injection of methyl-prednisolone: It is the most common approach. The rationale is given by the high level of prostaglandins in the cystic fluid, like an inflammatory process. The results are conflicting. Scaglietti, et al. showed a cyst healing with less morbidity than resection or curettage and bone graft, while others did not show important efficacy and reported the need of multiple injections and anesthesia with a relapse rate ranging from 15% to 88% after an average of 3 injections (3-6).

Injection of saline solution at high speed with intramedullary reaming: The bone cyst is a cavity isolated in the venous intramedullary circle. The injection of saline solution at high speed with intramedullary reaming open the vascular channels and connect the venous system of the cyst with the endosteal circle. Gebhart, et al. showed a healing of 11 out of 12 patients underlining that mechanic action has a more important role than the substance injected (3, 7).

Injection of autologous bone marrow: The rationale is given by precursors put on the bone inside the lesion. It proves to be effective but requires multiple injections (5, 8-11). Lokiec, et al. showed a healing rate of 100% with multiple perforation. Yandow, et al. showed a healing in 67% of cases, a partial healing in 17% and a non-response in 16%, all without multiple perforation, suggesting a higher success rate with a single injection, a faster healing, a higher efficacy in young adults and a higher incidence of fracture than the other study (3).

Injection of demineralized bone matrix: Killian et al showed a healing in 82% of cases after a single administration (3).

Injection of autologous bone marrow and demineralized bone matrix: This technique has shown a failure rate ranging from 11% to 22% (3).

Injection of demineralized bone matrix and calcium sulphate: Wilkins et al., using this approach after the perforation and washing with saline solution with the intent of occluding cystic spaces and inducing a rapid bone growth, obtained 100% success in 11 cases (1 asymptomatic relapse) (3).

Injection of calcium sulphate graft: This technique showed a partial or total healing in 80% of cases after a single injection and of 100% after 3 injections (3).

Surgical Technique

Resection or curettage associated with bone graft: This technique was established in order to provide a treatment for unicameral bone cysts. However, even this type of treatment sometimes had high relapse rates (22% to 64%), which combined

with donor site morbidity, reduced these procedures, favoring less invasive techniques, given the benign and self-limiting nature of these lesions (1-3, 8, 10). It consists in an open access through the opening of a cortical bone window, access to the cavity, removal of the internal fluid and washing, curettage of fibrous membrane of the wall of the cyst and the bone graft (3). To avoid donor site morbidity, various methods have been proposed, such as a subtotal resection without bone graft, used by McKay, et al. in 21 patients, with satisfactory healing in 19 cases and a relapse rate of 9% (8), use of allograft, which demonstrated efficacy in various studies (3) or of bone substitutes such as calcium sulfate which has been used successfully and with low rates of recurrence (11%) (3, 12).

Intramedullary flexible nailing: This method is based on the same injection principle, which is to stop the wall of the cyst while leaving an opening. However, it presents variable failure rates (0%- 6%) and in addition, necessitates changing the nail as it becomes short during growth in 28% of patients (3, 13). De Sanctis, et al. showed complete healing in 65.9% and healing with a residual radiolucent with no recurrence of the injury in 34.1%, concluding that this method, over time, can solve, the disease and in experienced hands, is the best method of treatment for bone cysts in the long bones of children (13). Roposch, et al. reported 14 cases of complete healing and 16 cases of healing with residual radiolucency (3).

Multiple hole drilling: The trephination and drilling with or without Kirschner wires, showed failure rates after the first treatment (0%-52%) (3). Chigira reported 10 cases in which there was healing after drilling, demonstrating that leaving a K-wire inside the lesion proved to be a valid therapeutic method in the prevention of local recurrence because in some cases, the removal of the wire was accompanied by the recurrence of the cyst (3). Shinozaki, et al. treated 23 simple bone cysts with multiple drill-holes and observed a recurrence of the cyst in 15 cases after the initial operation of which 12 were re-operated. At follow-up, good bone formation with no sign of recurrence was seen in 15 cases. A residual cyst was found in 8 cases, but further treatment was not considered necessary (3).

DISCUSSION

Literature analysis showed multiple ways of treatment with good results but also with conflicting results. We observed the same contrasting results analyzing various treatment methods.

Sung, et al. showed a failure rate after initial treatment with steroids of 84%, 64% by curettage and 50% using a combination of steroid, demineralized bone matrix and autologous bone marrow (SDB). Steroids were associated with previous failure rate, while failure rate after the second treatment was of 76% with steroids, 63% with curettage and 71% with the use of SDB. In addition, 18% of patients initially treated with steroids had a subsequent pathological fracture against 2.6% of patients treated with curettage and 12% with SDB. Therefore, the authors concluded that SDB is more effective than steroids in initial treatment and is associated with lower morbidity than curettage, which should be considered as the first treatment option for humerus and femur SBC in patients under 20 years (5).

Chang, et al. compared the injection of SDB with steroids. Out of 79 patients analyzed, 14 received 27 injections of bone marrow and 65 received 99 injections of steroids. Repeated injections were required in 57% of patients after the use of bone marrow and in 49% after the use of steroids and concluded that no advantage could be demonstrated through the use of autogenous bone marrow injections compared to steroid injections in the management of unicameral bone cysts (14).

Wright, et al., in a randomized controlled trial, compared the rates of healing of simple bone cysts treated with intralesional injections of bone marrow with rates of healing of those treated with methylprednisolone acetate, showing a healing higher in the second group (42% vs 23%) (15).

Oppenheim et al, comparing the curettage associated to bone graft with steroid injections, reported a higher recurrence rate and complications in the surgery group, while Farber did not find differences between the two groups (3), which was in contrast with Glaser DL et al. who reported a greater success in the treatment of calcaneus bone cyst (16).

Canavese et al. compared three treatment

modalities, demonstrating that at 2-year follow-up, patients that underwent percutaneous curettage and intra-medullary decompression, had a healing rate of 70% compared to patients that underwent bone marrow injection (21%) and methylprednisolone injection (41%). This suggested that the mechanical destruction of the membrane and the decompression of the cysts could have a primary role in activating the osteogenic potential of the bone, leading to healing the SBC (9).

Di Bella, et al. demonstrated that the healing rate of patients treated with multiple injections of corticosteroids against a single injection of demineralized bone matrix associated with bone marrow, had a higher rate of healing through the second method (58% *vs* 21%) and a higher failure rate after a single injection of steroids (63%), compared to a single injection of demineralized bone matrix and bone marrow aspirate (24%) (17).

Hou showed that minimally invasive methods such as curettage, cauterization with ethanol, rupture of the cystic membrane, the addition of calcium sulfate and the placement of cannulated screws to allow drainage, have a more favorable outcome compared to injection of steroids and bone marrow, open curettage and grafting with a calcium sulfate bone substitute with or without instrumentation (12).

Brecelj, et al. compared steroid injection (n=33), curettage and bone grafting (n=8) and a specially designed cannulated screw (n=28) in a prospective comparative study with a follow-up of 5.8 years. All three groups required multiple courses of treatment. After two attempts, the cannulated screw group demonstrated the highest rate of healing (P=0.001) (65%) compared to 50% and 19% with open curettage and methylprednisolone injections, respectively (18).

Li, et al. compared fixation with elastic titanium intramedullary nailing (TEN) *vs* aspiration and injection of autogenous bone marrow (ABN). In the ABM group, 60.9% bone cysts healed completely, 26.1% healed with small residuals after two injections and 13.0% recurred. In the TEN group, 69.6% bone cysts healed completely, 17.4 % healed with small residuals and 13.0 % recurred. There were no significant differences between both groups (19).

While comparing injection of methylprednisolon acetate, intramedullary nailing (IN), IN steroids+ and curettage plus bone grafting, Traub et al., showed no significant differences between the treatment groups in secondary fractures, function, pain or complications, while the failure rate after initial treatment was 36.6% with steroids, 50% with intramedullary nailing, 21,4% with intramedullary nailing plus steroids and none in the remaining group (20).

According to Donaldson, et al. (10) the only evidence-based treatment of SBC is represented by steroid injections and it seems to be superior to bone marrow injection (6, 11). However, this method is associated to a high rate of failure, therefore other methods of treatment although more aggressive, should be considered. Percutaneous or open curettage associated to bone substitutes and/or Platelet Rich Plasma or Autogulous Bone Marrow or intramedullary nailing (in particular in fracture or impending fracture), can be a solution, but must to be used, in our opinion, only in selected cases. Literature analysis does not help; the management remains controversial due to lack of guidelines and there are only two studies of level I and II (15, 18) while there are many studies of level III-IV. Moreover, they have different methods (uncontrolled, not randomized, unblinded outcome assessment, or short-term follow-up), different localization, different algorithm treatment in similar cases and inconsistent radiographic outcomes. For these reasons, no single method has emerged as the standard of care (21). New techniques are also applied, such as the use of cryosurgery, freeze-dried radiation and sterilized bone allograft impregnate with autogenous bone marrow or endoscopic surgery and bioceramic bone graft (22). This does not appreciate the value and benefits of these newer strategies (6, 10); given the heterogeneity of the studies and the reporting bias, the interpretation of these findings should be handled with caution (23). Furthermore, we do not know in which measure our treatments influence the results of the natural evolution of the cyst (24).

In conclusion, literature analysis reported rates of healing using bone marrow injections ranged from 42-100%. Based on the early promising results,

many surgeons prefer this method rather than steroid injections although it is not based on a high level of evidence requires more surgery time and has a higher cost. Therefore, comparative and larger studies are essential (6, 11, 23-25).

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AUTOLOGOUS BONE MARROW CONCENTRATE COMBINED WITH PLATELET-RICH PLASMA ENHANCE BONE ALLOGRAFT POTENTIAL TO INDUCE SPINAL FUSION

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Bone marrow cells concentrate (BMCs) is a source of osteoprogenitor cells and platelet-rich plasma (PRP) is a source of growth factors. The objective of the study was to determine whether BMC and PRP could increase the potential of bone allograft to induce posterolateral-lumbar spinal fusion compared to the bone allograft alone. A prospective nonrandomized radiographic study has been conduced on 10 patients with posterolateral instrumented fusion for degenerative lumbar disease with 1-year follow-up using CT scan. A fresh frozen bone allograft alone and bone allograft with a mixture of autologous BMC and PRP blended with thrombin were apposed in the right and left posterolateral side, respectively. CT showed good right fusion masses (allograft alone) in 4 patients and poor in 6; good left masses (BMC and PRP plus allograft) in 9 patients and poor in 1. The differences detected between right-side and left-side masses show an advantage in adding BMC and PRP to the bone allograft to increase spinal fusion rate.

Lumbar spinal fusion with pedicle screw fixation is a widely used surgical procedure and the most common technique performed to obtain fusion in the lumbar spine. In the United States, the number of lumbar fusions doubled between 1998 and 2008 (1). It gives important advantages in patients affected by degenerative disc disease, and it is based on intertransverse or interarticular bone formation being protected by fixation (2). However, reported failure rates still range from 5% to 45% (3). Therefore, procedures that may enhance bone repair and fusion still arise great interest in the scientific community.

Autologous iliac crest bone graft (ICBG) has been considered the gold standard in lumbar spinal fusion because of its osteoconductive and osteoinductive potential (1). However, its harvesting is associated with morbidity including hematoma, fracture, impaired wound healing, infection and most of all donor site pain (4). Local bone harvested from the laminae and spinous processes during the decompressive maneuvers of a lumbar surgery is also widely used as a suitable autologous graft to avoid donor site morbidity (1).

Allograft may represent an alternative source of bone graft and a valid substitute to the autologous bone graft. However, it lacks osteoinductive potential and osteogenic cells because of the processing that it undergoes to decrease its antigenicity (5). Therefore, the bone formation by the use of allograft may be improved by adding both osteoprogenitor cells, such as bone marrow cells, and growth factors to the fusion site.

Bone marrow cells concentrate (BMC) is an option to promote bone formation in spinal fusion (6). BMC contains mesenchymal stromal/stem cells (MSCs), which have been demonstrated to be able

Key words: Bone marrow, Stem cell, platelet rich plasma, spine fusion, bone marrow concentrate, allograft

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0393-974X (2016)

to proliferate and differentiate into muscle, cartilage, adipose, and also into bone cells (osteoblasts) and, therefore, to regenerate different tissues of the musculoskeletal system (1, 7, 8). It is a fully autogenous product, derives from the concentration of the bone marrow aspirate (BMA) and contains an elevated number of MSCs. Therefore, it can enhance the osteoinductive potential of the fresh-frozen allografts.

Bioactive growth factors are presently being considered as therapeutic possibilities to enhance the healing of different mesenchymal tissues. Platelet-rich plasma (PRP) can be obtained from peripheral blood cells as source of growth factors. It has been shown to stimulate osteoblast-like cells *in vitro* and to enhance bone graft incorporation in maxillofacial surgery and trauma surgery (9, 10). PRP has also proved to promote lumbar spinal fusion (11). PRP can undergo gelation when combined with thrombin obtaining platelet-rich fibrin (PRF).

An addition of autologous BMC and PRP to bone allografts represents an option to deliver autologous progenitor cells with osteogenic potential and osteoinductive factors to the osteoconductive scaffold forming a 'tissue-engineered' construct ready to be used in the operating room (6).

The aim of this study was to investigate the efficacy of PRP and BMC addition to corticocancellus bone allograft in instrumented lumbar posterior fusion surgery, by comparing the construct with an internal contralateral side control of bone allograft alone.

MATERIALS AND METHODS

Case series

This prospective nonrandomized radiographic study enrolled 12 consecutive patients, aged 43 to 65 years, with the diagnosis of isthmic or degenerative spondylolisthesis, or stenosis involving the lumbar spine, with a maximum of 3 involved levels. One patient developed an aseptic abscess and required revision surgery and prophylactic removal of the graft on both sides after one month. The explanted graft was sent for histological examination. Exclusion criteria included any previous lumbar surgery, active systemic infection or neoplasia. The number of levels fused was 1, 2 or 3. Single-level fusion was conduced in 4 patients while two or more levels in 6.

Autologous BMC preparation

After general anaesthesia, 60 ml BM aspirate was drawn from the posterior iliac crest with multiple punctures without exceeding 3-5 ml each aspiration. A total of 60 ml of BM was collected. All aspirates were pooled in plastic bags containing anticoagulant solution (ACD: citric acid, sodium citrate, and dextrose). The ratio between ACD and blood was 1:5. BM was transferred to a centrifugation chamber containing a specific membrane for buffy coat separation and centrifuged using the Smart PReP2 system according to manufacturer's instructions. Using a two-step centrifugation process, mononuclear cells were separated from red cells and resuspended in plasma with a final volume of 10 ml. The procedure has been standardized and cell viability and immunophenotype characterization of the concentrated cells has been assessed (6).

BMC immunuphenotype characterization

In 8 out of the 10 study patients the immunophenotype profile and the proliferative potential of the hematopoietic (HeCFU) and MSCs were assessed on a sample of BMC and the cell recovery compared with cells content of bone marrow aspirate before the manipulation. The immunophenotype was evaluated by cytofluorimetric assay focusing on the detection of hematopoietic stem/progenitor cells (CD34+) and mesenchymal progenitors that due to the lack of a specific and unique marker, were characterized by the expression of several non-specific markers: CD45-90+, CD73+, CD105+, CD146+. The proliferative potential of hematopoietic progenitors was evaluated by clonogenic methylcellulose assay whereas mesenchymal progenitors were assessed as number of adherent fibroblastoid colonies (CFU-F) in liquid cultures.

Autologous platelet-rich plasma, platelet-rich fibrin and thrombin preparation

PRP with leucocytes was obtained from patients venous blood (16 mL) using Regenlab™ THT tubes. Blood was centrifuged at room temperature 1500 g for twelve minutes. Red blood cells and 2 mL of platelet poor plasma were removed from each tube of platelets concentrate to obtain a platelet concentration of 0.8-1.2 μL in PRP.

PRF was prepared using Vivostat System™

(Vivolution, Birkeroed, Denmark) medical device. The entire process generates approximately 6 mL of PRF from 120 mL of the patient's own blood in about 30 min. Autologous thrombin was generated by 8 mL of venous blood according to Regenlab™ ATS tubes.

During surgery in aseptic conditions, autologous PRP (7mL) was mixed with autologous BMC (7 mL), 4 mL of autologous Thrombin and 4 mL of calcium gluconate to obtain a composite gel of autologous cells. The composite gel was placed inside a scaffold of corticocancellous bone. Autologous PRF (6 mL) was then sprayed locally all over the graft to promote tissues adhesion. Samples of BMC were collected to perform cells count and characterization.

Surgical technique

All surgery was performed by the senior author (VD). At surgery, a standard midline approach was used and decompression performed. Instrumentation consisted of two titanium bars with lateral mass polyaxial screw fixation. On the left posterolateral side of the spine, allograft plus autologous BMC and PRP was used, while on the right side allograft alone was used.

All the graft beds were decorticated. A freshly frozen iliac crest bone allograft was cut into long bars of corticocancellous bone and placed alongside the instrumentation of both sides. Allografts were delivered by an authorized bonebank institution.

In aseptic condition, the BMC was then combined with the autologous PRP (16 ml), cryoprecipitate (8 ml) and thrombin (4 ml). A gel-like tissue was obtained by adding calcium gluconate (2.8 g). The obtained gel was used to cover the bone allograft on the left side of the spinal instrumentation (Fig. 1).

Clinical and radiographic analysis

The patients were followed clinically and radiographically for a minimum of 1 year. Posteroanterior and lateral radiographs of lumbosacral spine were obtained immediately after surgery and at 6 and 12 months. Because the sensitivity of X-ray interpretation for lumbar spine arthrodesis is up to 70%, the status of the fusion was additionally evaluated using computed tomography (CT) scans at 12 months after the surgery.

For this study, radiographic fusion rate was the primary outcome measure. An independent radiologist evaluated 6- and 12-month x-rays and 12-month CT scans of the patients.

Fusion success was defined at the presence of continuous trabecular bone connecting the transverse processes. In this case, fusion mass was considered "good". No intersegmental bony bridging fusion was considered "poor".

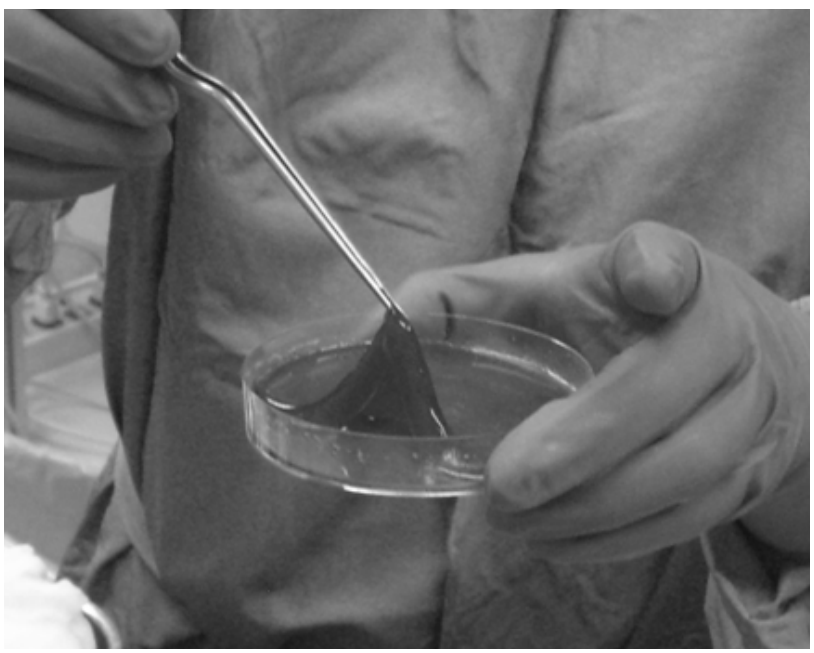

Fig. 1. *BMC and PRP blended with thrombin ready to be implanted on allograft.*

Histological staining

Bone biopsies of a patient who underwent revision surgery one month after postero-lateral fusion of the spine were obtained from both sides at the time of removal of the instrumentation. Histological samples were fixed 10% in neutral buffered formalin for 1 week, decalcified in ethylenediaminetetraacetic acid and processed for paraffin sectioning (5 μm in thickness). The sections were stained with both hematoxylin and eosin (H&E), using standard techniques and analyzed qualitatively under a light microscope (Nikon Eclipse E800, Nikon, Melville, NY), at 4 X magnification.

Statistical analysis

Statistical analysis was carried out comparing fusion rates for the 2 sides (Allograft *vs* Allograft + BMC + PRP) and for the immunophenotype data (BMC *vs* BMA) using Student *T*-test. All statistical analyses were made using commercially available software at the nominal 0.05 level of significance.

RESULTS

The study population consisted of 7 males and 5 females; the mean age was 59 years, with a range of 43 to 65 years. There were no significant intraoperative or postoperative complications or adverse effects due to the bone marrow aspiration. One patient developed an aseptic abscess one month after surgery and required revision surgery and prophylactic removal of the graft on both sides which was sent for histology analysis. One patient was lost at follow up.

The remaining 10 patients were followed up to one year and underwent X-rays at 6 and 12 months and CT scan at 12 months. The number of levels fused per patient was as follows: L4-L5: 1 patient; L5-S1: 3 patients; L4-S1: 2 patients; L3-S1: 2 patients and L3-L5: 2 patients. All patients underwent posterolateral fusion. Lateral X-rays showed good positioning and no mobilization of spinal instrumentation over time. CT scans showed good fusion on right masses (allograft alone) in 4 patients and poor in 6 patients; good left masses (allograft + BMC + PRP) in 9 patients and poor in 1 patient (Table I). Representative coronal and sagittal images of L5-S1 fusion at left side (allograft + BMC + PRP) and no fusion on the right side (allograft) are shown in Fig. 2.

After the concentration procedure (Table II), the number of BM white blood cells (WBC) was 3.4-fold higher than the cell number detected in non-manipulated BMA. The number of CD34+ cells was significantly increased (p=0.005) in BMC

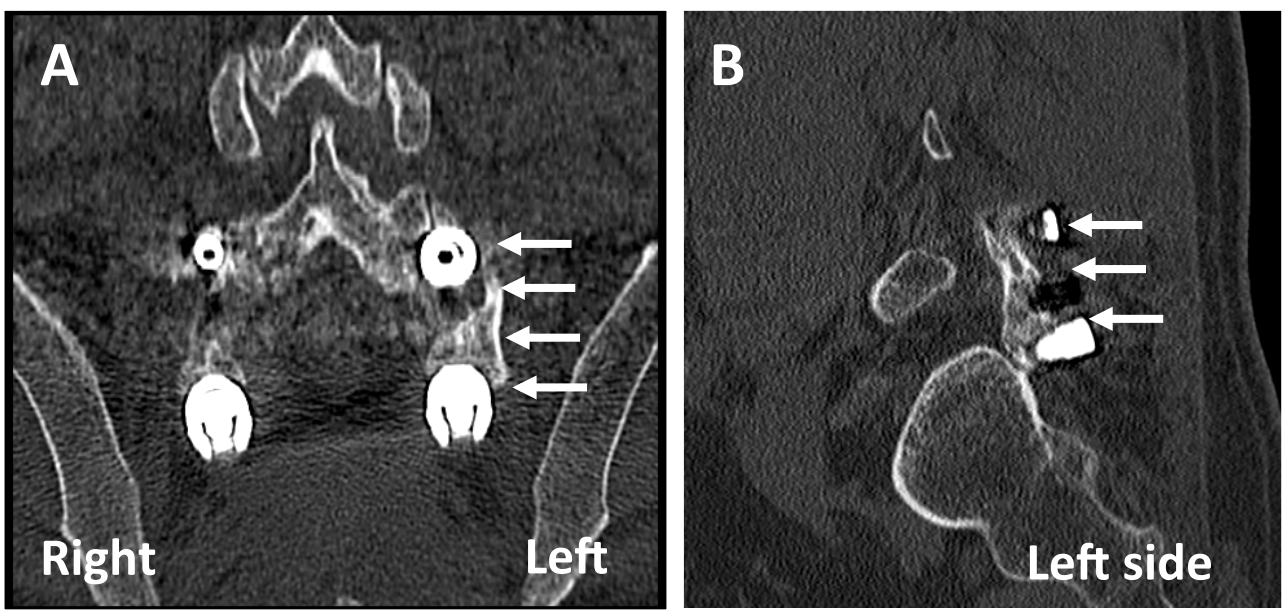

Fig. 2. *Representative CT images of patient who underwent posterolateral fusion showing instrumentation. Bone bridging along the instrumentation can be appreciated on the left side (Allograft + autologous BMC + PRP).*

Table I. *Numbers of patients and percentage of fusion success regarding results of CT imaging.*

	Allograft	Allograft +BMC +PRP
Good Fusion (n)	**4**	**9**
Poor Fusion (n)	**6**	**1**
Fusion Percentage (%)	**40%**	**90%**

Table II. *Characterization of bone marrow cells before and after Smart PReP2 concentration.*

	BMA	**BMC**	**p**	**FI**
WBC x 10^6/ml	33.0±25.8	83.9±46.4	0.01	3.4±1.7
$CD34^+$ x 10^3/ml	186.6±166.7	694.6±401.0	0.007	6.7±5.1
HeCFU x 10^3/ml	76.2±60.3	202.8±253.0	ns	3.3±2.8
$CD45^-90^+$	15.9±13.4	54.6±45.4	0,039	13.5±30.1
$CD73^+$ x 10^3/ml	489.6±589.6	1416.5±1072.2	ns	8.8±9.8
$CD105^+$ x 10^3/ml	450.5±581.6	1050.4±1991.3	ns	3.1±3.3
$CD146^+$ x 10^3/ml	88.6±162.2	169.0±49.2	ns	11.5±11.9
CFU-F x 10^3/ml	750.3±1239.6	2187.9±2603.1	0.044	4.9±2.5

Data are reported as mean±SD. ***BMA****: Bone Marrow Aspirate;* ***BMC****: Bone Marrow Concentrate;* ***FI****: fold increase calculated as number of cells in BMC/number of cells in BMA;* ***WBC****: white blood cells;* ***HeCFU****: Hematopoietic Colony-forming units;* ***CFU-F****: Colony-forming unit-Fibroblasts.*

compared to BMA. The mesenchymal progenitor cells (CD45-90+, CD73+, CD105+, CD146+) were respectively 13.5-, 8.8-, 3.1- and 11.5-fold higher than in the BMC compared to BMA. In agreement with the immunophenotype results, the mesenchymal progenitors (CFU-F) were significantly increased in BMC compared to BMA (p=0.044).

Histological analysis of the explanted graft from one patient 1 month after surgery showed fibrovascular tissue at the allograft with BMC + PRP interface, absent in the control graft (Fig. 3).

DISCUSSION

Bone regeneration involves many elements: (i) osteogenic cells capable to form new bone; (ii) osteoinductive factors (ie, growth factors and cytokines) that stimulate osteoblastic differentiation of MSCs; (iii) osteoconductive scaffold that supports bone ingrowth and facilitates neovascularization (1). Even though it has several disadvantages, the use of the autologous bone graft still represents the gold standard to achieve a solid fusion because it encloses all these factors (12, 13). Allograft may represent a valid alternative only when its limits are overcome, i.e., no osteoinductive potential or osteogenic cells (14). Many methods have been used to enhance allograft bone formation in spinal fusion, including magnetic fields (15, 16), direct current stimulation (17), bone morphogenetic protein and the addition of BMCs (10, 18, 19). Osteogenic, osteoinductive and osteoconductive elements have been used in our study to induce posterolateral lumbar fusion: the BMC as a source of osteoprogenitor cells, PRP to

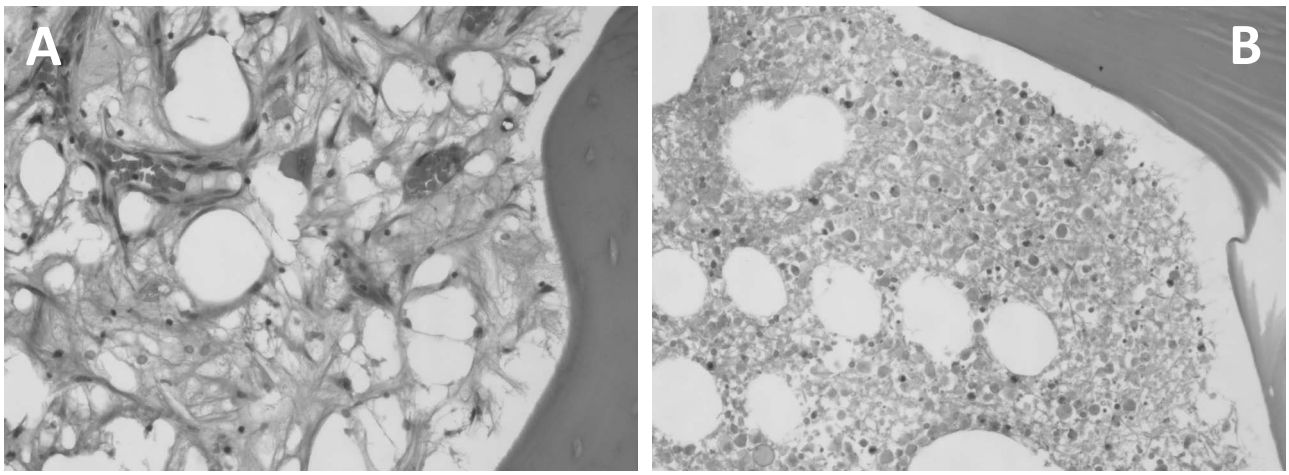

Fig. 3. *Representative images of histological sections of bone biopsies stained with H&E obtained from a patient who underwent elective removal of instrumentation. Allograft + BMC + PRP (**A**) show new vessels (arrows) formation while Allograft alone (**B**) is mainly necrotic tissue.*

add osteoinductive factors, and the corticocancellous bone allograft represents an osteoconductive scaffold.

Our study clearly shows how BMC and PRP increase the bone regenerative potential of the allograft. Using the posterolateral fusion model, we were able to test the effects of BMC and PRP on bone allograft in a side-by-side human model. The ectopic bone formation has been assessed by both CT scan and histological analysis. Moreover, the use of the device SmartPReP allowed a selective concentration of the mesenchymal progenitors, as assessed in 8/10 patients by the immunophenotype profile and the clonogenic potential.

BMA has been used as adjuvant for bone grafting procedures in traumatology (20) and spine surgery (21). Hernigou et al. demonstrated that the potential of BMA grafting in enhancing bone formation is related to the number of progenitor cells available at the graft site, which are capable of colony forming units (CFU-F) when placed in culture plates.

Progenitor cell numbers appear to be insufficient in the absence of the correct concentration (22). This evidence have been confirmed by our study where the process of concentration induced MSCs (CD146+ cells) increased 11-fold and the CFU-F increased 5-fold. The bone marrow concentration system used in the study allows to obtain intraoperatively a mononuclear cell concentrate from whole BMA, which is readily available for use in the surgical field.

Further advantages in the use of BMCs in orthopaedic procedures can be achieved by forming fibrin clots that encapsulate the progenitor cells, therefore avoiding the diffusion from the grafting site. Moreover, BMCs may be enriched with autologous growth factors form PRP, such as Platelet-derived Growth Factor, Transforming Growth Factor, Vascular Endothelial Growth Factor and Insulin-like Growth Factor (23). PRP itself has shown to drive osteoblast-like cells *in-vitro* and to boost bone formation mediated by grafting procedures in maxillofacial surgery *in-vivo* (9, 24). PRP promotes early maturation of bony fusion and is a good adjuvant in lumbar spine fusion (11).

Other studies have shown the effectiveness of the use of BMC and PRP with bone allograft to induce bone formation (11). We previously reported the effectiveness of this method in generating posterolateral cervical fusion in a difficult clinical setting where age and co-morbidities could compromise spinal fusion achievement (6).

The side-by-side human model to study effectiveness of bone grafts to induce posterolateral spine fusion has been used previously. Boden et al. compared an extract-containing bone morphogenetic proteins to an autologous bone graft form iliac crest on the other (25), proving the feasibility of the growth factors mixture in inducing spinal fusion.

There are several limitations to this study. First, the side-by-side model does not allow for any clinical evaluation based on the treatment. However, this model allows the evaluation of the spinal fusion by using CT scan to overcome the systemic variability among different patients that can affect outcomes. Secondly, the relatively small sample size still needs to be addressed. Indeed, randomized clinical trials comparing bone allograft + BMC + PRP to bone autograft (gold standard) may be helpful to confirm the effectiveness of the method. Thirdly, no cost analysis was performed in this study.

In conclusion, this simple and effective compound may improve the outcome of fusion rate of bone allograft in posterolateral spine fusion.

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MESENCHYMAL STEM CELLS FOR INTERVERTEBRAL DISC REGENERATION

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Low back pain (LBP) is one of the most common disabling symptoms affecting the adult population throughout the industrialized world. The main cause underlying this condition is intervertebral disc degeneration (IDD), which is characterized by progressive decrease of the proteoglycan content within the nucleus pulposus (NP), leading to disc dehydration and loss of its morpho-functional and biomechanical properties. To date, LBP treatment is based upon conservative and invasive procedures which are not capable of restoring the degenerative alterations of the disc, as they only help relieve the symptoms and/or slow down disc degeneration and are, nonetheless, characterized by significant comorbidities, costs and secondary risks. The potential use of different mesenchymal stem/stromal cells (MSCs) for treating IDD has been promisingly tested *in vitro* and *in vivo*. The combination of different cell types, preconditioning culture conditions, engineered scaffolds and delivery systems have yielded proof of disc matrix reconstitution, increased cell viability and tissue regeneration in several experimental settings. This article reviews the current literature on stem cell-based therapy for IDD and the outcomes that diverse approaches have achieved.

Low back pain (LBP) is a musculoskeletal symptom affecting more than 80% of the general population throughout their life, leading to high morbidity with great psychological, social and economic burdens. LBP attests itself as the first cause of disability in people under 45 years of age, especially among female individuals, hence resulting in huge national economic losses in developed countries (1).

LBP is mainly caused by degeneration of the intervertebral disc (IVD). The IVD consists of three highly specialized tissues: the inner nucleus pulposus (NP), the outer annulus fibrosus (AF) and the cartilaginous end-plate, which connects the disc with the adjacent vertebrae. The AF is a fibrocartilaginous ring, composed of concentric, dense lamellae of highly orientated type I collagen fibers which constitute an organized matrix hosting fibroblast-like cells. The NP is an amorphous, gelatinous matrix rich in proteoglycans (mainly aggrecan) and type II collagen. Aggrecan is a large-aggregating proteoglycan which is capable of interacting with hyaluronan and is provided with several negatively charged sulfated glycosaminoglycans (namely chondroitin sulfate and keratan sulfate) that are able to bind water. The high level of hydration produces a swelling pressure which is responsible for maintaining the disc height and contributes to provide the disc with mechanical resistance to twisting, bending and compression. Small chondrocyte-like cells can be found within the NP and are responsible for synthesizing the proteoglycans thus maintaining the matrix environment (2).

Intervertebral disc degeneration (IDD) in an age- and disease-related chronic process which is characterized by a progressive decrease of the proteoglycan (and consequently water) content in the NP, with the subsequent loss of the disc ability to resist compressive forces, leading to instability (3).

IDD may evolve to several spinal disorders such

Key words: stem cells, intervertebral disc degeneration, mesenchymal stromal cells, disc regeneration, spine surgery

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0393-974X (2016)

as disc herniation, degenerative spondylolisthesis and spinal stenosis associated with neurological symptoms including radiculopathy and myelopathy. Current treatment options for LBP and IDD range from conservative approaches such as rest, analgesic and anti-inflammatory medication and physical therapy, to invasive procedures, including epidural steroid injections and ablation techniques as well as surgical solutions, e.g. discectomy, total disc replacement, laminectomy and spinal fusion (4). However, these options have provided limited efficacy and do not produce preventable and reliable outcomes. Indeed, they act in order to relieve clinical symptoms instead of targeting the pathophysiology that underlies the degenerative process. For this reason, there is an emerging need to find an early treatment for LBP that may prevent, slow down, halt or even reverse the degenerative alterations of the IVD.

Significant advances in the fields of stem cell therapies have helped spine to develop innovative regenerative therapeutic approaches aiming to alter the natural course of IDD and possibly leading to disc repair/regeneration and recovery of function. The aim of this review is to discuss the potential use of mesenchymal stem/stromal cells (MSCs) from bone marrow for disc regeneration.

Pathophysiology of intervertebral disc degeneration

The detailed aetiology and pathophysiology of IDD still remains poorly understood. However, the progressive loss of aggrecan and free water content in the NP is known to be the major structural change in the IVD involved with the degenerative process (2). This seems to alter the normal balance between anabolic and catabolic functions of the NP cells, resulting in decreased synthesis, increased turnover or a combination of these two processes within the NP matrix (5).

In addition, the progressive decrease of cell number and density in the NP has been proven to be associated with both aging and IDD. This has been hypothesised to compromise the disc ability to compensate for these changes by producing and maintaining a functional extracellular matrix (6).

The direct consequence of the reduction in proteoglycan content within the NP is dehydration, which decreases disc height and alters its load-bearing capacity (7). Anomalous distribution of forces across the disc results in cracking and tearing of the AF, disc herniation and vertebral body pathological changes including subchondral sclerosis, end-plate ossification and osteophyte formation. The relatively isolated anatomical location, the inherent avascularity and low metabolic activity of the IVD may be the key reasons for the apparent disc inability for self-repair after injury and degeneration (1).

Stem cell-based therapy

Recovering the disc potential to restore the matrix and re-establish the original proteoglycan content may therapeutically lead to increased disc hydration, thus improving its biomechanical properties (1). The progressive cell loss seen in IVD aging and degeneration may be treated by injecting exogenous cells in order to supplement and restore the original disc cell population. This cell therapy approach has been investigated using different type of cells, such as disc cells (8) and progenitor cells (9).

Autologous disc chondrocyte transplantation is currently under clinical evaluation as a possible approach for preventing degenerative sequelae after lumbar disc surgery. To date, this method has been shown to be safe and to decrease LBP, to reduce disc narrowing and partially restore the physiological NP environment (10). However, this approach is only feasible after performing discectomy and is limited by low expansion rates and the loss of phenotypic characteristics when expanded in a monolayer cell culture. A recent study has demonstrated that this latter limitation could be overcome by coculturing autologous NP cells with bone marrow mesenchymal stem cells. This process allows to obtain activated NP cells that have shown the ability to improve type II collagen and proteoglycan synthesis and restore the disc height (11).

On the other hand, stem cell therapy is characterized by low harvest site morbidity (which is a major concern in spine surgery), ease of *ex vivo* expansion and favourable phenotype modulation before or after transplantation.

Stem cells are defined as undifferentiated and

uncommitted cells characterized by high proliferation rate, self-renewal ability and multilineage differentiation (1). Adult stem cells can be harvested from completely differentiated tissues, including bone marrow, adipose tissue, muscle, skin, periosteum, blood, pericytes, synovial membrane and trabecular bone (12). Their main function is to replace existing senescent and/or damaged cells following a lineage-committed differentiation pathway, in order to guarantee and maintain the physiological homeostasis of the tissues in which they reside. As they can be isolated from the patient's tissues, the application of adult stem cells in regenerative medicine does not raise any ethical concern.

The potential use of adult stem cells, such as bone marrow-derived MSCs, muscle-derived stem cells (MdSCs), adipose tissue-derived stem cells (ASCs), hematopoietic stem cells (HSCs), olfactory membrane stem cells (OFs), synovial stem cells and disc stem cells has been taken into account for IVD regeneration (12). All these cell types have demonstrated differentiation towards mesenchyme-like tissues lineages, including fat, cartilage, bone and muscle. In addition, the aforementioned cytotypes have all been shown to originate from the cells residing within the perivascular wall, such as pericytes and bone marrow sinusoidal adventitial reticular cells (12).

There are several methods to utilize adult stem cells for IVD regeneration: (i) they can be harvested, minimally manipulated in the operating room and injected; they can be isolated and expanded *ex vivo* and then transplanted into the IVD as (ii) undifferentiated cells or (iii) pre-differentiated using growth factors or co-culture methods; (iv) they can be engineered and combined with a visco-elastic hydrogels; (v) they can be transfected with genes of interest and then injected into the IVD. (Fig. 1). Stem cells can be also transplanted allogenically as a product off-the self (13).

Systemic delivery of bone marrow-derived MSCs has also been tested in an animal model: although no significant changes were reported in the extracellular matrix composition, this approach resulted in diminished hypoxic response, risk of herniation and increased synthesis of cytokines, that may help disc degeneration treatment (14).

Therapeutic effect of stem cells for disc regeneration

In the past ten years numerous *in vitro* studies have raised the possibility to evaluate the use of adult stem cells for the treatment of IDD. This has been postulated after adult human MSCs were acknowledged to be able to differentiate towards NP chondrocyte-like cells if cultured under discogenic conditions (i.e. providing cultures with chondrogenic growth factors and/or coculturing MSCs with NP cells) (15). Coculture studies have demonstrated a synergistic effect and a cross-talk between MSCs and NP cells that may enhance NP extracellular matrix protein synthesis and induce MSC differentiation toward NP cells (15, 16).

Our group studied the mechanism of interaction between MSCs and NP cells harvested from a degenerating disc by coculturing them in a 3-dimensional culture system characterized by such a short distance that allowed paracrine interactions between cells, which is typical of the NP. Gene expression profiles were analysed on both cell types and showed that MSCs acquired a chondrocyte-like phenotype and influenced mRNA levels in human NP cells, which resulted in an increase of type II collagen expression by NP cells, while aggrecan synthesis was downregulated (15).

A recent study confirmed our previous data (15) that NP cells alone and MSCs cocultured with NP cells showed higher expression of type II collagen and aggrecan, as well as increased GAG content, if compared to MSCs-only cultures. However, the presence of degenerative media conditions dramatically upregulated the expression of catabolic genes in NP cells-only groups, while this detrimental effect had only a slight influence on coculture groups, which proved to be more resistant to inflammation and hypoxia. Configuring a coculture micropellet instead of culturing individual cells showed to decrease the expression of catabolic genes despite hypoxic and inflammatory media conditions (17).

Shim *et al.* further investigated this paracrine interaction by isolating MSCs, AF and NP cells from the same donor's vertebrae and then culturing them together. Cocultures were characterized by increased mRNA levels of matrix components (including type II collagen, aggrecan and versican), growth

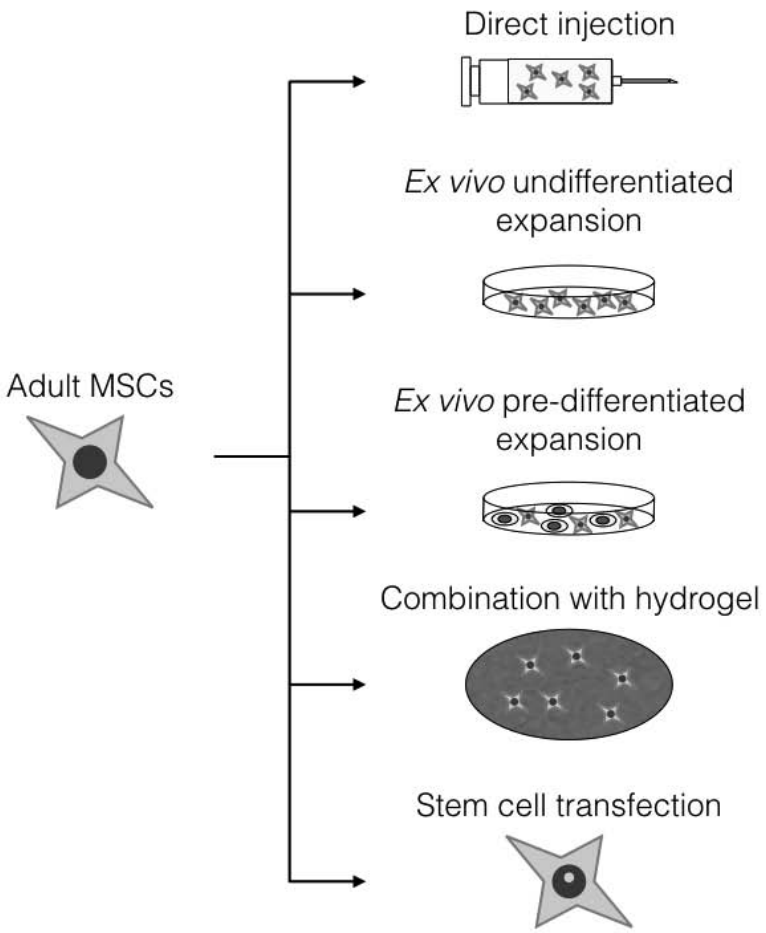

FIG. 1. *Different approaches adopted in the stem cell-based therapy of IDD. Once isolated, MSCs can be either directly intraoperatively injected into the injured disc, expanded ex vivo as undifferentiated cells or in coculture with other cytotypes or growth factors, combined with engineered biocompatible hydrogel scaffolds or transfected with target genes (from top to bottom).*

factors (EGF, IGF-1, TGF-α etc.) and decreased expression of pro-inflammatory cytokines (namely IL-1α, IL-1β, TNF-α and IL-6) when compared with monocultures (18).

Efficacy evidences in preclinical models

The IVD is characterized by avascularity, hypoxia, low pH, hyperosmolarity and low glucose content. All these features make it a harsh environment that may exhaust its intrinsic potential for self-repair and antagonize the engraftment of exogenously delivered stem cells. MSCs long term survival after disc injection was reported by several studies. Among those studies, our group assessed MSCs presence in the lumbar IVD of a healthy rabbit until 6 months after transplantation (16).

In order to evaluate the role of stem cell therapy for driving IVD regeneration, it is crucial to test regenerative approaches on animal models that may realistically resemble the human condition. Sakai *et al.* were the first ones to report that bone marrow-derived MSCs embedded in a type II collagen-based carrier and injected in a rabbit model of IDD led to increased proteoglycan content, disc hydration and height, which were maintained at 6 months after MRI evaluation (9).

Since then, significant advances in animal controlled trials based on stem cell therapy have been made. A systematic review and meta-analysis showed that 22 studies reported statistically

significant enhancements in disc height, MRI T2 signal intensity, type II collagen synthesis and decreased degenerative alterations by histological evaluation (19). These results are characterized by statistical heterogeneity due to different animal types (small versus large ones), cell types (NP cells, MSCs etc.) and injectable carrier (aqueous solution versus biocompatible scaffolds) (20).

A recent study from Maidhof *et al*. showed that not only cell type and delivery should be considered, but also planning a suitable timing for transplantation may influence the clinical outcomes. They administrated MSCs at 3, 14 or 30 days post injury and then assessed the effect of timing on the biochemistry and biomechanics of the disc; cells delivered at 3 days led to a significantly improvement on cell retention, compressive biomechanical properties and matrix reconstitution, while cells administered later did not yield comparable result, thus showing that cell therapy may be decisive if delivered at an early stage of degeneration (21).

A preclinical study performed by our group showed a potential side effect of stem cell-based therapy. MSC transplanted in a stab model of IDD in rabbit led to osteophytes formation due to MSC leakage from the injection side (22). In order to overcome the problem of AF damage and cell leakage, our group introduced a new method to deliver cells and hydrogels into the NP via the endplate with a small tunnel that can be repaired after delivery. This could be a promising regenerative approach to treat latest stages of IDD (23).

Clinical translation

The first phase I clinical study has been published by Orozco *et al*. who studied the transplantation of expanded autologous bone barrow MSCs in the NP of ten patients suffering from lumbar disc degeneration with intact AF. At 1 year after surgery, safety has been shown and improvement of pain and disability index was observed (24).

Elabd et al. reported that autologous bone marrow-derived MSCs cultured in hypoxic conditions and then injected in the discs of five patients led to an overall quality of life improvement at 4-6 years after surgery (25).

In a randomized controlled trial using allogeneic MSC, Noriega et al. treated 24 patients with LBP diagnosed with IDD divided into 2 groups. The test group received an intradiscal injection of 25×10^6 MSCs per segment. The control group received a sham infiltration of paravertebral musculature with the anesthetic. This preliminary phase II study showed clinical efficacy in terms of significant pain reduction in the treatment group compared to control. Moreover, IDD, quantified by Pfirrmann grading, improved in the MSC-treated patients and worsened in the controls (13). Although encouraging, these studies were including a small number of patients.

CONCLUSION

Significant advances towards the knowledge and the comprehension of the IVD biology and biochemistry, as well as regarding IDD pathophysiology, have been made so far. The progressive cell loss that characterizes disc aging and degeneration, together with the apparent inability of NP residing cells to self-repair intrinsic damage, have raised the possibility to replenish the native tissue with adult stem cells transplantation. This approach has repeatedly shown to help restore the disc microenvironment, thus leading to tissue regeneration. However, more effort is needed in order to assess efficacy of adult stem cell therapy for treating IDD in large controlled clinical studies. MSCs transplantation has been recognized to be promising and safe in both animal studies and clinical trials, which may make this approach a powerful tool for treating IDD in the near future.

ACKNOWLEDGMENTS

We gratefully acknowledge the support by the Research Grant for Young Investigator of the Italian Minister of Health (GR-2010-2318448), the BIOSPINA award of the Italian Society of Spine Surgery (SICV&GIS), and Università Campus Bio-Medico di Roma within the project START-Disc.

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SIMULTANEOUS DOUBLE ROD AND EN-BLOC DIRECT VERTEBRAL ROTATION TECHNIQUE FOR CORRECTION OF MAIN THORACIC ADOLESCENT IDIOPATHIC SCOLIOSIS: RETROSPECTIVE ANALYSIS OF 14 CASES

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Adolescent idiopathic scoliosis (AIS) is a triplanar deformity associated with rib hump, especially when a principle thoracic curve is present. The aim of this study is to evaluate the results of AIS correction retrospectively, using simultaneous double rod derotation manoeuvre technique followed by en-bloc direct vertebral rotation (DVR). Fourteen patients were included in this study. Coronal and sagittal thoracic Cobb angle, global coronal balance, sagittal balance, rib hump prominence, Scoliosis Research Society outcome instrument score (SRS-22) and Walter Reed visual assessment scale (WR-VAS) values were recorded pre- and postoperatively and evaluated. Results were evaluated at a mean follow-up of 2 years. Good to excellent radiographic and clinical results were obtained in all patients. No major perioperative complications occurred. This technique has proved to be effective for surgical correction of the deformity in Lenke type 1 AIS with good clinical and radiological results and low rate of complications.

Adolescent idiopathic scoliosis (AIS) is a triplanar deformity on coronal, sagittal and axial plane. In the majority of cases this leads to a hypokyphosis associated with a rib hump especially for severe Lenke type 1 curve or main thoracic form (1, 2).

Harrington first introduced deformity correction in 1962 using only concave distraction and convex compression forces. Although this technique achieved considerable coronal correction, it did not affect neither the sagittal deformity nor the rotational components (3). Subsequently, in order to obtain a better deformity control, Cotrel et al. developed the Cotrel-Dubousset (CD) multihook segmental instrumentation which allowed a superior coronal plane Cobb angle correction but also a partial sagittal curvature improvement (4).

Today pedicle screw constructs are considered the mainstay for scoliosis correction surgery because of their three-dimensional deformity correction capacity (5, 6). Pedicle screws are the only available instrumentation able to transmit forces onto the entire vertebra so as to allow direct vertebral rotation (6).

Based on implant density, pedicle screw constructs are considered segmental if more than 60% of the available anchor sites are fixated. Non-segmental pedicle screw fixation showed to be equivalent to segmental fixation in case of mild and flexible curves but for rigid and severe curve, segmental fixation is preferred to avoid excessive concentration of mechanical forces around the screws (1, 6, 7).

The direct vertebral rotation technique (DVR) was first introduced by Lee in 2004 aiming to restore the axial plane deformity by applying direct rotational forces on apical vertebrae (AV) (1). Both segmental and en-bloc DVR have been described based on

Key words: stem cells, intervertebral disc degeneration, mesenchymal stromal cells, disc regeneration, spine surgery

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0393-974X (2016)

the extension of the maneuver. The latter, acting on the whole deformed curve, is theoretically related with a decreased risk of lateral screw breakout or screw pullout due to the distribution of forces across multiple levels (8).

The sagittal plane alignment was closely related to clinical outcome and quality of life in patients with scoliosis (9). Vertebral rotation has been considered as predictor of deformity progression, long term decompensation and moreover, it is the primary cause of the thoracic rib hump and lumbar prominence that are the major cosmetic concerns of patients (9-11).

In light of these facts, the purpose of the surgery in these patients should be to obtain a triplanar deformity correction with good self-image satisfaction in a stable balanced spine. The aim of this study is to evaluate the results of AIS correction retrospectively using segmental uniaxial pedicle screws instrumentation and simultaneous double rod derotation manoeuvre technique followed by en-bloc direct vertebral rotation (DVR).

MATERIALS AND METHODS

Thirty-six patients recruited from a consecutive cohort of subjects affected by AIS and operated on between January 2012 and July 2015 at our institution were retrospectively evaluated by review of the medical records and radiographs. Inclusion criteria were Type 1 AIS according to Lenke classification, main cobb angle less than 100 degrees, patients operated with described surgical technique in this article without any additional procedure. Exclusion criteria were congenital, syndromic or neuromuscular scoliosis and less than 1 year follow-up. Fourteen patients were considered eligible for this study and were retrospectively analysed. There were 11 females and 3 males with a mean age at the time of surgery of 14.6 years (range 11-19 years).

Standing long-cassette radiographs on both antero-posterior and lateral plane were evaluated for coronal Cobb angle and T5-T12 thoracic kyphosis angle. Passive bending radiographs were used to evaluate the rigidity of the curve, to assess the fusion area and eventually classify the patients according to Lenke. Sagittal balance was evaluated using the sagittal vertical axis (SVA) as the distance of the C7 plumb line to S1. For global coronal balance the C7 coronal plumb line from the central sacral vertical line (CSVL) was measured.

Rib hump prominence measurements were obtained using an inclinometer during the Adam's forward bending test and the largest values were recorded. Scoliosis Research Society outcome instrument score (SRS-22) was used for clinical evaluation. Aesthetic impact was assessed using Walter Reed visual assessment scale (WR-VAS).

All patients were subjected to posterior correction of the deformity by facetectomy, segmental uniplanar pedicle screw instrumentation, simultaneous double-rod derotation technique, en-bloc direct vertebral rotation and fusion.

Pre- and postoperative radiographic measurements and clinical outcome were compared using matched-pairs analysis. A p-value threshold <0.05 was chosen to define statistical significance.

Surgical technique

Intraoperative spinal cord monitoring by somatosensory evoked potentials (SEPs) and motor evoked potentials (MEPs) was provided in all patients throughout the operation.

A standard posterior midline incision was made from one level above and one below the uppermost- and the lowermost-instrumented vertebra. The transverse processes were then exposed bilaterally. The facets and their articular process included in the fusion area except the uppermost level were removed in order to facilitate the identification of entry points, promote arthrodesis and allow an easier deformity correction. Uniplanar pedicle screws were placed at each level on both sides of the curve using drill-assisted technique. Poliaxial pedicle screws were used in case of severe rigid deformity when the rod resulted too far to be engaged. Two precontoured rods were placed on both sides of the curve and then simultaneously and gently rotated in a counter clockwise direction by 90° by two surgeons transforming the deformity on the frontal plane into the desired kyphosis on the lateral plane (7, 12). When the most proximal and distal anchors were fixed, the screw derotators were placed at each level of the deformity on both sides of the curve and then rotated in a clockwise direction using the neutral vertebrae as anchor points (1). No compressive or distractive forces were applied because there are no

proven deforming forces acting in this direction in scoliosis (1). Screws heads were tightened followed by decortication. Finally, bone graft was applied.

RESULTS

Results were evaluated for a mean follow-up of 2 years (range 1-4 years). Mean preoperative Cobb angle was 68.2° (range 50°-85°). Average post-operative Cobb angle measured at the last available follow-up was 16.7° (range 10°-35°) with a deformity correction of 75.5% (p=0.01).

Mean preoperative thoracic kyphosis was 14.1° (range 5°-19°). At the last follow-up average postoperative kyphosis value was 16.4° (range 10°-26°) with a no statistically significant increase of 2.3° (p=0.18).

Nor the sagittal balance measured with SVA (mean preoperative value 3.1 ± 8.7 mm) or the global spine balance measured by the C7 translation from CSVL (mean preoperative value 16.4 ± 7.3 mm), showed statistically significant changes after surgery (postoperative value respectively 2.5±6.8 mm with p=0.21 and 9.8±4.3 mm with p=0.15).

Mean preoperative rib prominence measured by the inclinometer was 16.4° (range 7.2°-26.5°). Average postoperative rib prominence was 6.2° (range 4.0°-10.2°) (p=0.03). Mean total SRS-22 score showed a statistically significant improvement at the last available follow-up (pre- and post-operative values respectively 2.8±0.6 and 4.2±0.5, p=0.01). The WR-VAS improved from 18.2±2.5 to 14.2±3.0 (p=0.05).

No major perioperative complications occurred. One superficial wound infection was reported and resolved with debridement and antibiotic therapy. At the last available follow-up no deformity progression, nor screw pull-out or non-union were recorded (Table I).

DISCUSSION

To our knowledge, this is the first study to investigate clinical and radiological results after treatment of AIS with both simultaneous double rod rotation and direct vertebral rotation technique. This procedure improved radiographic parameters on coronal and sagittal planes. In addition, the rib-hump prominence on the inclinometer reduced and good clinical results on SRS-22 score and WR-VAS were achieved.

Pedicle screw constructs have been related to the best corrective results for AIS surgery (1, 5, 6, 13-15), despite some authors reported a decreased kyphosis of

Table I. *Bold values are staticaly significant.*

	Pre-operative	Post-operative	p-value
Cobb Frontal	68.2° (50°-85°)	16.7° (10°-35°)	**0.01**
Kyphosis	14.1 (5°-19°)	16.4° (10°-26°)	0.18
Sagittal balance	3.1±8.7 mm	2.5±6.8 mm	0.21
Global spine balance	16.4±7.3 mm	9.8±4.3 mm	0.15
Rib prominence	16.4° (7.2°-26.5°)	6.2° (4.0°-10.2°)	**0.03**
SRS-22	2.8±0.6	4.2±0.5	**0.01**
WR-VAS	18.2±2.5	14.2±3.0	**0.05**

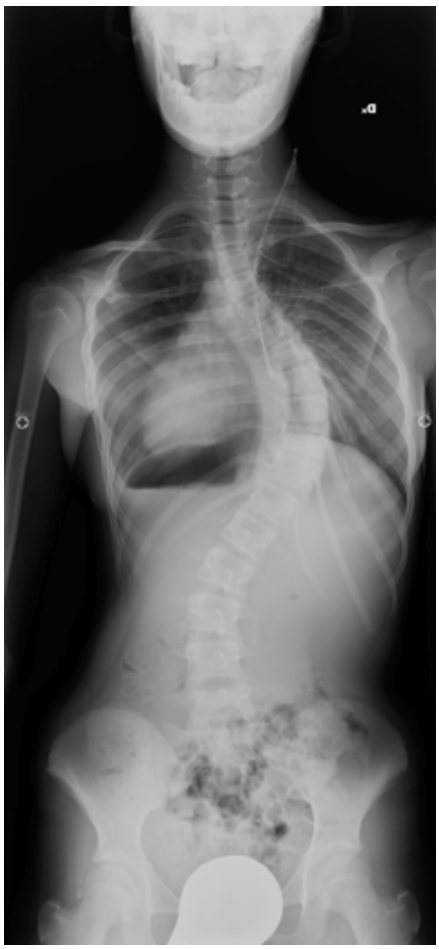

Fig. 1. *Preoperative standing long cassette radiograph of Type 1 AIS.*

the thoracic spine after corrective surgery for AIS with pedicle screws (13, 16, 17). In our study, we found a small increase of the thoracic kyphosis after corrective surgery but with no statistical significance.

Uniplanar pedicle screws were always used in the present study unless in vertebrae that were too far from the rod. In these rare cases, poliaxial pedicle screws were preferred in order to engage the rod easily on the screw heads. However, we believe that if the same technique is used for pedicle screws insertion and the curve is followed in a uniform manner, screw-rod coupling, even in severe deformity, can be easily achieved also. Kuklo et al. compared monoaxial and poliaxial pedicle screws in AIS correction, finding that although excellent coronal plane correction obtained with both type of screws, the monoaxial types were able to achieve a better apical vertebral rotation (18). However, it is clear that for severe rigid scoliosis monoaxial pedicle, screws are difficult to engage with the rod. The uniplanar pedicle screws were recently introduced in order to allow easier engagement of the rod while maintaining enough strength to adequately rotate the vertebrae (19). Simultaneous double rod rotation were first described by Ito et al (7) to avoid forces pushing down the spine around the apex during corrective manoeuvres. Similarly to our results they investigate only Lenke type I scoliosis achieving a correction rate on the frontal plane of 72.7% (from 67° preoperative to 17.8° mean postoperative Cobb angle) and an increase of kyphosis values in all patients. Similar results were later reported by Sudo et al (12) in a retrospective analysis of 32 patients with main thoracic AIS treated by simultaneous double rod rotation technique. The major presumed advantage of this procedure is the wider forces distribution during the corrective manoeuvres, that could reduce the risk of lateral screw breakout or screw pullout.

The rod rotation manoeuvres have been related to some rotational efficiency on the vertebrae since CD method (20-22). But, as later established, these results seemed more related to an overall correctional effect rather than a true derotation on each single vertebra (1, 22). Recently, Pankowski et al. showed that single concave rod manoeuvres do not reduce the deformity, but even worsen it (23). The recent literature reports very favourable results related to DVR on axial deformity correction. Lee et al. reported 42.5% of correction (1), Asghar et al. achieved correction rate values of 60% (24) and Kuklo et al. obtained up to 77.9% of correction using DVR (18).

According to recent literature, we believe that DVR manoeuvres is the only available instrument able to modify the vertebral rotational deformity. This technique also offers the possibility to avoid thoracoplasty procedures and related complications such as prolonged surgical time, hemothorax and pleural effusion (1). One of the main limits of the present study is related to the absence of a validated and reproducible procedure to evaluate rotational deformity correction. We only used indirect parameters such as inclinometers values and results derived from SRS-22 score and WR-VAS. However, we are aware of the superiority of CT scan derived measurements

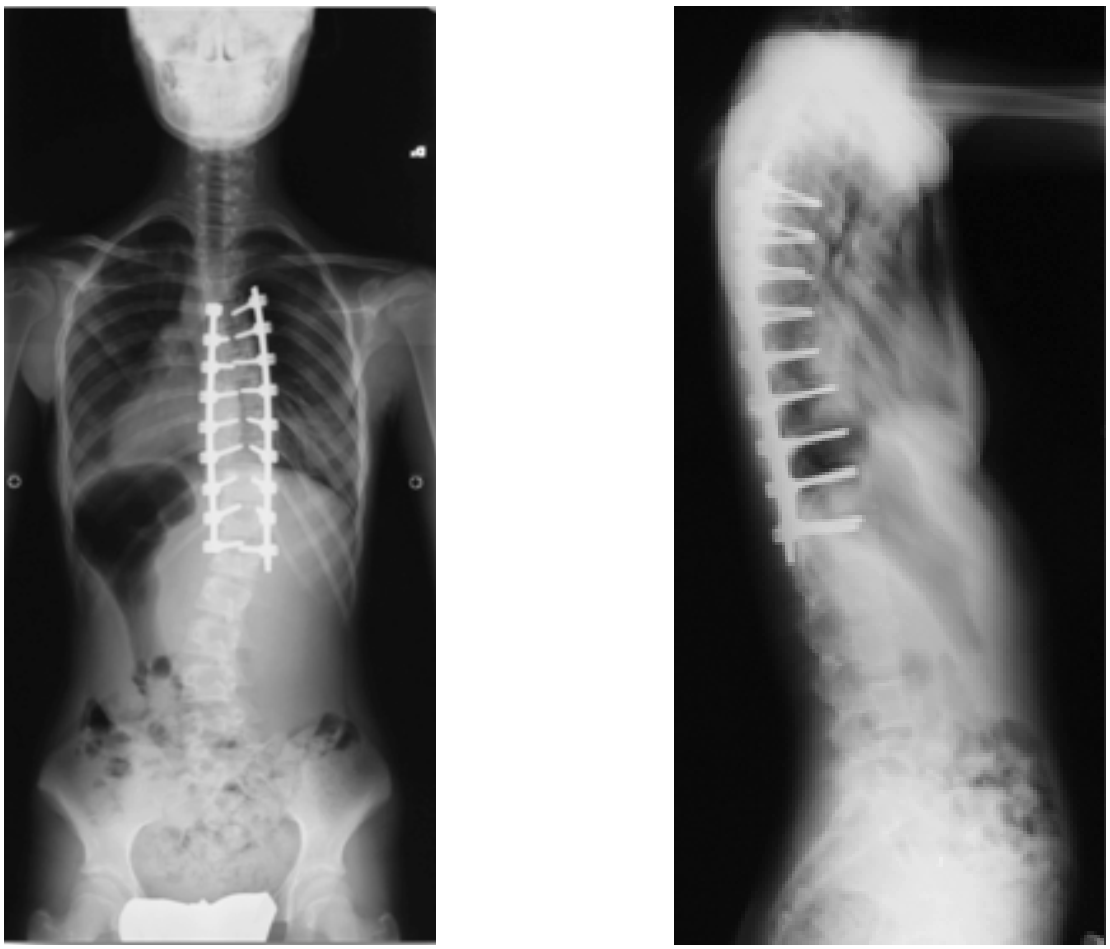

Fig. 2. *Postoperative radiograph on frontal (**a**) and sagittal (**b**) plane.*

for a correct assessment of DVR results, as reported by Aaro and Dahlborn (25). This procedure however, exposes the paediatric population to ionizing radiation and increases hospital costs without significant clinical benefits (19, 24, 26).

Others limits of the present study are: the small number of patients, which could reduce its statistical power; The short follow-up period that can conceal late complications such as hypokyphosis; the retrospective nature and the absence of a control group.

Despite the aforementioned limitations and considering the uniqueness of this study, we can affirm that AIS correction, using segmental uniplanar pedicle screws, simultaneous double-rod derotation manoeuvre technique followed by en-bloc DVR, seems to be an effective procedure for surgical correction of the deformity in type 1 Lenke AIS with good clinical and radiological results and low rate of complications. Further studies with greater statistical significance are required to better investigate indications and results for AIS corrective surgery, with all the available procedures.

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THE CORRELATION BETWEEN PREOPERATIVE LEVELS OF ALBUMIN AND TLC AND MORTALITY IN PATIENTS WITH FEMORAL NECK FRACTURE

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A femoral neck fracture in an elderly patient often represents a major challenge for the orthopaedic surgeon who has to face not only the fracture, but also all the multiple issues related to age. Among others, malnutrition has been recognised as an important factor associated with severe aggravation in these patients. One-hundred-and-forty-seven patients were enrolled to investigate the use of two markers of patient nutritional status, i.e. serum albumin level and total leukocyte count (TLC), as predictors of mortality in the elderly patient suffering from proximal femur fracture. We found that low preoperative values of serum albumin and TLC proved to be directly related to worse outcomes. Therefore, these exams can be useful to identify patients with a femoral neck fracture that have higher risk of malnutrition and consequent higher mortality and that can benefit from some measures, such as albumin or protein nutritional supplement.

Proximal femur fractures are one of the most common causes of hospitalization for elderly patients over the age of 65, with a lifetime risk of 18% in women and 6% in men (1). Every year 4 to 5 million new cases are reported worldwide with a growing trend that will reach around 6.5 million in 2050. In Italy, in 2008, we passed the 100,000 annual hospitalizations with an expenditure of about 1,000,000,000 Euros for our National Health System (2, 3). Femoral fracture is associated with a risk of death close to that of breast cancer and with an estimated mortality range of about 5% in the acute phase and 14-47% within a year (4). Some of the main risk factors associated with increased mortality after femoral neck fracture are age, sex, ASA (American Society of Anesthesiologists) score, nutritional status, the type of fracture and the delay in surgery.

Malnutrition is common in the elderly, particularly in hospitalized patients or patients in nursing homes. Patterson et al. reported that 60% of the patients he studied with femoral fracture were in a protein-energy malnutrition state during the first week of hospitalization (5). Therefore, protein-energy malnutrition (PEM) is an extremely important condition that must not be underestimated because it directly correlates with a worsening of outcome, increasing the risk of complications and even death (6-9).

In this scenario, it is important to recognize the increased mortality risk factors, due to fracture of the proximal femur, in order to allow the orthopaedic surgeon an optimization of the management of these patients and a consequent improvement of the outcome. With this aim, different scoring systems such as E-PASS (Estimation of Physiologic Ability and Surgical Stress), the NHFS (Nottingham Hip Fracture Score) and POSSUM (Physiological and Operative Severity Score for Enumeration of Mortality and Morbidity) are frequently used in clinical practice, but they are not free from limitations, particularly in terms of validity, specificity and simplicity.

Key words: femoral neck fracture, elderly, albumin, TLC, mortality

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0393-974X (2016)

In addition to these clinical scoring systems we have simple biological markers readily available at admission like haemoglobin (Hb), albumin, total leukocyte count (TLC), creatinine, BUN (Blood Urea Nitrogen) and parathyroid hormone (PTH) (10, 11). In particular, albumin and TLC are two indicators of the patient's nutritional status that, as we have pointed out, is a frequent condition in these patients, and directly related to a negative outcome.

The aim of this study was to investigate the role of serum albumin and total leukocyte count (TLC) measured at admission as predictors of mortality after proximal femoral fracture with the aim to validate the use in clinical practice since they represent two easily and cheaply available parameters, routinely detected at the entrance in the emergency room.

MATERIALS AND METHODS

Between January 2013 and August 2014, at the first Orthopaedics and Traumatology department of Pisa University Hospital, 233 patients were admitted with the diagnosis of proximal femoral fracture.

A retrospective study was therefore designed with the following inclusion and exclusion criteria. The inclusion criteria were:

- Age greater than 65 years;
- Diagnosis of proximal fracture of the femoral neck;
- Surgically treated patients with prosthetic replacement or endomedullary nail.

The exclusion criteria were:

- Patients hospitalized for pathological fractures;
- Patients with polytrauma;
- Patients with lack of data.

Patient data were collected from medical records and they were organized in Microsoft excel files. Preoperative serum Albumin and Total Leukocyte Count (TLC) were detected upon entering the emergency room while data regarding mortality were obtained through the consultation of death records. We divided the patients into four groups with the following criteria:

- Group A: Albumin≤3.5 g/dl and TLC≤1500 cel/ml
- Group B: Albumin≤3.5 g/dl and TLC≥1500 cel/ml
- Group C: Albumin≥3.5 g/dL and TLC≤1500 cel/ml
- Group D: Albumin≥3.5 g/dL and TLC≥1500 cel/ml

Data analysis was performed by SPSS (Statistical Package for Social Science) to study the survival curve through to February 2016. The relationship between the characteristics of the patients and the survival time was studied by means of Kaplan-Meier estimator and Cox regression model. The comparison between the different Kaplan-Meier survival curves was performed using the log rank test (Mantel-Cox), the Breslow test (Generalized Wilcoxon) and Tarone-Ware test. According to the regulations of our institution, the retrospective nature of the current study did not require ethical approval from the local ethics committee.

RESULTS

Following the inclusion and exclusion criteria, 147 patients were enrolled. Thirty-six were male and 111 female, with a M:F ratio of about 1:3. The overall average age was 84.41 years. The average age was 84.16 among women and 83.83 years among men. Surgical implants included 85 intramedullary devices, 11 total hip arthroplasty and 51 endoprosthesis.

The 147 patients enrolled were divided in four groups. Group A: Fifty-six patients presenting serum albumin and TLC below the standard; Group B: Nineteen patients presenting serum albumin below the normal values and TLC above normal; Group C: Fifty-one patients presenting serum albumin higher than the normal range and a low TLC; Group D: Twenty-one patients with both albumin and TLC higher than the normal range. During follow-up, there were 24 deaths in Group A, 16 deaths in Group B, 8 deaths in Group C and only one died in Group D. The number of deaths on total patients entered into the study was 49 with a mean survival time of about 1 year and 7 months (626.59 days).

We plotted the survival curves using the Kaplan-Meier estimator whose statistical validity was confirmed through the log rank test (Mantel-Cox) whose p-value was equal to 0.010, the Breslow test (Generalized Wilcoxon) whose p-value was equal to 0.014 and Tarone-Ware test whose p-value was equal to 0.013) (Fig. 1, Table I).

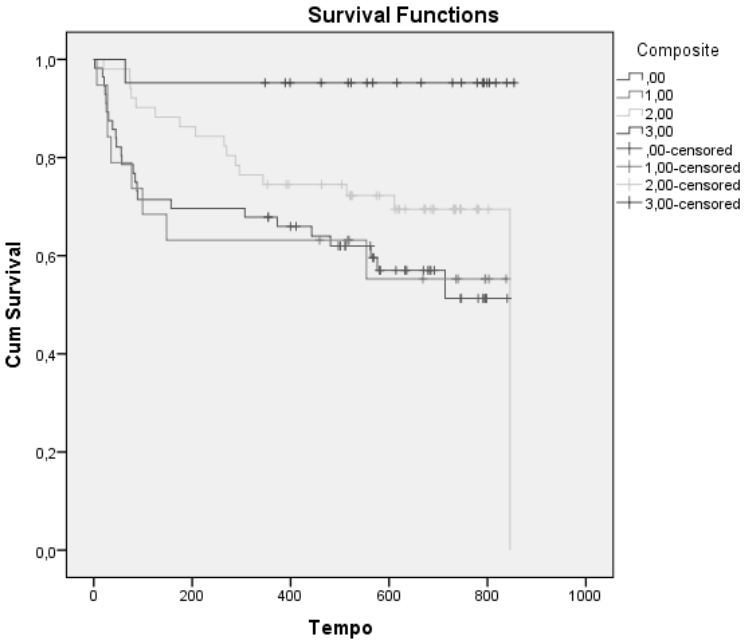

Fig. 1. *Survival curve of the four groups of patients (A-D).*

Table I. *Comparison of the Kaplan-Meier survival curves.*

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	11,428	3	,010
Breslow (Generalized Wilcoxon)	10,615	3	,014
Tarone-Ware	10,806	3	,013

DISCUSSION

The treatment of proximal femur fractures always represents a major challenge for the orthopaedic surgeon because of the complexity of the patients suffering from this type of pathology. Generally patients are elderly with a considerable number of concomitant medical comorbidities (66% of patients have an ASA score≥3) (12). Consequently, the need for an appropriate preoperative stabilization, the recognition and elimination of negative prognostic variables are crucial to optimize the clinical management of these patients.

Currently there is no universally accepted definition of protein-energy malnutrition (PEM). With reference to the orthopaedic branch, we believe that

early identification of elderly patients with femoral neck fracture and a concomitant status of PEM and/or patients who are at increased risk of malnutrition at admission would allow a stratification of the risk of mortality and morbidity.

In common with previous literature, we have used combinations of serum albumin and TLC at admission as surrogate markers of PEM (6, 13). These markers represent two inexpensive and quickly detectable parameters that every laboratory can easily provide. In this way, we used serum albumin and TLC as independent prognostic factors. In our study we specifically found that the lower the levels of serum albumin and TLC are at the time of admission, the lower the CSR (Cumulative Survival Rate) is. The survival curves for Groups A-D are shown in Fig. 1.

From the graph of Fig. 1, we note that the lowest survival time is found in patients in Group A, while the highest in Group D. The parameter associated with the worst postoperative outcome is serum albumin level as shown by the fact that Group B CSR value is lower than that of Group C. An important point to emphasize is that Group A has a higher CSR than Group B during the first year but then collapses in the following months. Mortality rates observed in this study are comparable with those reported in literature. Koval reported that preoperative serum albumin levels lower than 3.5 g/dl correlate with an increased likelihood of extending the duration of hospitalization, an increased rate of hospital mortality and a delayed return to ordinary activities after proximal femoral fracture (6). Similarly other studies conducted by Pioli et al. and Incalzi et al., demonstrated the value of albumin as a strong independent predictor of hospital mortality and long-term survival in elderly patients with proximal femur fracture (14, 15).

Albumin is the only variable significantly associated with mortality that can be potentially modified with an appropriate intervention, but there is only limited evidence that nutritional supplements in patients with femoral neck fracture can improve the outcome (16). On the other hand, some studies experimented nutritional supplements based on protein preparations or the use of a nasogastric tube to reduce the onset of long-term complications. They showed that patients who received oral nutritional supplements reduced hospitalization time and were less likely to have a potential major complication (17, 18)

Despite the evidence of this study, we have to recognize some limitations. Firstly, the serum albumin and TLC were used as unique markers for the assessment of nutritional status; secondly, albumin values can be lowered because of multiple acute events so that its use as a nutritional marker is not reliable when detected after admission (19, 20). Finally, in this study we have not included a comparison between the use of albumin as a nutritional marker and other tests more accepted and validated in this area, such as the SGA (Subjective global assessment) and the MNA (mini-nutritional assessment), due to complexity, high costs and subjectivity that these tools possess, preventing their use in clinical practice for this purpose (21).

CONCLUSION

Serum albumin and TLC are two fast and accurate PEM markers in patients with proximal femoral fracture. The value of protein-energy malnutrition (PEM) in the elderly is well known both as a causal factor of femoral fracture and as a negative prognostic factor for outcome (6, 22). In our study, serum albumin values and TLC at the time of admission are recognized as clear and significant predictors of mortality at 6 and 12 months. These two markers have multiple clinical utilities, in fact, they provide valuable prognostic information and if combined with a complete clinical evaluation, they may help clinicians to identify patients with proximal femoral fracture that can benefit from some measures, such as albumin or protein nutritional supplement.

In conclusion, our results suggest that routine measurement of serum albumin and TLC at the time of admission provides prognostic information useful for the treatment of this fragile population. Therefore, it is recommended that the levels of serum albumin and TLC should be regularly checked before surgery in elderly patients with proximal femoral fractures.

Additional work is still required to identify the precise causal link between the reduction in serum albumin, TLC and mortality in this population and to understand how albumin and TLC at the time of

hospitalization may be influenced by the presence of the fracture.

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IS A MINIMALLY INVASIVE ANTERIOR APPROACH EFFECTIVE IN OLD PATIENTS? A PILOT STUDY

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Minimally invasive approach to the hip is a blood preserving surgery, with rapid rehabilitation, and low dislocation rate. Intuitively, these characteristics render this approach extremely suitable in the elderly patient. The aim of this study was to analyze the early clinical and radiographic results in the first 30 consecutive patients above 70 years of age undergoing THR through a minimally invasive anterior approach. Clinical evaluations showed an improvement of the Harris Hip Score and WOMAC score after surgery. Radiographic assessment showed cup orientation averaging 47° (range 40°–59°) and no valgus stem aligned. Allogeneic blood transfusion was required in only 6 patients (19.8%). One patient experienced an intraoperative fracture of the greater trochanter. No early implant dislocation was observed in the study population. In conclusion we advise a minimally invasive anterior approach for THR in older patients when a careful patient selection has been done.

Minimally invasive procedures for total hip replacement (THR) gained increasing popularity over recent years in an effort to reduce soft tissue damage and improve patients' recovery. Focusing predominantly on early rehabilitation and quick recovery following THR, the majority of these techniques have been proposed for young and active patients (1-4). The direct anterior approach to the hip is a tissue sparing procedure that allows the surgeon to reach the joint using an intermuscular and internervous plane and reducing soft tissue traumatization (5).

However, minimally invasive approach to the hip is also characterized as a blood preserving surgery and is related to an early rehabilitation and recovery of ambulation. Intuitively, these characteristics render this approach extremely suitable for the fragile elderly patient, in which multiple comorbidities are associated to intra and postoperative complications. Elderly patients in fact are more likely to suffer cardiac complications due to postoperative anemia and neurological or vascular complications for delayed weight bearing. Furthermore, implant dislocation rate is higher in elderly patients especially using a traditional postero-lateral approach (6). The theoretical advantages of minimally invasive anterior approaches are the reduced postoperative blood loss, the early weight bearing on first postoperative day and the very low dislocation rate (7, 8).

The higher complication rate and the longer learning curve compared to traditional approaches to the hip have been shown to counterbalance the advantages of the rapid rehabilitation using this approach (9, 10). In this context, because of the aging of the population, it can be hypothesized that an increasing number of elderly patients should be scheduled for THR in the future and take advantage of a minimally invasive approach to the hip (11, 12-14) but at present very little has been published on the results of minimally invasive procedure in this

Key words: total hip replacement, minimally invasive anterior approach, old age, complications

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0393-974X (2016)

population.

The aim of this study was to analyze the early results in the first 30 elderly patients undergoing THR through a minimally invasive anterior approach (AMIS). Particularly, we asked whether this minimally invasive anterior approach is effective in reducing postoperative blood loss, general complications related to anemia, and the incidence of early implant dislocation.

MATERIALS AND METHODS

Thirty-two patients aged 70 years or older, who underwent cementless THR in our department between April 2012 and July 2012 with an AMIS approach, were included in the study. Two patients did not complete the postoperative self-assessment questionnaire and though clinically well performing, were excluded from the study, leaving thirty patients under observation. All patients were aware that collected data could be used for publication and signed an informed consent.

The preoperative diagnosis was primary osteoarthritis (OA) in 25 hips and secondary OA in 5 hips. There were 20 males (70%) and 10 females (30%). Surgery was performed on the right hip in 13 cases and on the left hip in 17 cases. The average age of patients at the time of surgery was 75 years (range 70 to 80 years) and the average body mass index (BMI) was 29.5 kg/m^2 (range 26.3-33,3 kg/m^2).

The senior Author performed all surgeries through an anterior minimally invasive (AMIS) approach using a dedicated mobile leg positioner (Fig. 1) and a dedicated surgical instrumentation (Medacta, Switzerland).

A cementless elliptical hydroxyapatite coated cup (Versafitcup®, Medacta, Switzerland) and a cementless straight hydroxyapatite coated short stem (AMIS® stem, Medacta, Switzerland) with ceramic on ceramic couplings were used in all cases. The patient was placed supine on a standard surgical table. The leg was secured on a mobile leg positioner (AMIS mobile leg positioner, Medacta, Switzerland) that can be easily fixed to the surgical table. The leg positioner allows the mobilization of the operated leg in extension, traction, abduction, adduction and rotation.

The anterior superior iliac spine (ASIS) and the lateral border of the patella are the landmarks for the surgical incision. A straight incision starting about 1 cm distal and 1 cm lateral to the ASIS was prolonged for about 7 cm toward the lateral border of the patella. The intermuscular plane between the tensor fasciae latae and the sartorious was developed and the lateral circumflex vessels were ligated. The rectus femoris and the iliopsoas tendon were

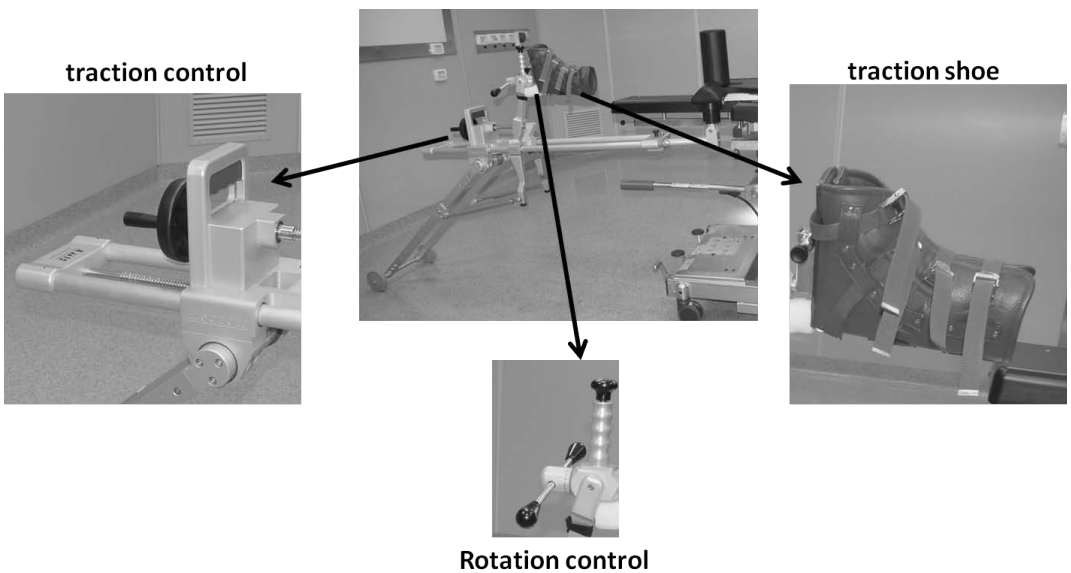

Fig. 1. *The mobile leg positioner: the leg is secured on a traction shoe; the traction system and the rotation control allow a third operator to modify limb position during surgery.*

released and an "H" shaped capsulotomy was performed.

The head of the femur was resected *in situ* and then removed. The leg was placed under traction and a slight external rotation was performed to improve acetabular exposure before the cup was implanted. Femoral exposure was achieved by placing the leg in extension, adduction and external rotation. After the stem was implanted, the hip was reduced using the mobile leg positioner and the capsule was repaired. Standard fascial and skin closure were performed over a suction drainage.

All patients received a short-term antibiotic prophylaxis with third-generation cephalosporin and low molecular weight heparin was used within the first post-operative day, up to full weight bearing according to institutional and international protocols. Patients achieved a standing position and began to ambulate the day after surgery. Partial weight bearing with two crutches was allowed for the first three weeks after surgery and then full weight bearing as tolerated.

Clinical evaluations were held preoperatively and postoperatively, at 30 days and 3 months using the Harris Hip Score (HHS) (15) and WOMAC score (16). Questionnaires were administrated by two authors that were not involved in the surgery who collected and gathered the data. Radiographic assessment was performed preoperatively and at the latest follow-up on standard anteroposterior and lateral views of the affected hip. Cup and stem orientation, and leg length discrepancy were evaluated.

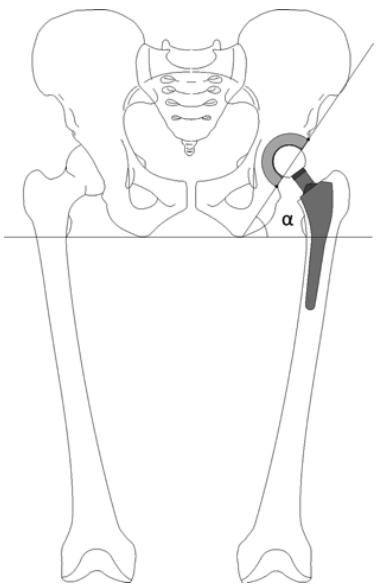

Fig. 2. *Cup abduction measurement*.

Cup orientation was measured by one experienced surgeon not directly involved in the surgery on an anteroposterior radiographic view of the pelvis as the angle between the inter-teardrop line and the edge of the cup (Fig. 2) (17).

A stem was considered varus or valgus if the angle described by the intersection of the axis of the stem and the axis of the femoral shaft was greater than 5°. Subsidence of 2 mm or more and/or varus or valgus tilting was considered as a sign of stem failure.

Leg-length discrepancy was measured on an anteroposterior radiographic view of the pelvis, comparing the distance between the inter-teardrop line and the middle of the lesser trochanter on both sides; the major diameter of the prosthetic head was used as a reference to standardize radiographic magnification.

The level of haemoglobin during the first postoperative day and at discharge were recorded, as well as the total amount of perioperative blood loss, evaluated according to the total amount of postoperative drainage, intraoperative loss and transfusion need.

Descriptive statistics were performed on the study population. Data are reported as mean and standard deviation. When considered appropriate, full data ranges were reported.

RESULTS

The average duration of follow-up was 5 months (range 3-8 months) and the only complication recorded (3.3%), was an intraoperative fracture of the greater trochanter, immediately treated with cerclage wire.

At the latest follow-up, the Harris Hip Score averaged 89.2 (range 70-97) with a marked improvement in respect to preoperative score (average 43.3, range 24-73); the WOMAC score improved from 66.3 (range 55 to 85) preoperatively, to 26.7 (range 19 to 35) postoperatively. Cup orientation was on average 47° (range 40°-59°) and in seven hips the abduction angle was out of the optimal range of 40°±10° (18). Two stems were aligned in varus (6.6 %) and 28 were neutral (93.4%).

There was no evidence of periprosthetic osteolysis or signs of migration in any of the cups. All the stems appeared to be stable without signs of subsidence or tilting. The mean preoperative leg length discrepancy was 12 mm (range 0-24 mm), with a difference greater than 1 cm in 8 hips. Postoperatively, the mean leg length discrepancy was 4 mm (range 0-12 mm), with a difference greater than 1 cm in one hip.

The mean preoperative Hemoglobin was 12.3 g/dL (range 8.6-15.4); on the first postoperative day the mean hemoglobin level was 9.1 g/dL (range 7.2-11.4), whereas the mean value at discharge was 9.8 g/dL (range 9-13.5).

The blood loss was on an average of 423.5 mL (260 to 1200 mL), requiring allogeneic blood transfusion in only 6 patients (19.8%) and 2 of these patients were treated with anticoagulant agents before surgery for cardiovascular prophylaxis.

We did not record any early implant dislocation. On the first postoperative day, 19 patients were able to partially weight bear the operated hip, the average patients length of stay in our department was 5.6 days (range 4 to 9 days).

DISCUSSION

Minimally invasive techniques for THR are usually reserved for young and active patients in which these tissue sparing procedures allow a better functional recovery (1-3).

Nonetheless, these theoretical advantages could also be useful in old patients as in this subset of patients the control of post-operative anemia and the early weight bearing could reduce general cardiovascular complications. In fact, a higher incidence of deep vein thrombosis following THR has been reported in old patients (19), leading to a postoperative mortality rate ranging from 1% to 1.3% within 90 days, even with the use of antithrombotic prophylaxis (19-21). Furthermore, a readmission rate has been reported for symptomatic thromboembolic complications within 28 days of discharge after THR, ranging from 0.73% to 1.39% (10, 22) thus, consistently increasing the costs of the procedure.

The results on our cohort of patients aged at least 70 years, showed excellent short-term clinical outcomes using the AMIS approach for THR (Fig. 3, 4).

The AMIS approach ensure less surgical trauma, reduces muscular trauma and allows for a straightforward rehabilitation protocol. With the AMIS approach, associated with an early weight bearing, we did not observe any deaths in the first

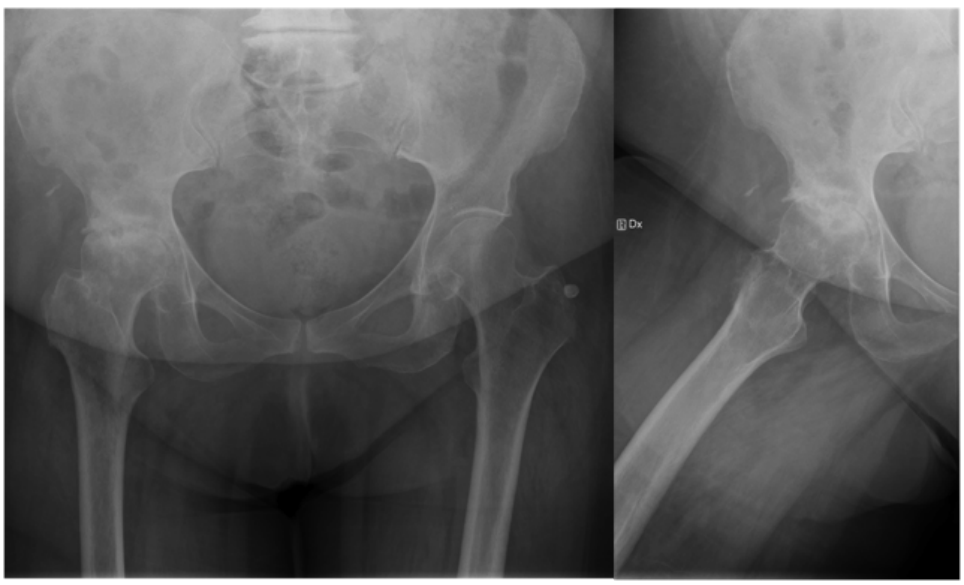

Fig. 3. *Preoperative radiographs of a 73-year-old female patient with severe osteoarthritis of the hip.*

90 days and through the use of low molecular weight heparin as antithrombotic prophylaxis, none of the 30 patients required readmission to our department.

The mean length of hospital stay after THR in this series was 5.6 days and this is consistent with the data reported for younger populations in other series (23, 24), therefore these promising results seem to support the hypothesis that tissue sparing procedures can allow a faster rehabilitation protocol. An enhanced recovery program after THR also reduces the rate of 30-day myocardial infarction and stroke (25) and increases the cost-effectiveness of hip replacement surgery.

Rates of blood transfusions between 20% and 22.4% and a total blood loss between 1000 and 2000 ml have been reported following THR (26) whereas a 19.8% rate of transfusion and an average 423.5 ml of total blood loss have been recorded in this study.

In addition to the reduced incidence of important general complications, the AMIS approach allows to reduce considerably the use of blood transfusion in our patients, enhancing the patient's compliance for the procedure and reducing the overall costs. Finally, we did not record any implant dislocations within the first 90 days after surgery, a very important achievement considering the usually higher rate of this ominous complication in old patients (6) that usually requires a second admission and a reoperation in a third of them (27).

The higher risk of intraoperative complications such as femoral fracture or acetabular protrusion counterbalances the advantages of AMIS approach to the hip and these complications could be theoretically higher in osteopenic patients. In this series, we recorded a greater trochanter fracture requiring cerclage wire fixation, probably both due to the low bone quality of the femur and the high BMI of the patient (39.6 kg/m^2) that lead to a misprocedure broaching the femoral canal. Learning from this complication, we now perform and suggest a more careful patient selection in terms of BMI, and we usually prefer stem designs with a reduced shoulder.

The major limit of this study is the very short follow up and the reduced number of patients enrolled. However, the follow up was congruent to evaluate the effectiveness of the procedure in terms of post-operative blood loss and general complications related to anemia and incidence of early implant dislocation. To our knowledge, this study is the first to evaluate the effectiveness of a minimally invasive anterior approach in old patients.

In conclusion, we advise a minimally invasive anterior approach for THR in older patients when a

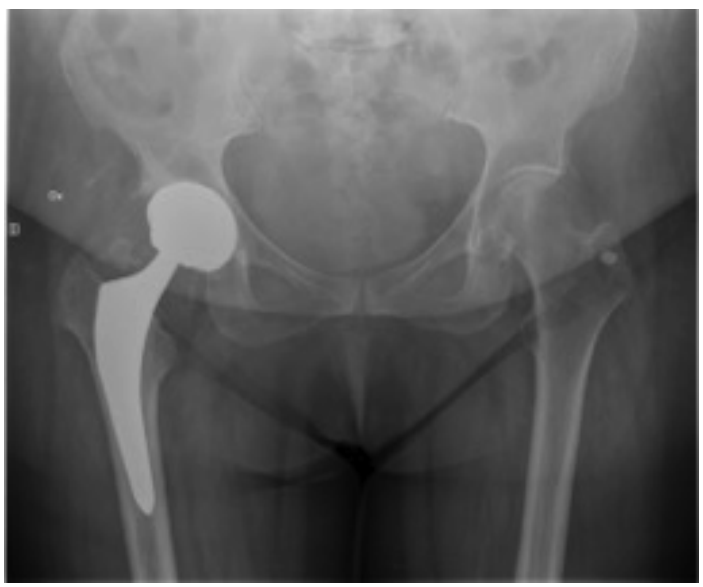

Fig. 4. *Eight months follow-up films.*

careful patient selection has been done in terms of BMI and a low profile stem is employed.

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ISOLATE ACETABULAR CUP REVISION THROUGH THE DIRECT ANTERIOR HIP APPROACH: SURGICAL TECHNIQUE, EARLY EXPERIENCE AND REVIEW OF THE LITERATURE

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Direct anterior approach to the hip allows perfect exposure of the acetabulum and an easy proximal and medial extension that makes it eligible for isolate acetabular cup revision although it is seldom used and there are only few published studies. On 23 consecutive acetabular revision (16 cases Paprosky grade 1 or 2, 5 cases 3A, 1 case 3B and 1 case 4) at an average 28-month follow up, we did not record failures or major complications. Early complications included prolonged wound healing in 4 cases and transient femoral cutaneous nerve palsy in 2 cases, the mean postoperative Harris Hip Score was 82.2 with 82.5% of excellent and good results. Our results are consistent with those reported in the literature with similar techniques. The direct anterior approach has shown excellent results for isolated cup revision, though is probably better suited for surgeons that have some experience with the same approach for primary cases.

The direct anterior (DA) approach through the Smith-Petersen or Heuter interval has been gaining popularity for primary hip arthroplasty and while some limitations are reported for femoral exposure, none are reported for the acetabulum (1). The DA allows a perfect exposure of the acetabulum and an easy proximal or medial extension that could be very useful in case of acetabular protrusio to isolate and protect the iliac vascular bundle (Fig. 1). Furthermore, the DA approach has the potential to decrease the postoperative dislocation rate since the dislocation rate with the same approach for primary hips is known to be low (2, 3). However, few papers have described acetabular revision through this approach. Thus, the aim of this paper is to describe our midterm experience of acetabular revisions through the DA approach.

MATERIALS AND METHODS

Twenty-three consecutive acetabular revisions operated in our Department between 2012 and 2015 were performed through a DA approach. Sixteen cases were rated grade 1 or 2 according to Paprosky, 5 cases 3A, 1 case 3B and 1 case 4. All cases underwent a preoperative protocol with bone scan, RCP and biopsy to exclude infection. Clinical and radiological 28-month follow up was performed in each case except one.

Surgical technique

As DA is usually performed on a patient that has undergone primary surgery through another approach, a long incision for a good exposure is not usually required. Since the femoral component is not subject to revision, special leg positioners or dedicated devices are not required. In case of cup revisions with an acetabular Paprosky 1 and 2 bone loss, we usually perform a simple tissue sparing Heuter approach as for primary surgery (Fig. 2).

After the administration of general or spinal anaesthesia and third generation cephalosporins prophylactic, the patient is placed supine on a standard table with the

Key words: hip, revision, anterior approach, acetabulum, failure

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0393-974X (2016)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

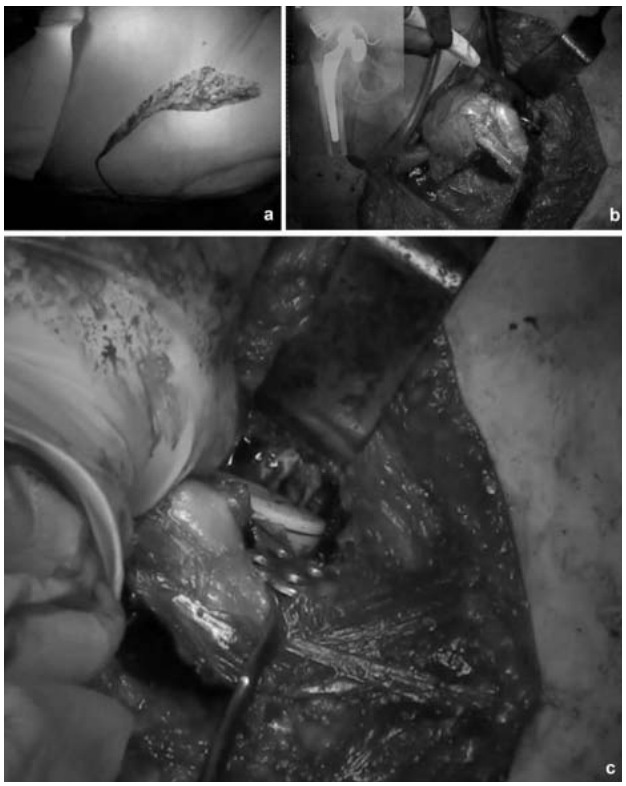

Fig. 1. ***a)****: Proximally extended DA approach in case of cup and ring intapelvi protrusion;* ***b)****: The approach was extended medially in between the iliac wing and the iliac muscle. This approach allowed a direct explant of the implant dislocating medially the iliac vascular bundle with a low risk procedure.*

affected hip in neutral rotation and extension. The anterior superior iliac spine (ASIS), umbilicus, greater trochanter and the patella are identified, taking care to include them in the operative field. The positioning of the patient and the surgical bed are placed so that the affected hip is fully accessible by the C-arm intensifier.

A straight 7-8 cm incision is carried out, starting 1 cm distal and 1 cm lateral with respect to the ASIS and prolonging toward the Gerdy's tubercle. The interval between *tensor fasciae latae* and *sartoriuos* is identified, the fascia is incised over the medial border of the *tensor fasciae latae* and the plane is bluntly developed in between the muscle fibers and the muscle fascia. During this phase, the hip could be lightly flexed and abducted to reduce muscle tension but should be held with a neutral rotation.

The lateral femoral cutaneous nerve is protected by the *tensor fascia latae* muscular fascia that is gently retracted medially with the *Sartorius*, while the *tensor fasciae latae* muscle (without its fascia) is retracted laterally. This approach is more lateral than the classic Heuter approach in order to avoid injuring the femoral cutaneous nerve and its branches, but care should always be taken for anatomical variations. The fascia of the *rectus femoris* muscle is identified and gently dissected. When present, the lateral circumflex bundle is carefully isolated and tied or cauterized. A Beckmann retractor is positioned to retract the Sartorius and direct head of the *rectus femoris* muscles medially and *tensor fascia latae* muscle, laterally.

Using a Cobb elevator, the fatty tissue over the capsule, the reflected head of the rectus femoris muscle and the iliocapsularis muscle are gently detached from the capsule and finally visualized. A Hohmann retractor is placed under the reflected head of the *rectus femoris*, the second Hohmann retractor is placed over the supero-lateral border of the acetabulum and a third Hohmann retractor is placed inferiorly, surrounding the posteromedial aspect of the femoral neck and retracting downward and laterally to the gluteus medius. Once the capsulotomy is completed, the hip dislocation can be performed safely and easily by applying a slight traction and external rotation and using a hook. To expose the acetabulum 2, Hohmann retractors are placed on the acetabular rim at 10 and 2 o'clock and 1 is placed on to the neck of the prosthesis at 7 o'clock.

When the acetabular bone loss is higher and ancillary procedures are required (such as for Paprosky 3A or 3B), in case of acetabular protrusio or when a pelvic discontinuity is present (Paprosky 4) we prefer to extend the described approach into a traditional Smith Petersen approach. In these cases the proximal insertion of the tensor fasciae late and of the rectus femoris can be released in order to have a complete and easy access to the acetabulum; sometimes for the protrusio we do extend the approach medially through the iliac muscle and the iliac wing in order to isolate the inner part of the cup with direct view of the iliac vessels. Occasionally, also in a very stiff hip the mini invasive DA Heuter approach is converted into a traditional Smith Petersen in order to provide an easier dislocation of the femoral component and an adequate exposure of the entire rim of the acetabulum retracting postero-superiorly the femoral component. After removal of the existing acetabular component and any residual scar tissue or bone cement, the acetabulum is carefully evaluated for pelvic discontinuity. If such discontinuity is

present, through the same approach a pelvic ring (such as the Burch-Schneider ring) is usually used.

RESULTS

At the last follow up, we did not record any failure. No septic or thromboembolic complications occurred. Early complications included prolonged wound healing in 4 cases and a transient palsy of the femoral cutaneous nerve in 2 cases. The Harris Hip Score revealed 82.5% of excellent and good results (mean preoperative score 39.9; mean postoperative score 82.2). Four patients were scored fair. Neither the preoperative nor the postoperative HHS had a significant correlation to the preoperative acetabular defect. In 21 cases the implants were related as absolutely stable and osseointegrated in the radiological evaluation. In two cases at the latest follow up a radiolucent line greater than 2 mm in 1 zone was visible but without signs of gross cup mobilization. No heterotopic ossifications were seen in all cases

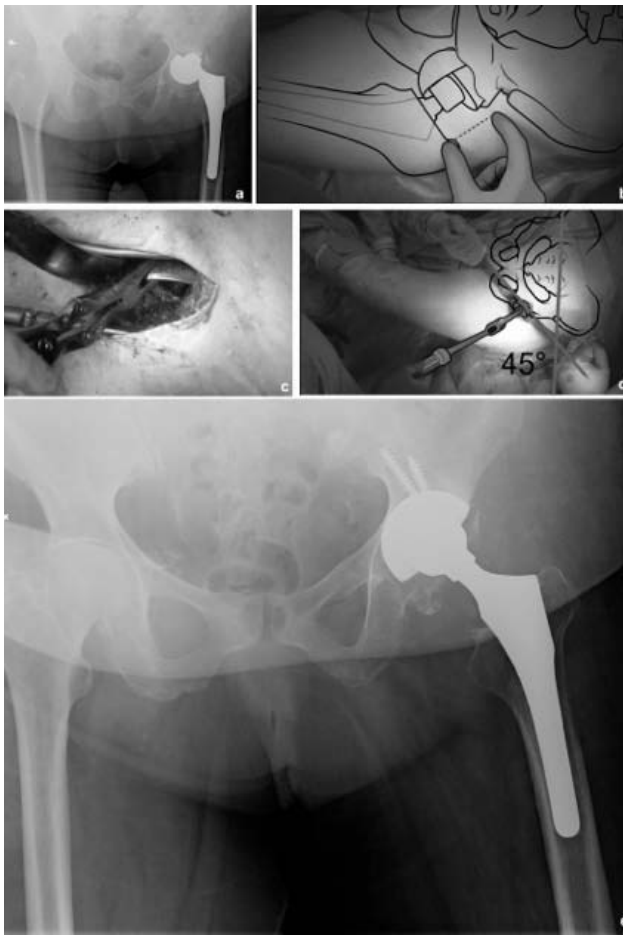

Fig. 2. ***a****): Preoperative radiograph of a isolate cup mobilization in a Paprosky 3A acetabular bone loss;* ***b****): The surgery was performed through a standard DA approach;* ***c****): Cup explant was easily performed even through a small incision;* ***d****): Good implant stability was achieved with a standard porous hemispheric cup and 2 cancellous bone screws. Good cup orientation is done with the same reference point of a direct lateral approach;* ***e****): postoperative radiographs showing the god implant osseointegration at 38-month follow up. The DA approach allowed an easy loosened cup explant and a good revision cup orientation.*

DISCUSSION

The concept of approaching the acetabulum through a DA is based on the belief that the surgeon could solve any possible problem related with a cup revision in an easy and effortless manner and a simple proximal extension of the approach could allow to solve any possible acetabular problem (4). In fact, the only concern related to the DA approach is the intraoperative need of managing the femoral component also (5). It has been shown that a distal extension of the DA approach in a "lazy" s shape allows to access the femur and perform all necessary reconstructive procedures on it without damaging the surrounding nerve structures(6).

Manrique et al. reporting on the DA approach stated that there are no absolute contraindications for performing revision THA through the DA approach, as this approach can virtually be used in every case and that to their knowledge, there is no published data in regards to complications using the DA for revision arthroplasty (7). However, on their review of the surgical technique they do not present any personal results. Ziran et al. (8) stated that in most cases the revision of the acetabular cup through a DA approach is not as challenging as the femur and they propose an iliac crest osteotomy to expose the femur in case a revision of the femoral component in necessary through this approach. However, in the case of revision of the femoral component, we did not use the osteotomy technique but extended the approach proximally, more distally and laterally. We believe that a good femoral exposure is possible just

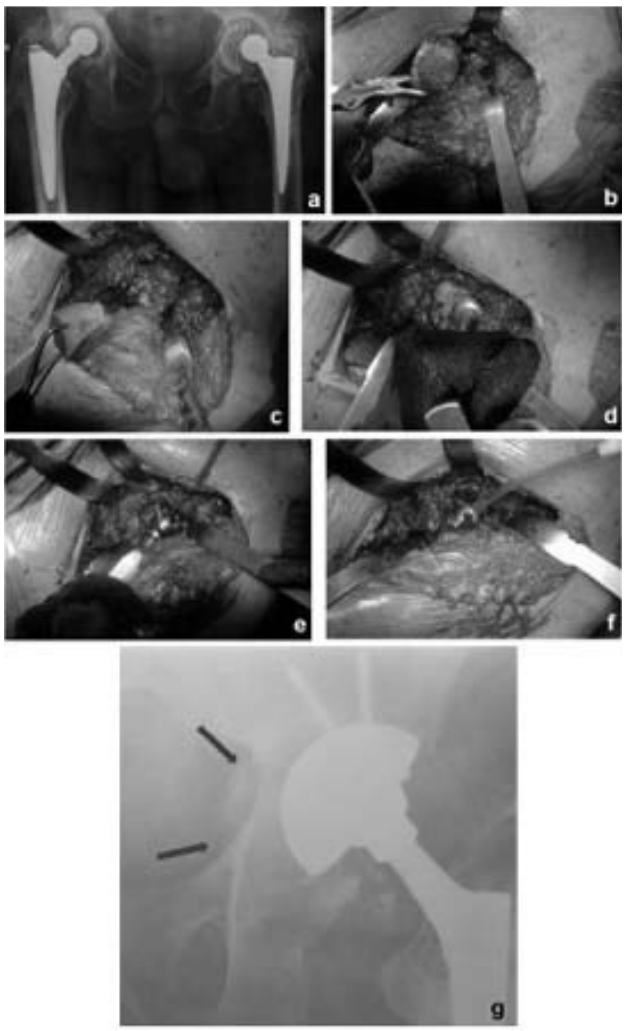

Fig. 3. ***a****): Preoperative radiograph of an isolate cup mobilization in a Paprosky grade 3B acetabular bone loss;* ***b****): The surgery was performed through a proximally extended DA approach;* ***c, d****): Medial bone stock deficiency was repaired with omolougus bulk bone graft;* ***e, f****): Good implant stability was achieved with a standard porous hemispheric cup and 2 cancellous bone screws;* ***g****): postoperative radiographs showing the god implant osseointegration at 28-month follow up. The proximally extended DA approach allowed an easy acetabular bone stock reconstruction and a good implant positioning.*

releasing the soft tissues while ancillary techniques such as osteotomies are not necessary (9). Spanyer at al. proposed that the DA approach extend proximally to treat all cases with anterior column deficiency (10). The Authors, as with our experience, use the same approach also in cases of protrusio acetabuli and Paprosky 3B cases. Releasing the *rectus femoris* from its proximal insertion onto the anterior inferior iliac spine (AIIS), they accessed the true pelvis and as with our experience, they removed the cup through direct approach. Their experience supports the versatility of the DA approach for acetabula cup revision and their proposed surgical technique is in line with ours. Cogan et al. (2), reviewing 61 cases of isolated cup revision through a DA approach, showed that the rate of dislocation for revision THA by DA approach was higher than when the approach was used for primary THA but similar to the dislocation rate reported in published series. In their experience, the Hueter anterior approach was a viable option for isolated cup revisions as long as the presence of femoral loosening has been excluded and the orientation of the intact femoral component is known. In contrast with their experience, we did not have any dislocation following revision but our cohort of patients is smaller and this better result could be also related to different populations. York et al. (11) suggested the DA approach for acetabular cup revision, as they do not consider this approach as safe as a direct lateral approach to manage the femoral side. Their approach is very similar to ours; we think that if a femoral revision is for any reason necessary, with some shrewdness surgery is also safe. Mast et al. (12), in a series of 51 patients operated for acetabular loosening through a DA approach, reported low rate of complications (9.8%) and good functional outcomes at an average follow-up of 54.5 months. To have good access to acetabulum in case of isolated cup revision the Authors suggested the removal of the femoral head and a pocket in the superolateral capsule. By flexing the hip 30° and externally rotating the femur 45°, the femur can be subluxated into the previously prepared pocket. This technique is safe and effective and we usually do the same. Finally, Kennon et al. (13) have reported their experience on 119 isolated cup revisions with DA approach on 3 cases of dislocation (2.5%), 2 infections, zero femoral cutaneous nerve problems and 3 intra-operative acetabular fractures. They suggest using the DA approach for revision surgery because of the low blood loss and low complication rate reported in their series and they believe that these very good results were due to the

muscle-sparing nature of this approach, which keeps soft-tissue trauma to a minimum and leads to faster postoperative mobilization and rehabilitation.

In all cases of our series, we preferred to use a hemispheric cementless cup with holes for additional cancellous screw fixation with a polyethylene liner in order to avoid possible ceramic fractures due to micro-instability (14-22). In case of acetabular bone loss Paprosky 2C-3A-3B, we used bulk bone graft to restore the bone stock through a previously described technique (23) (Fig. 3).

As the previously implanted femoral stems were all monoblock and did not allow over-correcting of the femoral leg length and offset as modular stems (24-26), we tried to fix the acetabular component in the natural center of rotation and in some cases we used a dual mobility acetabular component.

All the studies published on DA approach for acetabular revision supported our short-term results. In our series, we had very few complications and very good clinical and radiological results. The anterior approach requires very good comprehension of surgical anatomy of the hip and is probably better suited for skilled surgeons that have some experience with the very same approach for primary cases.

In conclusion, we consider the DA approach the natural approach to the acetabulum and a safe and effective approach for isolated cup revisions.

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RESTORING THE FEMORAL OFFSET PREVENT EARLY MIGRATION OF THE STEM IN TOTAL HIP ARTHROPLASTY: AN EBRA-FCA STUDY

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The use of modular stems is still debated and controversial. Some authors have highlighted a number of disadvantages of modular prostheses including high costs, the tendency to fracture, the fretting and corrosion and the increased production of debris. Other authors have emphasized several advantages to adapt the prosthesis to the morphometric differences of patients, to allow better accuracy in restoring the anatomy and biomechanics of hip joint. The advantages of the modular devices appear to be more evident in patients with developmental dysplasia of the hip (DDH). In our study we compared 96 patients, operated for arthritis of the hip with 55 modular neck prostheses (PROFEMUR®, Wright® Arlington, Tennesse, USA) and 41 standard femoral stems (SYMAX®, Striker® Kalamazoo, Michigan, USA). The precision of restoring the natural offset during surgery was correlated with the clinical outcome and the radiological early migration of each stem measured using the computer-assisted EBRA-FCA method. The average preoperative HHS (Harris Hip Score) was 44 (23-66); the postoperative 86.56 in the 55 patients operated with modular prostheses and 81.70 in the 41 patients with monoblock stem. The worst HH Scores were seen in patients in whom the offset was not restored properly. On the contrary, the best scores have been reached in patients in which that value is closer to the "target" value (offset value of the contralateral hip). Restoring the proper offset seems to determine an appropriate tension of the abductor muscles of the hip and implies a better functioning of the joint and a better primary stability of the implant, with less early migration. This has to be a primary objective of THA surgery.

The hip is one of the most replaced joints in the world. Total Hip arthroplasty (THA) is a worldwide recognized treatment in patients suffering from arthritic disease of the hip, reducing pain and improving function (1).

The first problem of a total hip arthroplasty (THA) is the longevity of the implant. Infection, polyethylene wear, aseptic loosening, design faults, material mismatch and poor surgical technique cause failures. Being able to control the orientation of the prosthetic components is of critical importance in normalising the biomechanics of the hip (2-4). It is important to achieve a stable joint with the ideal range of motion for patients, in order to fulfil their daily activities (5).

The primary stability and early migration of the prosthesis components can be measured on plain radiographs, with Roentgen stereophotogrammetry (RSA) or Ein-Bild-Roentgen-femoral component analysis (EBRA-FCA) (6, 7). RSA is the most precise option with an accuracy of 0.1-0.4 mm but unfortunately needs tantalum markers and special instruments (8). The accuracy of EBRA-FCA to detect implant migration has been reported to be ±1

Key words: total hip arthroplasty, femoral offset, primary stability, longevity of the implant, early migration, EBRA-FCA

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0393-974X (2016)

mm with a specificity of 100 % and sensitivity of 78 % to detect (9).

Restoration of the femoral offset (FO), limb lengths discrepancy (LLD) and rotation centres appear to be crucial to the patients function and to improve the long-term survival rates of THA (10-12). Apparently, restoring the normal anatomy of the hip not only avoids weakness of the abductor muscles (13) but reduces the risk of limping and early dislocation (14). A precise and careful preoperative planning with intra operative verification tests are essential for restoration of the femoral head centre and correct hip biomechanics (15).

The main aim of this study was to compare the accuracy in primary THA surgeries in restoring the normal FO using modular femoral stems compared to monoblock prosthesis. The second aim was to evaluate the influence of correct restoration of the FO on the early migration pattern measured with EBRA-FCA software.

MATERIALS AND METHODS

Study cohort

From 2011 to 2013, 124 THA in 148 patients were performed. Exclusion criteria was bilateral hip disease, previous contralateral hip surgeries, neck fractures, developmental hip dysplasia (DDH).

In total, 96 patients (mean age 68.7) were evaluated in our retrospective observational study. Thirty-one of 148 patients were excluded due to previous contralateral hip arthroplasty, 15 for femur neck fracture preoperative diagnosis and 8 for bilateral developmental hip dysplasia. The age of the patients ranged from 32 to 88 years. Sixty-one patients were female and 31 were male. According to the implants, patients were assigned to:

- Group A**:** 55 patients treated with modular neck prosthesis (PROFEMUR Z® Microport Inc);
- Group B**:** 41 patients received nonmodular neck implants (SYMAX Stryker®).

An accurate clinical examination and radiographic measurements were performed on pre and day 1 (T0), 6 months (T1), 12 months (T2), 2 years (T3) and 3years (T4). Preoperative Harris Hip Score (16) was calculated for each patient. The minimum follow-up was 24 months (mean 32; range 24-48). Maximum follow-up occurred in 52 cases.

Surgical procedure and implants

The same senior surgeon (B.M.) in our university hospital performed surgery. The direct lateral approach according to Hardinge (17) was performed with the patient in the lateral decubitus.

Standardized peri- and post-operative protocol was used in both groups, including short antibiotic therapy (Cefazoline 2g i.v. x 3), as tolerated and low molecular weight heparin LMWH for five weeks postoperatively, as well as prophylaxis for deep vein thrombosis. Two different type of cementless titanium implants were used with the following stem designs:

The PROFEMUR Z is an evolution of the Zweymüller stem. Made in titanium alloy, it has a rectangular cross-section with a particular rough surface. The neck is fabricated from titanium alloy and has two lengths (short and long), two options for neck-shaft angle (8 varus and 8 valgus), and two options for femoral-neck version (8 anteverted and 8 retroverted) (18).

The SYMAX-HA Femoral stem has a CCD angle of 128°. Each implant size (8 sizes) has a unique built-in neck anteversion (from 9.29° to 13.34°), offset (from 33.3 mm to 45.2 mm) and neck length, increasing progressively with each size (19).

Tables I, II, III, and give details of the different sizes and prosthetic components used. The research of a secure press-fit fixation, equal leg length and restoring the FO were the ultimate goals of the surgeon in each operation.

Clinical and radiological evaluation

The patient demographics, comorbidities and patient reported outcome measures were recorded at the pre-operative assessment clinic.

Clinical evaluation includes apparent or clinical limb-length discrepancy measurement, Harris Hip score. The evaluation was repeated postoperatively and at 6 months and 1-, 2- and 3-year follow-ups.

A standardized radiological protocol included an anteroposterior film of the pelvis and an anteroposterior and lateral film of the operated hip. The distance of the X-ray tube-film was 110 cm. All plain films were taken according to the method that Lindgren introduced (20). The femoral offset was measured as the distance from the centre of rotation of the femoral head to the long axis of the femoral shaft. Radiographs were taken postoperatively and at every clinical examination appointment at 6, 12, 24

Table I. *Profemur-Neck types used.*

	SHORT	LONG
8° Retroversion	14	9
15° Retroversion		5
STRAIGHT	6	4
VARUS	4	8
VALGUS		5
Total	24	31

Table II. *Profemur Z Stem used.*

Size	Female	Male
1	4	
2	3	1
3	13	3
4	8	2
5	4	8
6	1	6
7		2
Total	33	22

Table III. *Nonmodular neck implants used.*

Size	Female	Male
1	**5**	
2	**4**	**1**
3	**5**	**1**
4	**7**	**2**
5	**4**	**2**
6	**3**	**5**
7		**1**
8		**1**
Total	**28**	**13**

and 36 months. Three of us (GV, GS, AS) measured all radiographs for native and reconstructed femoral offset with the help of an informatics technician using MATLAB (The Math-Works Inc, Natick, MA) software. The cohort of study was divided in 3 subgroups according to the following FO restoration accuracy:

- SUBTYPE I: within 5mm
- SUBTYPE II: 5 mm<Δ<15mm
- SUBTYPE III: Δ>15 mm

Implant migration was determined using the EBRA-FCA software. One independent observer (senior ortho-radiologist) performed all measurements. Nineteen reference points were defined after the calibration of the images, with the diameter of the prosthetic head (Fig. 1):

- 7 on the femoral head
- 2 on the stem
- 8 on the femoral cortex
- One at the minor and major trochanter respectively.

The statistical analysis for comparison was performed using R Project Software version 3.1.0 for macOS. Student's *t*-test and *Chi*-squared test were performed.

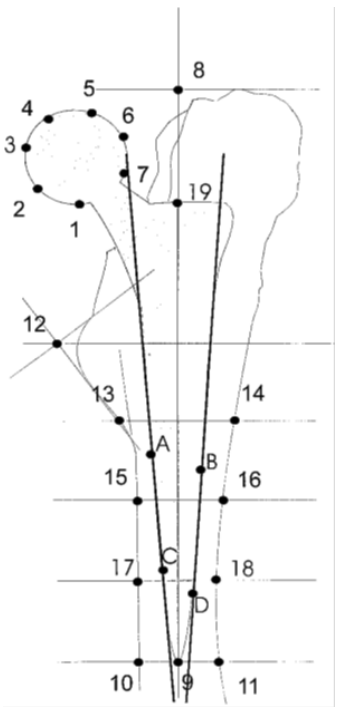

Fig. 1. *Scheme of the reference points of the EBRA-FCA software.*

RESULTS

Demographic data and preoperative radiological differences relative to FO, LLD with regard to the contralateral healthy hip were comparable between groups A and B. In the modular neck cohort, a greater proportion of patients had equal (within 5mm) or more accurate restoration of the femoral offset (81.82%), compared to the non-modular cohort (36.58%) (Table IV).

As described by other authors (21), we found a greater range of external rotation in patients with greater femoral offset. Three-hundred-and-fifty-six digitized radiographs were analysed by one independent observer. The software automatically rejected 3.6% of the films.

Using EBRA-FCA software, the mean distal migration was:

- Group A -0.23mm (±0.3 mm) at 6 months, -0.41mm (±0.3 mm) at 12 months, -0.56mm (±0.3 mm) at 24 months and -0.71mm (±0.5 mm) at 36 months;
- Group B -0.27mm (±0.3 mm) at 6 months, -0.47mm (±0.3 mm) at 12 months, -0.68mm (±0.3 mm) at 24 months and -0.82mm (±0.5 mm) at 36 months;

The higher migration rates were identified in the patients with less accuracy in FO restoration (Table V). In subtype I of both Group A and B, no differences were identified.

Table IV. *FO restoration accuracy.*

				Total
Subtype	**I**	**II**	**III**	
Group A	45(81.82%)	9(16.37%)	1(1.81%)	55
Group B	15(36.58%)	19(46.34%)	7(17.08%)	41

DISCUSSION

The main aim of this study was to compare the accuracy in primary THA surgeries in restoring the normal FO using modular femoral stems compared to to monoblock prosthesis.

As described in the Table IV, in group A, a greater proportion of patients had a more accurate restoration (within 5 mm) of the femoral offset (81.82%) compared to the nonmodular cohort (36.58%). A similar result was described by Duwelius et al. (22). In their study, the authors highlight the accuracy of the modular neck in restoring the head centre and the femoral offset, although it did not translate into better short-term (1-2 years) outcome scores, revisions or complications. They conclude that in their reported higher risks, there is no clear indication for modularity with a primary THA, unless the hip centre cannot be achieved with a nonmodular stem, which is rare.

The superiority of modular prosthesis in outcome scores, hip biomechanics and complications, compared to conventional implants, have not been

Table V. *Correlation FO restoration - early migration using EBRA-FCA.*

	T1	**T2**	**T3**	**T4**
GRUPPO A	-0.23mm (±0.28mm)	-0.41mm (±0.32mm)	-0.56mm (±0.3mm)	-0.71mm (±0.5mm
Subtype I	**-0.18mm (±0.23mm)**	**-0.27 (±0.21mm)**	**-0.40 mm (± 0.37mm)**	**-0.58mm (±0.5mm**
Subtype II	-0.26mm (±0.31mm)	-0.33mm (±0.35mm)	-0.48mm (±0.19mm)	-0.77mm (± 0.5 m
Subtype III	-0.29mm	-0-38mm	-0.56mm	-0.83mm (± 0.5 m
GRUPPO B	-0.27mm (±0.3mm)	-0.47 (±0.37mm)	-0.68 mm (±0.39mm)	-0.82mm (±0.5mm
Subtype I	**-0.19mm (±0.22mm)**	**-0.27 (±0.20mm)**	**-0.40 mm (± 0.34mm)**	**-0.63mm (±0.5mm**
Subtype II	-0.26mm (±0.31mm)	-0.43mm (±0.35mm)	-0.48mm (±0.19mm)	-0.78mm (± 0.5 m
Subtype III	-0.29mm (±0.28mm)	-0-49mm (±0.31mm)	-0.71mm (± 0.36mm)	-0.83mm (± 0.5 m

shown. Gerhardt et al. (23) observed no significant differences in restoration in arm, leg length and cupangle in two groups of patients treated with modular and nonmodular stems. A borderline significant difference was revealed in abductor moment arm restoration. They registered the same number of dislocations within one year and observed an unclear benefit in the restoration of hip geometry and dislocation rate after THA with modular necks.

In our case series, we registered an easier restoration of the femoral offset with the use of the modular neck compared to the nonmodular neck, but no significant differences could be established.

The ability to adjust version and offset is useful in the prevention of impingement between the socket and neck and bony and muscular impingement (24). In a study of Monobloc stems, femoral offset and limb length were not restored in 36% of patients (28 of 79) (25).

We hypothesize that nonmodular neck implants permit the modification of the femoral offset with less options compared to the modular neck implants. The offset can be modified only in relation to the different stem size, although the modular neck implants allow more options and adjustments for restoration of the anatomy of the hip.

Many studies evaluate the early migration of the implant which is supposed to be the best indicator for mechanical failure of femoral stems (9, 19, 26-28). As shown in Freitag et al., axial migration of more than 1.5 mm within 2 years after implantation is associated with a higher risk of implant failure (29). They conclude that there are no statistical differences of early micromotions according to gender, BMI and femoral offset value.

Our retrospective evaluation is the first in literature that correlates the accuracy of femoral offset restoration to the possible early distal migration of the femoral stem. Although there was no statistical significance, we recognized different trends: the subtype I of our cohort of study (more accurate FO restoration within 5 mm) present a lesser early distal migration than the subtype III. Restoring the proper offset appears to determinate an appropriate tension of the abductor muscles of the hip, which implies a better functioning of the joint and a better primary stability of the implant with less early migration; this has to be a primary objective of THA surgery.

The present study has several limitations. One limitation is the rather short retrospective observation period of 3 years. Moreover, the two different implants evaluated present different designs and technologies. The limited number of cases did not allow us to evaluate and analyse other different factors that could influence the early migration of the femoral stem, such as Bone Mass Density, Bone Mass Index and Vitamin D blood level.

New clinical retrospective investigation using EBRA-FCA and prospectively using RSA need to be performed in order to clarify the possible correlation between FO restoration accuracy and primary stability of the implants in primary THA surgeries.

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THE ADULT ZEBRAFISH AS POLYHEDRIC MODEL FOR SKELETAL STUDIES

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In the last decade, several examples have been produced by scientific literature about zebrafish as a model to study human bone diseases. In fish, bone turnover, reparation and remodeling of the adult bone tissue cannot be studied in embryonic or juvenile stages. In addition, fins and scales represent unique anatomical features useful to study adult bone metabolism and diseases. For these reasons, the adult zebrafish represents an innovative and readily available resource for studying the bone metabolism at cellular and molecular level. Although the adult fish is less used than the embryo, several applications have been found in the last years with the production of innovative pathological models in adult zebrafish, helpful to understand the mechanisms of bone physiopathology. The use of mutants, regenerating organs, transgenic fish and scales have increased the power of this model in the last years.

The bone tissue from Danio rerio to Homo sapiens

Danio rerio (zebrafish) is an elective model organism for the study of vertebrate development. This is due to the unique characteristics of the embryo such as large clutches (up to 250 embryos/ week), small size, rapid external development and transparency of the larval body. Such advantages encourage the use of live imaging and powerful genetic tools based on mutagenesis. Moreover, automated systems have been coupled with zebrafish embryo to create one of the most important *in vivo* methods for drug screening, drug discovery and toxicity testing. The combination of these characteristics makes zebrafish an excellent animal model for basic biomedical research, drug development and translational medicine studies (1). Bone is a heterogeneous tissue composed by a mineral phase, hydroxyapatite, organic phase (type I collagen, other structural proteins, lipids) and water. Bone tissue is not a simply protective and static scaffold for the body but a dynamic organ that is constantly remodeled. Moreover, bone stores crucial nutrients, proteins, minerals, and lipids. In addition, in recent years has emerged the endocrine role of the skeletal tissue because of its implication in the hormonal network, energy metabolism and the physiological regulation of the several organs such as kidney, bone marrow, muscles etc. (2).

All these functions are common in the vertebrates, from human to fish. The similarity of the skeletal structure between *Danio rerio* and *Homo sapiens* has hired zebrafish as animal model to study different aspects of skeletal physiology: osteogenesis, bone metabolism, tissue turnover, resorbing activity etc. (3). In the last decade, several examples have been produced by the scientific literature about the introduction of zebrafish as model to study human bone diseases. Different and useful approaches can be used in zebrafish field, from embryo to adult.

Key words: zebrafish, animal model, bone, cartilage, regeneration

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0393-974X (2016)

The adult zebrafish as model for skeletal studies

In fish, some characteristics such as bone turnover, reparation and remodeling of the adult bone tissue cannot be found in embryonic or juvenile stages. In addition, fins and scales represent unique anatomical features with undoubted advantages like transparency and the presence of mineralized tissue similar to human lamellar bone. The same dyes used for live embryo bone tissue staining can be used in adult fish, on both live fish and fixed sample to highlight scale mineral matrix (3) or caudal fin rays (4). For these reasons, the adult zebrafish represents an innovative and readily available resource for studying the bone metabolism at cellular and molecular level. Although the adult fish is less used than the embryo, several applications have been found in the last years with the production of innovative pathological models in adult zebrafish, helpful to understand the mechanisms of bone physiopathology. The use of mutants, regenerating organs, transgenic fish and scales have increased the power of this model in the last years.

Adult zebrafish mutants and human bone diseases

The identification of adult zebrafish mutants that recapitulate human mineralized craniofacial, dental and skeletal system disorders can be used as models to study the pathogenesis of the human bone diseases. Recently, a large-scale forward-genetic chemical mutagenesis screen led to the identification of 7 adult homozygous recessive mutants with skeletal disorders including craniofacial defects, suture fusion, morphological or numerical alteration of skeletal elements, scoliosis (5).

Years ago, zebrafish mutant *chihuahua* was isolated in a forward-genetics screen of adult fish searching for skeletal abnormality using X-ray. Heterozygous fish was characterized by defective bone growth, altered vertebral shape, reduced mineralization and frequent rib fractures. The same clinical evidences can be found in human patients affected by heterozygous Osteogenesis Imperfecta (OI). It has been proved that *chihuahua* mutant carries the heterozygous missense mutation G390D in *col1a1a* gene. The Glycine residue changed in the mutated fish is located in the conserved Gly-X-Y motif, the same residue mutated in *col1a1a* gene in human OI patients (6). Thus, the adult zebrafish mutant *chihuahua* can be considered as model of human OI useful to elaborate new therapeutic strategies.

Moreover, the recessive non-lethal zebrafish *leviathan/col8a1a* mutant (see embryo section) lacks collagen type VIII alpha1a (COL8a1a) and shows defects in assembly of the embryonic notochord. Such alteration produces vertebral column malformations at the adult stage which resemble to human scoliosis (7).

An adult zebrafish mutant can be also generated by large scale mutagenesis programs such as Zebrafish Mutation Project (ZMP) of the Sanger Institute (8). One example is Bruck syndrome (BS), a human bone disease similar to OI and characterized by joint flexion contractures and skeletal defects. A double mutation in FKBP10 and PLOD2 genes is the primary genetic defect associated to the disease. PLOD2 encodes the lysyl-hydroxylase 2 (LH2) which is responsible for the cross-linking and the organization of collagen fibrils in the bone matrix. A zebrafish mutant *plod2*$^{sa1768/sa1768}$ (TL) carrying homozygous plod2 nonsense mutation has been recently generated resulting in reduced stability of bone type I collagen (9). *plod2* mutants show at the adult stage severe skeletal abnormalities associated with bone fragility and frequent fractures. In conclusion, plod2 mutant zebrafish show molecular and clinical characteristics of the human BS patient

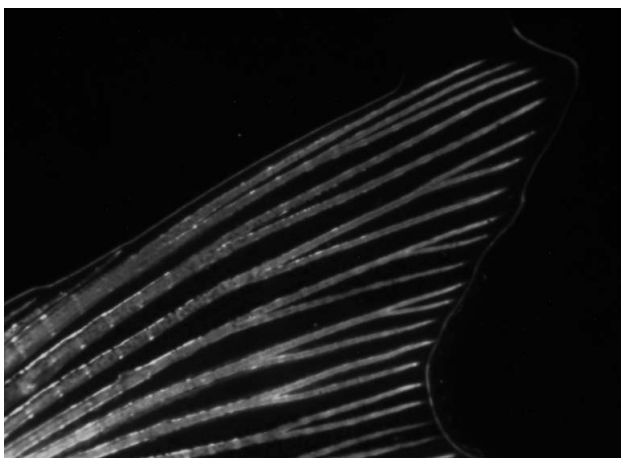

Fig. 1. *Calcein staining of zebrafish caudal fin.*

therefore it may be considered a promising model to study the pathogenetic mechanisms leading to BS and new therapeutical approach against the disease.

Several zebrafish mutants display skeletal defects starting from the end of the juvenile stage and show characteristics which, in humans, are evident in the adult bone only. These examples underline the importance to produce adult zebrafish mutants able to elucidate the mechanisms of the pathological alterations in the adult bone physiology.

Different resources for regenerative medicine: fin, skull and jaw

Regenerative medicine represents the more powerful approach to cure organ dysfunction, injury and trauma. The goal is to reconstitute organ structure and function after injury stimulating or re-establishing organ-specific repair processes. The de-differentiation process is unique to animals like zebrafish and drives the perfect restore of a lost or damaged tissue. The knowledge of the regeneration mechanisms could support the development of new instructive therapies directed toward human cells. Different anatomic parts of the zebrafish body can be informative for bone regeneration studies.

Caudal fin

Since Zebrafish caudal fin possesses unique characteristics of accessibility and transparency, it has been introduced to investigate the reparative capacity of adult bone tissue (figure 1). Indeed, zebrafish fin ray, an example of intramembranous ossification, is able to regenerate together with the whole fin structure (10). The transparency of the caudal fin allows the use of live staining like calcein and alizarin red (11) to mark mineralized tissue in the regeneration process.

The fin amputation model has been recently used to elucidate the behavior of osteoblasts during fin ray regeneration. After injury, they de-differentiate to form the "blastema", proliferate and re-differentiate to produce new bone (12).

Although there are important variations in timing and anatomical organization between species, the main molecular regulators of the regenerative process are well conserved.

Skull

Skull injury represents an alternative model to fin ray amputation. Frontal and parietal bones of the zebrafish cranial vault could be injured following the protocols already used for calvarial rodents (13). In particular, a circular piece of bone is explanted by using a microdrill generating a hole of 0.5 mm diameter. Interestingly, the drill injuries are almost completely filled in 7 days post injury (dpi) whereas in 14 dpi the regeneration process is complete. In conclusion, the skull model has contributed to demonstrate that mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull.

Jawbone

In mammalian bone fractures, the periosteum also appears to contribute first to cartilage and then to bone during repair (14). The regeneration of zebrafish lower jawbone involves the formation of a cartilage intermediate, before to mineralize the final bone tissue. The studies have found that, upon injury, the resident stem cells are instructed by the expression of *indian hedgehog a* (*ihha*) to make cartilage instead of bone (15). A similar behavior is present in mice during the large-scale bone repair. The jawbone injury model could be helpful to understand the mechanisms and the role of cartilage deposition in human bone fractures repair.

Fin ray fracture

A caudal fin ray crush injury has been used to

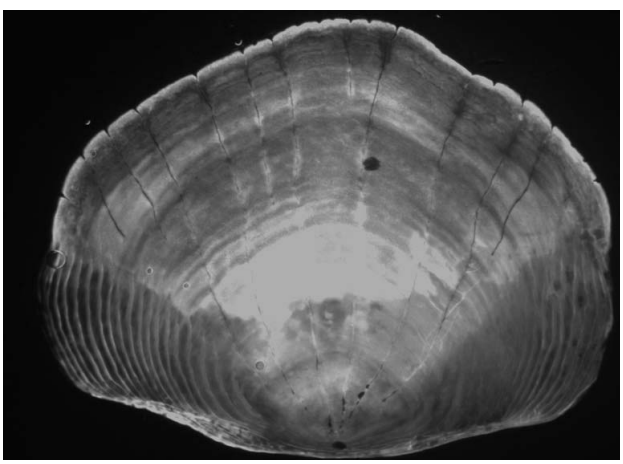

Fig. 2. *Calcein staining of an adult zebrafish scale.*

develop a model of vertebrate long-bone fracture (16). The regeneration program, typical of the extensive fin injury, should not be activated for a single ray fracture but rather a tissue repair process, much more similar to a wound healing. This model will help to elucidate the mechanisms involved in zebrafish adult bone repair because the transparency of the adult caudal fin encourages the use of imaging techniques to easy visualize bone matrix, bone cells and specific molecules. The identification of the key processes that regulate bone fracture in zebrafish fin ray may contribute to improve the bone repair medical research in humans.

Whole skeleton analysis

Several staining and radiographic techniques can be used to visualize the whole skeleton in the adult stage. Chemical, pharmacological or mechanical treatments may have effects on bone remodeling and integrity. The gravity, for example, is a mechanical force having severe effects on skeletal structures. Progressive bone-loss is a serious side-effect of the long-term permanence in absence of gravity. Clinical studies on astronauts revealed that an average of 1 to 2 percent of bone mass can be lost each month (). Several data have been produced examining the effects of simulated microgravity on different animal models. In particular, the skeleton of adult fish exposed to simulated microgravity resulted in growth alteration, reduced ossification and distortion of some skeletal elements (17). The whole skeleton analysis may provide a precise examination of single bones of the fish body.

Transgenesis in adult fish

Genetic techniques such as conditional transgenesis can be used to produce specific skeletal defects in adult fish. Recently, an osteoporotic transgenic fish has been made using GFP under control of the *cathepsin K* promoter in osteoclasts and *Rankl* together with CFP under control of a heat-shock promoter (18). After increase of temperature and *Rankl* induction, the in vivo formation and activation of osteoclasts has been reported with time-lapse fluorescent imaging. This is an example of how the genetic plasticity of zebrafish can be used to study in the adult fish the pathogenesis of human bone disease.

A non-invasive read-out system: the scales

Adult zebrafish scale has been recently introduced as read-out system for bone metabolism studies (19). The transparency and the size of the scale facilitates manipulation, treatment and observation of the results under a basic stereomicroscope (figure 2). In addition, several parameters such as biochemical and cellular markers, bone matrix architecture, bone deposition, bone resorption and behavior of bone cells (scleroblasts and osteoclasts) can be easily analyzed in the scale (20). These advantages make the scale an elective system to study mineralization and remodeling mechanisms in the pathological models.

Recently, secondary osteoporosis has been reproduced in adult zebrafish after treatment with prednisolone (21), a glucocorticoid known to have the same effects in humans. The osteoclast-dependent resorbing activity has been observed in explanted scales from treated fish using the specific staining for mineralized matrix and histological staining to highlight osteoclast activity. The treatment with alendronate, an anti-osteoporotic drug, has surprisingly resulted in a significant protective effects in prednisolone-treated fish (22). These data confirm the power of the scale as "read-out" system to study human bone metabolism and diseases.

Transparent adult fish and imaging resource

The fish mutant named *casper* has been generated from the breeding of two different mutants defective for pigment synthesis. The *nacre* mutant grows without melanocytes whereas the spontaneous mutant *roy orbison (roy)* shows translucent skin and absence of iridophores. *Casper* exhibits a ghost-like phenotype thus it appears entirely transparent at the adult stage (23). A live staining for mineralized tissue in *casper* can be used to visualize spontaneous or induced skeletal malformation in adult fish during screening experiments.

Several imaging resources may be used to facilitate the investigations on the adult zebrafish models of bone disease contributing for the knowledge of the pathogenic mechanisms of human counterpart. A lot

of imaging resources can be adapted for small animal models like rodents or fish.

Recently, Time-Gated Optical Projection Tomography (TGOPT) has been used in adult zebrafish to visualize in 3D the anatomical body structure without chemical contrast (24).

In addition, whole adult zebrafish can be scanned by Micro Computed Tomography (μCT), permitting the analysis of the skeleton and the determination of body mass index (BMI) for each bone scan section. Total-body μCT scans can be applied in pharmacological studies where the 3D reconstruction of the zebrafish bone system is used to detect alterations in bone structures after particular treatments like dietary supplementation (25). X-ray radiography have been already introduced in adult zebrafish mutant screening to detect abnormalities in skeletal morphology (4).

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TENDINOPATHIC SUPRASPINATUS TENOCYTES MAY HAVE A NEUROENDOCRINE-LIKE FUNCTION, SECRETING CGRP, SP AND VEGF: A PILOT IMMUNOHISTOCHEMISTRY STUDY

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We wanted to observe and compare the appearance of neurovascular tissue from tendon *ex vivo*, in patients with and without painful rotator cuff tendinopathy. Supraspinatus tendons were biopsied from 5 participants with painful tendinopathy and normal tendon from a young male. Slides were stained with haematoxylin & eosin and toluidine blue for histological assessment. Immunohistochemical markers for general nerves (protein gene-product 9.5 and synaptophysin), sensory nerves (calcitonin gene-related peptide; substance-P) and vascularisation (vascular endothelial growth factor) were used. PGP9.5 and CGRP-immunoreactive fibres were associated with vessels in cases and control. Synaptophysin-labelled fibres were observed in close relation to vessels in tendinopathy. PGP9.5, CGRP, SP and VEGF-immunoreaction also labelled tenocyte-like cells in degenerative areas and fibres in regions of fat and collagen. Sensory innervation and vascularity are increased in tendinopathy. The evidence for innervation and vascularity of symptomatic rotator cuff tendon may aid the development of novel investigations and therapies in the management of patients with this ailment.

Rotator cuff tendinopathy is a common cause of pain and disability (1). Its pathogenesis of rotator cuff tendinopathy is unclear and the response to intervention is frequently slow and often incomplete (2), the result of a combination of extrinsic or intrinsic factors.

Symptomatic and pain-free Achilles tendons, patellar tendons and the common extensor tendons of the wrist are supplied by general and sensory innervation, intimately associated with blood vessels (3). Using immunohistochemical techniques (3), general nerves are revealed by staining tissue with the neuronal marker, protein gene-product 9.5 (PGP9.5), and sensory fibres labelled with neuropeptides calcitonin gene-related peptide (CGRP) and substance-P (SP). Pathological tendons demonstrated an increased immunoreactivity for sensory neuropeptides which can cause pain (3). The innervation patterns in the supraspinatus tendon are unclear.

Additionally, neovascularisation has been associated with Achilles tendinopathy (4). Vascularised tendons may maintain an inflammatory state by supplying cytokines to the injured area (5). Angiogenesis is also associated with proliferation of sympathetic nerve fibres, promoting neoinnervation (6). The role of neovascularisation remains unclear. Some studies (7) reported hypovascularity in torn tendons, while others evidenced florid neovascularity (8). At ultrasound, Lewis et al (9) demonstrated a greater degree of neovascularity in rotator cuff tendinopathy compared with asymptomatic tendons.

Key words: tendon, shoulder, rotator cuff, supraspinatus, immunostaining

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0393-974X (2016)

Lakemeier et al (10) found an upregulation of vascular endothelial growth factor (VEGF) in torn supraspinatus tendon using immunohistochemistry. However, the findings were limited due to a lack of controls. Expression of VEGF has been described in the painful subacromial bursa (11), but there is limited evidence relating neovascularisation to painful rotator cuff tendinopathy. A comprehensive understanding of the vascularity, innervation and biochemicals involved in pain could be used to guide effective treatment modalities in patients with painful rotator cuff tendinopathy.

This preliminary study compared the histological features of normal and symptomatic supraspinatus tendons, focusing on fibre arrangement and vascularity. It also explored the angiogenic features and innervation patterns of suprasinatus tendons using immunohistochemical markers for angiogenesis (VEGF), general (PGP9.5 and synaptophysin) and sensory innervation (CGRP and SP).

MATERIALS AND METHODS

Participants

Five patients were included in the study and divided into case or control groups (subjects described in Table I). Patients underwent arthroscopy by the same orthopaedic surgeon. Participants were provided with an information booklet and signed informed consent documents prior to participation. Ethics approval was obtained by the NHS Research Ethics Committees. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Full written informed consent was gained from patients involved in this study.

Selection criteria

Skeletally mature adults aged between 18 and 70 were recruited if they fulfilled the following: 1): shoulder pain for at least three months; 2): scheduled for shoulder arthroscopy; 3): positive lag signs on examination; 4): MRI or 5): ultrasound evidence of a supraspinatus tendon lesion. Participants were excluded if they had: 1): a diagnosis of autoimmune disease (such as rheumatoid arthritis) or diabetes; 2): previous surgery on the affected shoulder; 3): corticosteroid injection in the affected shoulder less than 6 months before surgery; 4): history of smoking; 5): taking medication for chronic conditions; 6) reproduction of shoulder pain with cervical spine region assessment; 7): restricted passive shoulder external rotation and elevation range of movement; 8): pain extending beyond the elbow, and 9): abnormal limb power, reflexes or sensations. Control patients were subject to similar selection criteria, except that patients had: 1): no shoulder pain and 2): were not undergoing rotator cuff surgery.

Table I. *Participant demographics and associated rotator cuff symptoms.*

Participant	Gender	Age (years)	Diagnosis	Pain duration (months)	Pain severity (/10)	Previous therapy
1	M	20	Instability	n/a	n/a	n/a
2	M	53	Partial Tear/ Impingement	18	8	Exercise
3	F	45	Partial Tear/ Impingement	36	5	Exercise Cortisone
4	M	39	Partial Tear/ Impingement	18	7	Exercise
5	F	42	Partial Tear/ Impingement	12	5	Exercise Cortisone

M, male; F, female; n/a not applicable

Tendinopathic tendons

Four patients (2 males, mean age 46 years; 2 females, mean age 43.5 years) experienced painful rotator cuff tendinopathy for an average of 21 months prior to surgery, with clinically diagnosed supraspinatus tendinopathy (Table I). Prior to surgery, participants reported a mean shoulder pain score of 6.3 (based on a VAS (pain) where 0 represented no pain and 10 the worst imaginable pain). All participants experienced night pain. Prior to surgery, all had failed exercise therapy and 2 patients received local corticosteroid injection more than 6 months prior to surgery.

Non-tendinopathic tendon

A control supraspinatus tendon was biopsied from a male with 5-month history of atraumatic shoulder instability who underwent arthroscopic re-stabilisation.

Sampling & Preparation

During arthroscopy, a partial thickness biopsy of supraspinatus tendon was taken measuring 4x4 mm from within 1 cm of insertion into the humeral head (control) or from a 1 cm margin of the tear edge (cases).

Samples were immediately fixed in 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) for 48 h and then immersed in 20% sucrose in the same buffer for 48 h. Once flash-frozen in OCT Embedding Medium (CellPath, UK) and frozen at -80°C, sections were cut at 10 µm using a cryostat (Leica CM1900) and thaw-mounted onto Superfrost-Plus slides (VWR International, UK).

Histology

Slides were stained with haematoxylin & eosin (H&E) and toluidine blue (TB) following established protocols. The most pathological areas were assessed under a Nikon Eclipse 80i light microscope (Japan; with a CX9000 camera, MBF Bioscience, Vermont) for fibre structure and arrangement, tenocyte rounding, cellularity and vascularity.

Indirect-immunohistochemical staining

Slides were immersed in 10% donkey serum (Chemicon, Billerica, MA) for 60 minutes to block non-specific binding sites. Sections for immunoperoxidase staining were pre-treated with 1% hydrogen peroxide for 30 min before blocking to quench endogenous peroxidase activity. Incubation in these procedures was conducted in a humidified box at room temperature.

Immunofluorescence technique

Sections were incubated in primary mouse and rabbit

Table II. *Characteristics and preferred staining methods for all antibodies.*

Antigen	Host	Dilution	Source
Primary Antibodies			
CGRP	Rabbit	1:2000	Biomol, Switzerland
PGP9.5	Rabbit	1:4000	AbD Serotec, Oxford, UK
Substance-P	Rabbit	1:1000	Chemicon (Millipore), Billerica, MA
Synaptophysin	Mouse	1:200	DAKO, Denmark
VEGF	Mouse	1:200	Chemicon (Millipore), Billerica, MA
Secondary Antibodies–Biotin-Conjugated			
Anti-rabbit	Donkey	1:400	Jackson ImmunoResearch, West Grove, PA
Anti-mouse	Donkey	1:800	Jackson ImmunoResearch, West Grove, PA
Seconadary Antibodies–TRITC			
Anti-rabbit	Donkey	1:400	Jackson ImmunoResearch, West Grove, PA
Anti-mouse	Donkey	1:400	Jackson ImmunoResearch, West Grove, PA

antibodies overnight (at dilutions described in Table II). After washing in PBS (3 lots of 10 min washes), slides were incubated with tetramethylrhodamine-isothiocyanate (TRITC)-linked secondary antibodies (appropriate to species of primary) for 4 h before being washed and coverslipped using PBS-glycerol (1:8 mixture). Sections were examined with fluorescence under a Leica Leitz DMRB photomicroscope (Wetzlar, Germany) and a Hamamatsu C4742-95 camera (Japan).

Immunoperoxidase technique

Immunoperoxidase staining was employed because of its high sensitivity for localising antigens and for the stability of the reaction product. Sections were pre-treated, blocked and incubated in primary antibodies as described above. These were then treated for 2 h with appropriate biotin-conjugated secondary antibodies (mouse or rabbit, as per Table II). An avidin/biotinylated-enzyme reagent (VECTASTAIN® Elite ABC-Peroxidase kit, Vector Laboratories, CA), diluted 1:2 with PBS, was applied to sections for 1 h before developing in a fresh DAB solution (3.3'-diaminobenzidine; EnVision™ Peroxidase, DAKO, Denmark) for up to 5 min and then washed. Finally, slides were dehydrated through graded alcohols, cleared in xylene and coverslipped with DPX medium. Examination was performed using the same microscope under white light.

Negative controls of each tendon were also performed to demonstrate background staining. This was done following similar methods but without incubating sections in primary antibodies.

Following initial assessment, the identifier on each slide was covered with a sticker and re-assigned a random number. Sections were re-assessed by the same investigator. Discrepancies between the findings were resolved by a senior histopathologist and a final outcome was decided.

RESULTS

Normal tendon

Collagen fibres were organised in tightly cohesive, parallel bundles. Spindle-shaped tenocytes were seen to course inconspicuously between fibres, which displayed some crimping (Fig. 1a). Large, well-demarcated vessels were found in clusters (Fig. 1b). Infiltration of tissue was observed at the tendon-bursa junction.

Nerve bundles exhibiting PGP9.5-immunoreactivity were seen both in adventitia and immediate regions around blood vessels (Fig. 1c). To a lesser extent, fibres were also observed in the presence of adipocytes. CGRP-immunoreactive nerve fibres were only seen around vessels (Fig. 1d). VEGF labelling was evident in tenocytes surrounding vessels compared to avascular regions.

Tendinopathic tendons

All tendinopathic tendons exhibited collagen fibre fragmentation and disruption. A loss of the normal parallel architecture, fibre separation and oedematous expansion of collagen bundles occurred in areas of greatest degeneration (Fig. 2a). Tenocytes in hypercellular regions were more numerous and took on a more sinuous, larger and rounder appearance. Abnormal tenocytes in specimens 2-4 resembled chondrocytes, with substantial rounding and swelling (Fig. 2b).

Tendinopathic specimens expressed similar or fewer (specimens 3 and 5) blood vessels compared to healthy tendon. However, their presence alongside fatty infiltration appeared to interrupt local collagen arrangement, disrupting tendon architecture. Some vessels were considerably smaller in diameter, less demarcated and surrounded by a thinner intima (Fig. 2c). Immunoreaction was more delineated after immunoperoxidase staining than with TRITC. Therefore, most the following results were obtained following immunoperoxidase treatment. Specimen 4 displayed the greatest immunoreactivity. No reactions were observed in negative controls.

PGP9.5-immunoreactivity was seen in most tendons, presenting as lighter, varicose nerve fibres and bundles which were not only associated with small blood vessels but also with fat (Fig. 3a) and collagenous tissue (Fig. 3b).

Synaptophysin-labelled nerves (Fig. 3c), including abnormal fibre bundles (Fig. 3d) were detected near some vessels and fat. Some SP-labelling occurred in fibres near vessels, fat and between collagen layers (Fig. 3e). Thinner nerve fibres and bundles expressing CGRP-immunoreactivity were also detected near collagen layers (Fig. 3f). Additionally, SP, CGRP and VEGF-immunoreaction was present

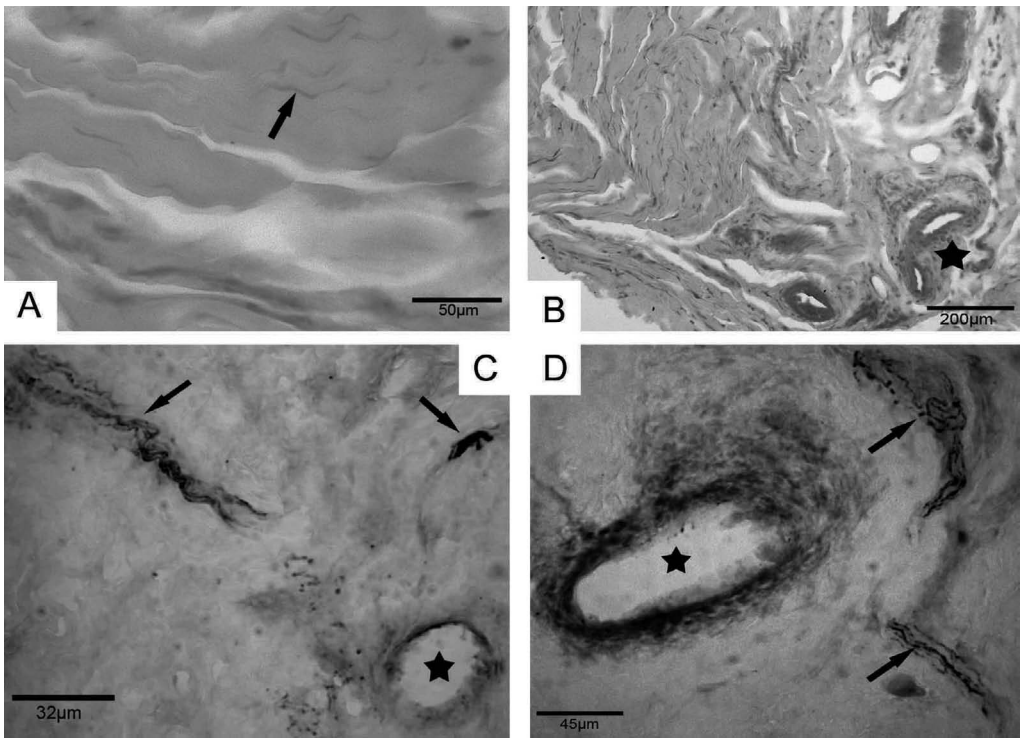

Fig. 1. *Photomicrographs of supraspinatus tendon taken from a 20-year-old male without tendinopathy. Haematoxylin and eosin stained sections showing collagen fibres are arranged in closely packed, parallel bundles interspersed with the flattened, darker nuclei of tenocytes (**Fig. 1a** arrow: x40 magnification). Bundles of well-pronounced arterioles and surrounding interstitium were found in regions near the tendon-bursa junction (**Fig. 1b** star: x10 magnification). Sections stained using an immunoperoxidase technique demonstrate both CGRP (**Fig. 1c**: x62.5 magnification) and PGP9.5 (**Fig. 1d**: x45 magnification) immunoreactive nerve fibres occurring in association with blood vessels (star in vessel lumen).*

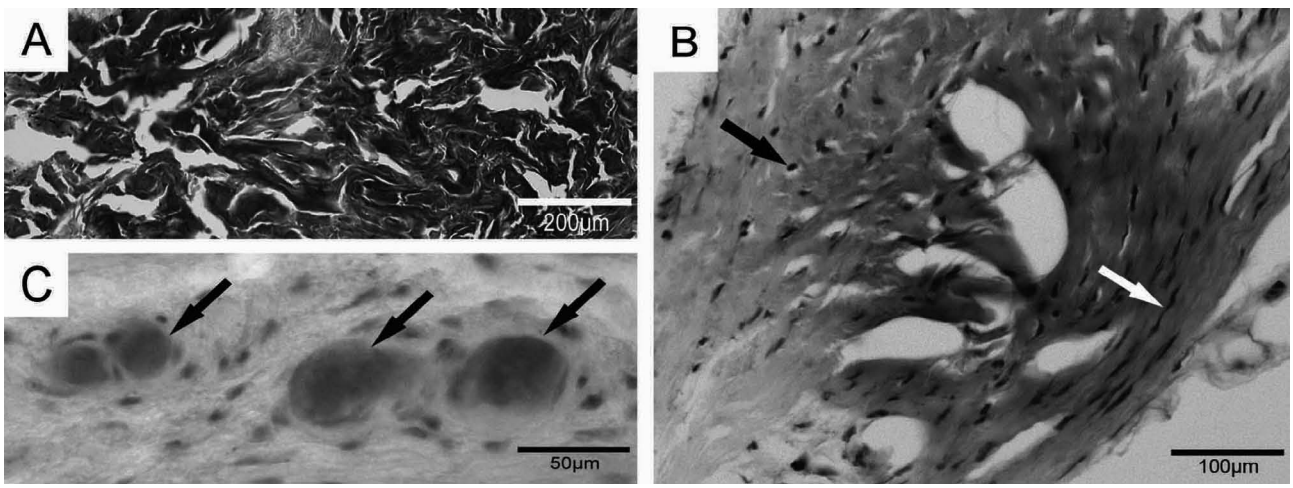

Fig. 2. ***2a)****: Photomicrographs of a ruptured supraspinatus tendon taken from a 53-year-old male. Note the gross disorganised distribution of collagen bundles and tissue separation caused by oedematous expansion (toluidine blue; x10 magnification);* ***2b)****: Section of tendon taken from the torn edge of supraspinatus in a 45-year-old female (toluidine blue, x20 magnification), illustrating the flattened spindle shaped nuclei of tenocytes (white arrow) in contrast to the rounded nuclei of chondroid-like cells with enlarged cytoplasm and surrounding lacuna formation (black arrow). Small, fragile and thin-walled blood vessels are a common feature of tendinopathic specimens (**Fig. 2c**, arrows: x40 magnification; haematoxylin and eosin).*

in both normal and abnormal tenocyte nuclei near the bursa, tear margins (Fig. 3g) and degenerative zones (Fig. 3h). There was further VEGF-labelling in cells surrounding vessels.

DISCUSSION

Sections from painful supraspinatus tendons revealed profound histopathological changes when compared to an asymptomatic control. Additionally, pathological changes were not limited to the tear margin but widespread throughout the tendon biopsy. This was characterised by degeneration and disorganisation of collagen fibres, a stark contrast to the polarized alignment of bundled fibres in healthy tendon.

Abnormal cells resembling tenocytes in tendinopathy were seen to gradually round and swell, undergoing chondroid metaplasia, as observed in other tendinopathies (12). Chondroid-like cells were seen along the torn, deteriorated edge of tendon, consistent with existing literature (13).

Normal and tendinopathic tendons displayed similar degrees of vascularity. However, the much smaller, immature vessels in tendinopathy may suggest a reparative response by injured tissue. Regeneration of damaged tissue relies on neovascularisation and fibroblast proliferation to promote collagen-rich granulation tissue (14). The current study reported VEGF-labelling in endothelial cells and tenocyte-like cells near these vessels and in areas of degeneration, which suggests tenocytes may themselves express VEGF when stressed (15). While VEGF-induced neovascularisation could be seen to instigate healing, it may also compromise the mechanical stability of tendon (16). Hypercellularity surrounding vessels in the current study may indicate the presence of an active inflammatory process. Furthermore, CGRP, PGP9.5 (a neuroendocrine marker) and SP immunoreactions also occurred in abnormal chondrocyte-like cells. Labelling of this nature has not been previously

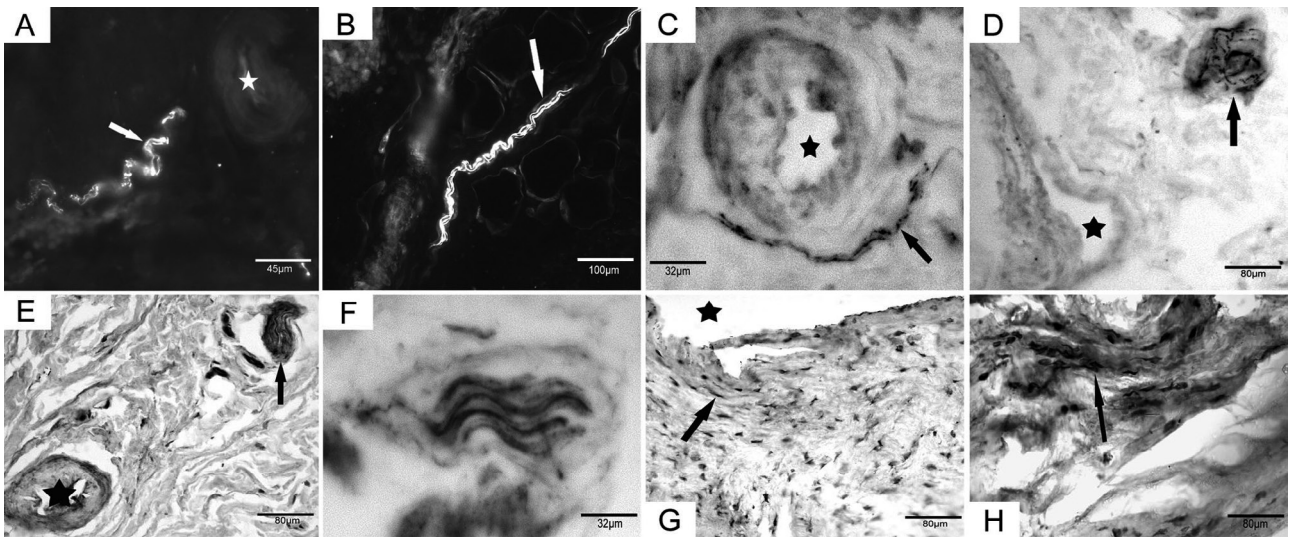

Fig. 3. *Sections of tendinopathic supraspinatus tendons stained with TRITC exhibiting PGP9.5-immunoreactive nerve fibres (arrows) in association with small blood vessels (**Fig. 3a**, star in vessel lumen: x50 magnification) and also with fatty infiltrate (**Fig. 3b**: x20 magnification). **3c-e)**: Immunoperoxidase-treated sections of supraspinatus tendon taken from patients with symptomatic rotator cuff tendinopathy; There is an intimate association between blood vessels (star in vessel lumens) and neural tissue (arrows). Whilst synaptophysin-immunoreactive nerve fibres are associated with smaller blood vessels (**Fig. 3c**: x62.5 magnification), there is also the appearance of an abnormal and disorganised bundle of nerve fibres that are also present in serial sections (**Fig. 3d**: x25 magnification). Nerve fibres, which are immunoreactive to substance-P also exhibited similar these characteristics (**Fig. 3e**: x25 magnification). **3f-h**) Immunoperoxidase-treated sections of supraspinatus tendon taken from a 45-year-old female stained with rotator cuff tendinopathy showing a CGRP-immunoreactive nerve fascicle (**Fig. 3f**: x62.5 magnification). Tenocyte nuclei (arrows) located within close proximity of the tear edge (star) exhibit significant CGRP (**Fig. 3g**: x25 magnification) and VEGF (**Fig. 3h**: x25 magnification) immunoreactivity.*

reported. This may suggest that stressed tenocytes may become neuroendocrine-like cells, secreting factors including CGRP, SP and VEGF into tissue, which can encourage local inflammation. Stressed tenocytes synthesising glutamate are involved in the development of painful Achilles tendinopathy (17).

Nerve fibres to the supraspinatus tendon are received from the suprascapular nerve (18). In both groups, the supraspinatus tendon was innervated by general and sensory fibres near vessels. In accordance with existing evidence, tighter varicose fibres and bundles were found closer to vessels, in fat, collagen and in the tendon-bursa junction of ruptured supraspinatus tendon, which were not seen in the control (3).

SP-labelled fibres, more intimately associated with vessels, were only detected in some tendinopathic tendons, which may suggest an inflammatory role in the regulation of local blood supply. This is more evident in an earlier study whereby intra-articular administration of SP in arthritic patients worsened inflammation and was then alleviated by infusion of an SP-antagonist (19). Whether SP has a similar function in tendon is as yet unknown.

There was a greater expression of SP in tendons that exhibited immature vessels. This may relate to the angiogenic properties of the neuropeptides CGRP and SP that have been previously identified (20). During specific stages in tendinopathy, neuropeptides stimulate neovascularisation (21) whilst mediating the repair process (22). The upregulation of CGRP and PGP9.5-labelled fibres in tendinopathy has been previously documented in experimental Achilles tendinopathy, yet associated hypervascularisation was not observed in that study (23).

Synaptophysin-immunoreactive nerves were closely related to vessels in tendinopathy. Despite being present in small concentrations in most presynaptic nerve endings as a neurotransmitter regulator, high concentrations of synaptophysin have been found in sensory nerves of tendon (24), in a similar fashion to the current study.

Limitations and directions for future research

Previous studies used qualitative methods for analysis, as performed here (3). A more quantitative approach would be desired. A sample size of 30 subjects was proposed (15 healthy; 15 symptomatic), based on similar studies (3). However, the significance of differences in innervation arrangements between both groups cannot be determined due to limited sample sizes, particularly in control cases. Also, the control was a male, 19 years younger than the tendinopathy group, so controls were unmatched for age or sex.

In our samples, there was no apparent association between age, pain score or symptom duration with histological and immunohistochemical features. Semi-quantitative scoring systems and modifications have been used in studies to classify tendinopathy (25). While a gold standard does not exist, these can be employed in future study with larger sample sizes.

Additionally, the healthy tendon was not necessarily a true control as it was from an unstable shoulder. It unknown whether instability causes pathology, as altered biomechanical loads may stress the rotator cuff (26).

There were difficulties with consistent sampling. Biopsies were excised from the superior aspect of tendon under the bursa. In 3 specimens, adherent bursa remained on the tendon. This could be overcome by sampling deeper within the tendon.

To our knowledge, this is the first immunohistochemical study of human supraspinatus tendon to employ CGRP, PGP9.5, SP and synaptophysin antibodies, yet further experimentation is required in order to optimise dilutions. Compared to PGP9.5, synaptophysin may not be a good general nerve marker. Detection of mRNAs using in-situ hybridisation (ISH) or reverse-transcriptase PCR using ISH for cellular localisation to confirm protein synthesis can be used to verify SP and CGRP production in tendon. Western-blotting could be used to ensure correct proteins are detected and absorption controls to demonstrate antigen-binding specificity. Due to non-specific staining with SP and VEGF, better antibodies may need to be identified.

From a clinical perspective, the observations of vascularity and innervation of the painful supraspinatus tendon is of interest, and its relevance need be determined in future research.

The current study has demonstrated new evidence of the general and sensory innervation patterns in the supraspinatus tendon using a reliable method of immunostaining with experimental antibodies.

ACKNOWLEDGEMENTS

We thank Mr Simon Owen-Johnstone and Mr Ali Noorani for their assistance in the surgical biopsy of tendon tissue.

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QUANTITATIVE STUDY ON VANCOMYCIN RELEASE FROM CEMENT IN 3 DIFFERENT FORMULATIONS: PRELIMINARY RESULTS AND ANTIMICROBIAL ACTIVITY

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The purpose of this study is to investigate the best preparation method of the cement powder mixture, solvent and antibiotic in order to obtain the greatest amount of antibiotic in the joint for the longest time as possible. At time T0 the three samples, packed in a sterile environment in different formulations, were placed in sterile tubes, adding to each one 5 ml of saline phosphate buffer solution (PBS) and put in a stove at 37°C for 24 h. A sample of PBS without cement (T control) was also created. Qualitative and quantitative assessment of the incubated liquid with cement was performed along with biochemical analysis with High Performance Liquid Chromatography (HPLC). The analysis of the raw data demonstrated that at T1 there was a prevalence of antibiotic release from sample , compared to sample 2 and 3. This difference was maintained until the T20; from T21 the antibiotic release gradually leveled in 3 samples. The elution of the antibiotic remained detectable up to T60. Our work shows that the sample preparation is decisive on the quantity of released antibiotic. These results are confirmed by microbiological tests. It is useful to know the actual kinetics of antibiotics in articulation. Further studies are necessary to determine the effectiveness of antibiotic against micro-organisms and how long it acts.

Periprosthetic joint Infections (PJI) are one of the most dangerous complications in orthopedics. Although over the years surgeons have tried to prevent them, these occurrences are still common. Nowadays the gold standard treatment is the "two-stage surgery" using an antibiotic-loaded acrylic bone cement (ALABC) spacer.

The function is double: keeping the length of the limb (while waiting for the definitive prosthesis) and resolving the infection, acting directly on the septic focus, keeping high local concentration of antibiotic and minimizing systemic side effects. Recently, attention was focused on the elution of the antibiotic by polymethylmethacrylate (PMMA), in particular on the amount of released antibiotic, on how long it is released and on its efficacy. However, there are no studies in literature to describe how different preparations of the mixture ALABC could modify the elution of the antibiotic.

The aim of this study is to verify *in vitro* the different elution of antibiotic from three different preparations of the mixture (ALABC).

MATERIALS AND METHODS

The antibiotic used was Vancomycin (Hospira,

Key words: cement, prosthetic joint infections, antibiotic elution, PMMA

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0393-974X (2016)

ABBOT), 1 g powder. The cement used was G1-40 (G21 Srl), 40g, standard viscosity; within each individual pack of cement there is a phial consisting of methyl methacrylate (MMA) and N,N dimethyl-P-toluidine (DMPT) and a bag of powder made from polymethyl-methacrylate (PMMA) and benzoyl peroxide (BPO). Sample preparation took place in the operating room in order to maintain maximum sterility of the compounds.

The various components were added to a bowl through 3 different mixing methods. Each sample was modelled using as 5 ml syringe mold cut into a cylindrical shape. All samples had equal shape and size. The three samples were mixed in this sequence:

1st sample: vancomycin (powder) 4gr + solvent (MMA+DMPT) + cement powder (PMMA+BPO) 40 gr.
2nd sample: vancomycin (powder) 4gr. + saline (4ML) + cement powder (PMMA+BPO) 40gr. + solvent (MMA+DMPT).
3rd sample: vancomycin (powder) 4gr. + cement powder (PMMA+BPO) 40 gr. + solvent (MMA+DMPT).

Conduct of the experiment

At time T0 the 3 samples were placed in 3 sterile test tubes (working under hood), adding 5 ml of phosphate-buffered saline (PBS) in each and then placed in an oven at 37°C for 24 h to replicate physiological conditions.

We proceeded to withdrawal of the cement cylinder with antibiotic and transferred it to a new sterile tube to which 5 ml of PBS were added and repositioned in stove at 37°C for 24 h. A qualitative and quantitative assessment of the incubated liquid with cement in the previous 24 hours was carried out we proceeded with the withdrawal for biochemical analysis with HPLC (High Performance Liquid Chromatography)

Determination of Antibiotic concentrations

Quantitative analysis of vancomycin released in PBS was performed by high performance liquid chromatography (HPLC) as previously reported by Chang et al. (2011) (1). At the end of each incubation period, tubes containing PBS were centrifuged to eliminate the remnants of cement. The concentrations of vancomycin were then determined using an Agilent 1100 chromatograph system (Agilent Technologies, Santa Clara, CA, USA) with a stainless-steel column (RP18 column, 100 mm by 4.6 mm, 5 µm particle size) maintained at a temperature of 30°C. The mobile phase consisted of water-acetonitrile-100 mM ammonium formate (composite ratio, 78/12/10). The UV detector was set at a wavelength of 220 nm. The HPLC system had a sensitivity of 0.5 µg/ml for vancomycin.

The concentrations of the antibiotic released in PBS from cement cylinders were determined using external standard curve that was prepared daily and the peak areas were correlated with antibiotic concentrations. The released antibiotic amounts have been expressed in percentage of the total amount of vancomycin in cement cylinders.

Microbiological part of the experiment

The biological activity of antibiotics released by the three cement specimens was studied with a standardized method of broth micro-dilution. We used two strains of *S. Aureus*: *Staphylococcus aureus* ATCC 25923 MSSA and ATCC 43300 MRSA and parts of the elution of the specimens for which we already knew the concentration of antibiotics. Moreover, we made bacterial suspensions with 0.5 McFarland turbidity. This bacterial preparation was then diluted in order to obtain the final concentration of 10^5 CFU (colony forming unit) for each cockpit. In each specimen, 100 microliters of the elution liquid was mixed from each specimen; 100 microliters of broth, 20 microliters of the bacterial suspensions (MSSA or MRSA) and the whole plate (formed by 96 cockpits) was incubated for 24 h at the temperature of 37°C. Every specimen was made twice in the plate.

The bacterial growth in the wells was determined comparing the turbidity between the experimental and the control ones, in which we put only broth and bacteria. The organization of the plate reflected the entire trend of the study. In fact, in lines A and B we determined the action of the elution fluid from the specimen number 1 during the different times (T1,

T2, etc.) which are represented in the columns 1-10. In lines C and D, we evaluated the action of the elution fluid from specimen number 2 and finally, in lines E and F, we determined the action of specimen number 3. We made controls in the H line. In particular, in the MSSA plate, from H1 to H4 we filled the wells with PBS and Bacterial suspension, from H5 to H8 with saline solution (used for the bacterial suspension) and bacteria and from H8 to H12 the cockpits were filled with broth and bacteria.

In the MRSA plate, although the organization of the whole plate was the same as the MSSA plate, we made a different H line. In fact, in this line only the first six wells were filled with broth and bacteria because, in our opinion it was not necessary making other controls wells.

RESULTS

The elution kinetic profiles of vancomycin in three different cement specimens are shown in Fig. 1. Overall, specimen 1 provided a better release efficacy during the 60-day study period in comparison to both specimens 2 and 3, which instead released a low amount of antibiotic.

In particular, specimen 1 showed a significant higher elution rate at day 1 (about 15%) in comparison to specimens 2 and 3, which at the same time released only 1.7% and 1.3% of total amount, respectively.

The release from specimen 1 rapidly decreased to 6% and 1.2% at day 2 and day 3, respectively. However, the elution rate in cement specimen 1 was higher up to day 20 when compared to other specimens. Indeed, after the first day the elution rate in both specimens 2 and 3 was lower than 1%.

During time-course between day 30 and until day 60, the release of vancomycin from cement specimens 1 and 2 was similar and slightly higher than specimen 3, however, in all specimens the elution rate was lower than 0.05%.

When logarithmic scales were used on both the horizontal and vertical axes, linear regression for the values obtained from the three specimens were obtained (Fig. 2). Based on these results we observed that intercept on the Y-axis is at the highest value for specimen 1. In addition, the slope value correspondent to specimen 1 is significantly higher than those observed in specimens 2 and 3, respectively 1.83 *vs* 1.16 (p=0.005) and 1.83 vs 1.30 (p=0.022).

In regards to the microbiological part, it is possible to divide the results in two different groups: the results obtained on MSSA and the ones obtained on MRSA. In the first group, the amount of antibiotic

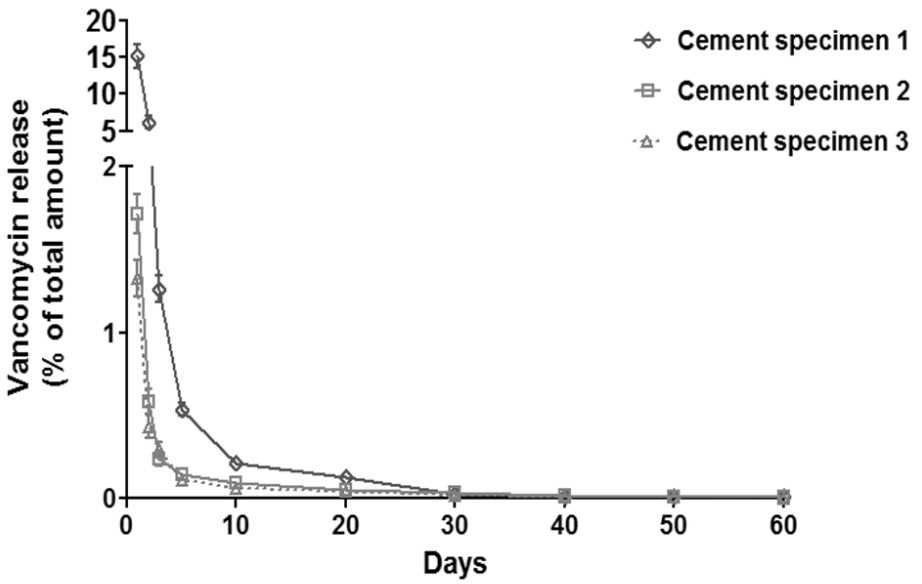

Fig. 1. *Elution Kinetic profiles of Vancomycin.*

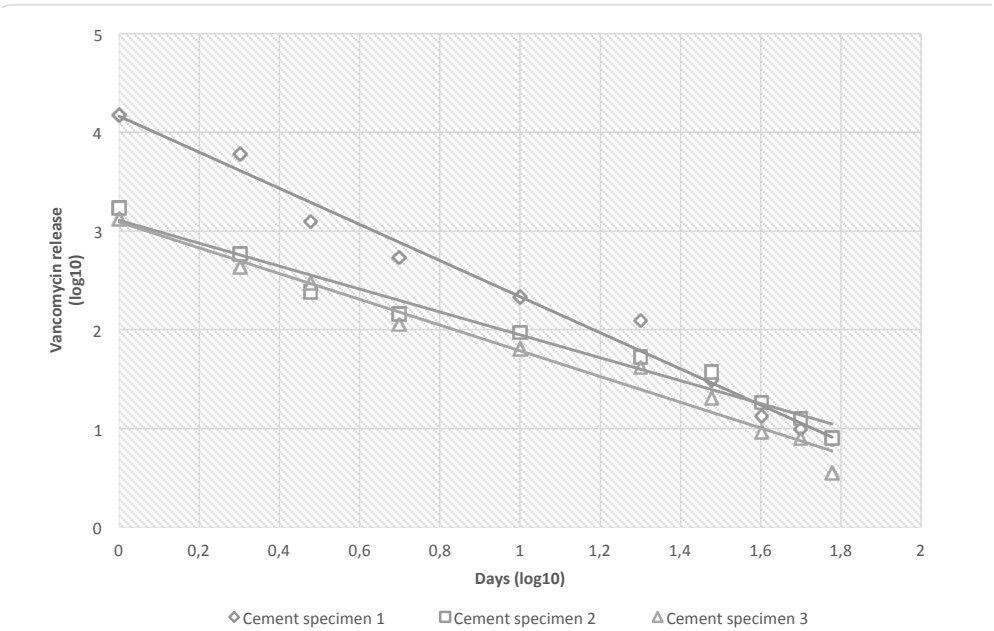

Fig. 2. *Linear regression of the obtained values.*

contained in all the elutions is more than the MIC of our bacteria so that every specimen is capable of inhibiting the bacterial growth until the day 60. Even in the second group (MRSA), all the elution fluid derived from the cement specimen was capable of inhibiting the bacterial growth until the day 60.

DISCUSSION

One of the first authors to report his experience on the ALABC as spacer during the treatment of PJI was Buchholz in 1981 (2). He treated 583 patients and, through the "two stage surgery", the rate of success was 90% after the second operation. The reason why cement is loaded with antibiotic is that the antibiotic is gradually released only inside the articulation, reaching high local concentration and low systemic concentration of the drug: this is not possible with the intravenous therapy. Since 1981, the "two stage surgery" is considered the gold standard for the treatment of PJI.

Over the years, surgeons focused their attention on the features and the time of release of the antibiotic loaded cements and on biomechanic and microbiological features, in order to obtain higher concentration of local release of the antibiotic, keeping a good mechanic resistance.

The porosity increase of ALABC, certainly determines a major elution of antibiotics. In order to increase the porosity, different strategies were tested in literature. McLaren (3), for example, made different studies using inert fillers such as glycine, sucrose and xylitol to enhance the porosity of the cement and make the antibiotic more bioavailable.

Another strategy proposed to increase the porosity, was to use liquid antibiotic such as gentamycin, although this method caused the decrease of biomechanic resistance. Chang et al. compared the elution of antibiotic in three different formulations: powder antibiotic, liquid antibiotic and mixed powder of antibiotic and xylitol (4). All the specimens showed a peak of elution during the first day, with a rapid decrease during the next days (the specimen with liquid antibiotic and the specimen with xylitol showed a major elution of the antibiotic).

In our study we compare 3 different formulations, thinking that the formulation with saline solution would have released a major quantity of antibiotic, according to the idea that the heat released by the

polymerization of the cement makes part of the water evaporate, giving more porosity to the cement and consequentially, more elution of the drug.

Moreover, lots of attention was paid to which antibiotic should be used. The most important features should be a broad-spectrum antibiotic, a good compatibility with cement, a good bioavailability and a good action into the bone in order to prevent side effects. The most used antibiotics are gentamycin, daptomycin and vancomycin (5, 6).

In our study, we used Vancomycin. It is an antibiotic with a linear glycopeptide structure of 7 aminoacids, 5 of which are aromatic. It has a tricyclic structure with the presence of sugars and was isolated in 1956 as a product of the fermentation of an actinomycete from Borneo (Streptomyces orientalis).

It is an antibiotic with bactericidal action that acts by inhibiting the synthesis of the bacterial wall of the gram-positive microorganisms, both cocci and bacilli, and aerobes and anaerobes. The most important action is on methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, which are the most frequent bacteria involved in PJI, although many strains are often resistant to various classes of antibiotics.

Many studies were also made in regards to the release of antibiotic both *in vitro* and *in vivo*, such as one by Bunetel et al. who studied the elution of gentamycin using specimens of blood, urine and post-surgery drainages (the concentration of gentamycin was 0.5g in 60 g of cement) (7).

Analyzing the specimens immediately after drawing and after two weeks from drawing (keeping specimens at 4°C), Bunetel et al. did not observe significant differences except those related to the different specimens (5). For example, in the blood specimens, gentamycin was not quantifiable while in the drainages there was a biphasic release with a mean half-life of 2.97 h and 13.5. In the urine specimens, the half-life was of 7.16 and 47.12 hours. Our study varies to this in diverse points. Firstly, we studied the elution of Vancomycin (not gentamycin) *in vitro*, simulating the human physiological turnover through the replacement PBS; secondly, we focused our attention to the capacity of elution of three different specimens of cement made with different procedures, studying also the microbiological features and the achievement of the MIC.

There are also many studies about biomechanical features of the ALABC, showing conflicting opinions. Dunne et al. affirms that in chronic PJI, the use of antibiotic makes the cement weaker without acting on the bacterial biofilm (8). On the other hand, Raimondi et al. (9), confirming the increment of the weakness of the cements, affirms that both ALABC and normal acrylic cement reach the standard mechanical features and that this is due to the basic cement.

Paz et al. evaluated the release of Vancomycin *in vitro*, comparing the results to those of cefazolin (10). According to his results, the specimen containing both antibiotics has the major elution in 28 days, highlighting the synergy between the two antibiotics. There are many differences between this study and ours. Paz uses different antibiotics and different composition of the antibiotic rate between cefazolin and vancomycin while we use only vancomycin in three different methods of cement mixing. Moreover, there are important differences in data recording: they made a table with the total release of antibiotic in 28 days while we recorded the exact quantity of antibiotic released at specified times (T1, T2, etc.) so that we could sum them up and obtain the total amount. Unlike them, we did not focus our attention on the mass gain of the specimens. In regards to the mechanical features, Paz evaluated not only the differences between the different specimens at compression and bending tests, but even after 1 month aging. Instead, we paid attention to mechanical differences between the three different mixing methods, compared to a control specimen not loaded with antibiotic.

In regards to the microbiological part, different authors in literature focused their attention on the antibacterial capacity of ALACB, proving *in vitro* the efficacy of elution liquid, obtained with a similar procedure to this study. For example, Sakoulas et al. in their study tested the antibacterial capacity of the cement on different strains of *S. Aureus* isolated from different infected patients (11). They divided the results in three different groups: group 1, 0% efficacy with Vancomycin; group 2, 23.1% efficacy;

group 3: 50% efficacy. Other authors, after mixing inert materials to increase the release of antibiotic, used disks to visually highlight the inhibition of the bacterial growth (6).

Moreover, Andollina et al. (12), evaluates the antibacterial capacity of cements with different concentration of vancomycin while Chang et al. (1), in a study more comparable to ours than previous ones, evaluates the action of the elution liquid from three different specimens with 1g of antibiotic on 40g of cement, 4g/40g and 8g/40g, by using three different antibiotics: Vancomycin, Teicoplanin and Daptomycin. We used the same technique, (based on wells and not on disks to highlight the bacterial inhibition) but our study is different both in the preparation of the specimen (we used the same quantity of vancomycin modifying just the preparation of the cement) and in the results. According to Chang et al., the specimens with Teicoplanin release a higher quantity of antibiotic than the others (1). Moreover, specimens with 8g of antibiotic showed an antibacterial action for all the 60 days towards all the strains (MSSA, MRSA, VISA). Specimens with 4g of antibiotic showed an antibacterial action up to day 21 (except specimens with Teicoplanin that had the antibacterial action until day 40) on MSSA, showing less positive results for MRSA and VISA. Specimens with 1g of antibiotic showed the antibacterial action just on day 1. In our study, we compared the action of Vancomycin according to the preparation of the cement rather than the quantity of antibiotic. Based on these premises, we found that all three specimens are capable of releasing a high quantity of antibiotic in order to exceed the MIC (MSSA) until day 60 and inhibit the bacterial growth. We think that our study shows how a different formulation of cement could modify the elution of Vancomycin. In particular, the first specimen has the higher elution and it was made in the theatre room mixing Vancomycin with the solvent and then with the cement powder. In our opinion, it is possible to verify that the concentration of the Vancomycin in PBS is high enough to be detectable and effective for all the specimens until day 20, although there are differences due to the mixing methods. In fact, testing the elution liquid on MSSA, MRSA and another strain of SA obtained in our hospital, it is evident that specimen 1 has a better antibacterial activity and therefore should be the best mixing method to use for PJI treatment.

Nevertheless, it is important to highlight that this is an *in vitro* study, so it is necessary to examine some variables that could influence the prognosis, *in vivo*, of the patients with PJI. Firstly, it is important to remember that in the articulation, we could find focuses (bone necrosis) that, if not reached by antibiotic could make the infection active. Secondly, some bacteria, such as *S. Aureus* or *P. Aeruginosa*, are able to switch to SCV (small colonies variant) whose form has an important DNA mutation that makes their metabolism much slower and can have an intracellular life, so that they cannot be killed by antibiotic. These could be the two motivations, together with the formation of a bacterial biofilm on the prosthesis, which explain why, even with two-step surgery, some patients do not eradicate the infection.

ACKNOWLEDGEMENTS

We would like to express my special gratitude and thanks to Prof. Daniela Iannello who handled microbiological analysis.

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