Article

J. Biol. Regul. Homeost. Agents. 2024; 38(5): 3751–3764
https://doi.org/10.23812/j.biol.regul.homeost.agents.20243805.296

In Vitro and *In Vivo* Evaluation of a Novel Intracanal Medicament for Delayed Teeth Replantation

Ying Wang[1], Han Deng[1], Yanping Hu[1], Shasha He[1], Shuying Hu[1], Xuyang Shen[1], Boyang Sun[1], Nannan Wang[1], Gang Cao[2,*], Ting Guo[1,*]

[1]Nanjing Stomatological Hospital, Affiliated Hospital of Medical School, Nanjing University, 210008 Nanjing, Jiangsu, China
[2]Department of Stomatology, Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University, 210002, Nanjing, Jiangsu, China
*Correspondence: m13770910192_1@163.com (Ting Guo); cyj690830@163.com (Gang Cao)
Published: 1 May 2024

Background: In dental avulsion, delayed replantation usually has an uncertain prognosis. After tooth replantation, complex inflammatory responses promote a return to periodontal tissue homeostasis. Various types of cytokines are produced in the inflammatory microenvironment, and these cytokines determine the periodontal tissue response. This study aims to conduct in-depth research on developing a novel root canal medicament, which employed methylcellulose hydrogel as a carrier system and was loaded with clindamycin, acetazolamide and triamcinolone, to determine its triple action of antibacterial, anti-inflammatory and anti-resorptive capabilities in delaying tooth replantation and to preliminarily explore its potential mechanisms of action.
Methods: The release concentration of the paste from human extracted root apices was determined using UV-spectrophotometry. The cytotoxicity of corresponding drug concentrations on HPDLFs (human periodontal ligament fibroblasts) was assessed on days 1, 3, 5, and 7 using the CCK-8 (cell counting kit-8) assay. The antibacterial activity against Fusobacterium nucleatum (*F. nucleatum*) was measured using the agar diffusion method. The gene and protein expression of inflammatory factors (IL-1β (interleukin-1β), IL-6 (interleukin-6), TNF-α (tumor necrosis factor-α) and bone metabolism molecules (RANK (Receptor Activator of Nuclear Factor-κB), RANKL (Receptor Activator of Nuclear Factor-κB Ligand), OPG (Osteoprotegerin)) in HPDLFs stimulated with Escherichia coli (*E. coli*) lipopolysaccharide were determined by ELISA (enzyme-linked immunosorbent assay) and RT-qPCR (real-time quantitative polymerase chain reaction), respectively. Furthermore, the rat delayed replantation tooth models were established and the resorption of incisors was analyzed by micro-CT (micro-computed tomography) after 60 days. The degree of root inflammation and resorption was evaluated by hematoxylin-eosin (HE) staining, and the expression of RANKL/OPG was assessed by immunohistochemical staining.
Results: The novel paste showed sustained slow release in root canals for 60 days. *In vitro*, the experiments demonstrated good cell compatibility and antibacterial activity ($p < 0.01$). The paste suppressed the expression of IL-1β, IL-6 and TNF-α, and down-regulated the RNA and protein levels of RANKL and OPG in HPDLFs stimulated by *E. coli* ($p < 0.05$). *In vivo* experiments revealed that the novel paste down-regulated the expression of RANKL and OPG, effectively preventing inflammatory root resorption in rat delayed replantation teeth ($p < 0.05$).
Conclusion: The novel paste can inhibit inflammatory root resorption by modulating the RANKL/RANK/OPG signaling pathway and has the potential to be used as an intracanal medicament for root canal treatment in delayed replantation teeth.

Keywords: intracanal medicament; delayed teeth replantation; inflammatory root resorption

Introduction

One of the most severe types of dental trauma is dental avulsion, which is characterized by the total separation of the tooth from the alveolar socket, leading to cementum damage, infection of the root surface, and necrosis of the periodontal ligament and pulp. The recommended course of treatment for an avulsed tooth is prompt replantation. In many cases, however, delayed replantation occurs. Severe periodontal damage in avulsion injuries may lead to inflammation or substitute root resorption after replantation [1], among which inflammatory root resorption is the most common type with the worst prognosis [2] his type of resorption may lead to early loss of replanted tooth (mean duration of 1.7 years) [3] and is the primary cause of premature tooth loss following replantation.

Inflammatory root resorption is triggered by abnormal osteoclast activity with root and periodontal tissue loss. The current consensus suggests that two premises are essential for its development: initial injury and subsequent constant stimulation, including bacteria infection and certain inflammatory mediators [4]. Traumatic injury damages the protective layer mainly consisting of cementum, leading to dentine exposure and bacteria infection, which finally causes the significant increase of inflamma-

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

tory factors and activation of osteoclasts that directly exacerbates resorption by producing more cytokines (e.g., IL-6 (interleukin-6), TNF-α (tumor necrosis factor-α) and IL-1β (interleukin-1β)), mostly affecting Receptor Activator of Nuclear Factor-κB Ligand (RANKL)/Receptor Activator of Nuclear Factor-κB (RANK)/Osteoprotegerin (OPG) system. RANKL is a ligand for RANK (Receptor Activator of Nuclear Factor-κB), which is required for osteoclast generation, and OPG is a decoy receptor for RANKL.

One of the most popular intracanal drugs is calcium hydroxide (CH) because of its outstanding antibacterial properties [5]. However, there are different opinions regarding its limited efficacy in preventing inflammatory root resorption [6]. Some studies suggest that calcium hydroxide increases ankylosis and replacement resorption, while other studies propose that due to its inherent toxicity and irritability, direct contact with tissues after resorption may exacerbate inflammatory responses [7–9]. Additionally, CH has the disadvantage of increasing tooth brittleness and requiring multiple dressing changes.

Given the limitations of the existing drug, the development of intracanal medicament has emerged as our top priority and osteoclastogenesis suppression remains a significant therapeutic strategy for this disease. According to Mohammadi *et al*. [10], the local administration of antibiotics as a more efficient drug delivery strategy in endodontics prevents the possibility of systemic allergic reactions. Following short-term treatment, clindamycin has been shown to effectively treat facultative and stringent anaerobic bacteria infestations in the root canal and dentinal tubules without tooth discolouration [11], making it a first-line antimicrobial agent for dental infections [12]. Corticosteroids play a crucial role in reducing inflammation and exerting immunosuppressive effects [13]. The activation and migration of inflammatory cells are all inhibited by corticosteroids, which also prevent the generation of these chemicals. Carbonic anhydrase is a key player in the activity of bone resorption, and can generate hydrogen ions via catalyzing reaction between carbonic acid and water, leading to a decrease in the pH of Howship's lacunae. Other resorption-related enzymes can be released and activated when the pH is acidic. Acetazolamide (ACZ), by inhibiting the activity of carbonic anhydrase, prevents the synthesis of hydrogen ions. This pH modulation helps alleviate the resorption reaction. Additionally, several investigations have shown that acetazolamide inhibits the resorption of tooth roots [14]. Methylcellulose is widely used in the pharmaceutical field for delaying drug release and reducing dosing frequency. Its hydrogel possesses excellent biocompatibility and injectability, making it extensively utilized for delivering low concentrations of intracanal medications in the oral field.

Therefore, this study employed methylcellulose hydrogel as a carrier system loaded with clindamycin, acetazolamide and triamcinolone to prepare an injectable paste, aiming at determining the triple action of antibacterial, anti-inflammatory and anti-resorptive capabilities of this novel paste in delaying tooth replantation and preliminarily exploring its potential mechanisms of action.

Materials and Methods

Medicament Preparation

The novel intracanal medicaments used in this study were prepared in methylcellulose hydrogels. First, 5% acetazolamide, 5% clindamycin and 1% triamcinolone acetonide were dispersed in 3 mL of sterile water and shocked for 90 minutes under continuous ultrasonic. Then, methylcellulose powder (C6333, Macklin, Shanghai, China) was gradually added to the medication solution under controlled vigorous stirring to create a creamy injectable texture of medicine paste. A methylcellulose placebo paste and a commercial calcium hydroxide paste (M813702, Macklin, Shanghai, China; C799356, Macklin, Shanghai, China) were also used as controls in this study.

In Vitro Study

Medicament Release through Apical Foramen

Fourteen single-rooted incisors were selected from the department's collection of teeth of patients. The removed teeth were maintained in saline at 4 °C before the study. Teeth with caries, fractures, resorptions, calcifications in the canal, and the slightest variant anatomy were excluded. A diamond bur with a cylindrical shape was used to remove the crowns from every tooth. Each sample had a specified length of 15 mm. Instruments made by Mtwo (VDW, Munich, Germany) with a 40.06 taper were used to create the root canals. After each instrument, the canals were alternatively irrigated with 2 mL 0.9% sterile saline and 2 mL 1% sodium hypochlorite solution. After flushing the root canal with 6 mL saline to eliminate the Ethylene Diamine Tetraacetic Acid (EDTA), the smear layer was eliminated by rinsing it with 2 mL 17% EDTA solution. Finally, a dental air-water pistol was used to dry the root surfaces, and then dental absorbent points were used to dry the canals. Except for the apical third region, the entire root was painted with nail polish. The coronal access cavities were sealed with temporary restorative material, then the root canals were filled with 0.03 mL unique intracanal medications. After that, a rubber ring was used to insert the apical third of the root into 24-well plates that contained 1 mL deionized water in each well. Up to the scheduled analysis time, the plate was kept in a 37 °C oven. After that, specimens of 1 mL deionized water were withdrawn periodically at 1, 5, 10, 15, 20, 30, 40, 50 and 60 days, replaced with fresh deionized water each time. The absorbance value of acetazolamide was analyzed by an ultraviolet spectrophotometer (Lanbda365, PerkinElmer, Waltham, MA, USA) at 265 nm. The concentration of acetazolamide was calculated by the standard curve method.

Cell Culture

Human third molar teeth were removed from three healthy individuals aged 18 to 24 years and their periodontal ligaments were used to create human periodontal ligament fibroblasts (HPDLFs). In STR (specialized technology resources) identification, a combination of PCR (polymerase chain reaction) amplification and electrophoresis analysis was used to analyze the DNA fingerprint of the target cell. We further compared and found that the standard human cell line did not have cross contamination within the species. The culture method was used for detection and was not contaminated with Mycoplasma. The Affiliated Stomatological Hospital of Nanjing University School of Medicine (Nanjing, China) approved the study (Ethical No. PY2017023). The patients and their families have signed informed consent and the research was conducted by the Declaration of Helsinki. The origin of HPDLFs is the periodontal ligament in the middle third of the root surface. The tissue was separated from the surface using a sterile blade, washed three times in PBS containing 2% penicillin and streptomycin and put into a culture container. The adhered tissue was dissolved in 5 mL Dulbecco modified Eagle medium (DMEM) at 37 °C with 5% CO_2 and supplemented with 20% fetal bovine serum (FBS), 1% penicillin and streptomycin. The media was replaced every five days once the cells attained around 80% confluence. Then the cells were separated with 0.25% trypsin and subcultured in DMEM containing 10% FBS, with DMEM replacements occurring every two days. Cells from passages 3 to 8 were employed in the research.

Cytotoxicity Test

The cell counting kit-8 (CCK-8) technique was used to determine the cytotoxicity of various doses of the new drugs using HPDLFs. The tested drugs were prepared by dilution in DMEM to various concentrations (180 μg/mL, 140 μg/mL, 100 μg/mL, 60 μg/mL, 40 μg/mL, and 20 μg/mL). 96-well plates were filled with 7103 cells. Following a 24-hour incubation period, 100 micro-litres of fresh DMEM and 100 micro-litres of media supplemented with various medication dilutions were introduced. After 1, 3, 5 and 7 days of incubation, 10 μL CCK-8 solution (C0038, Beyotime, Shanghai, China) was added to each well. Then the absorbance value at 450 nm wavelength was determined using a microplate reader (SpectraMAXM3, Molecular Devices, Sunnyvale, CA, USA). Five experiments were repeated per sample group. We measured cell viability (cell viability = (total number of cells – number of dead cells)/total number of cells × 100%).

Antimicrobial Assay

To evaluate the antimicrobial activity of the novel drug, the agar plate diffusion method was performed on Porphyromonas gingivalis (*P. gingivalis*) (ATCC: 33277). The bacteria were supplied by The Affiliated Stomatological Hospital of Nanjing University School of Medecine. The experimental drug was obtained by diluting the prepared paste with sterile water to the same concentrations as 2.2.4. Firstly, *P. gingivalis* was inoculated with BHI liquid medium (containing heme chloride and vitamin K3) for activation for 3 days. The medium was stored in an anaerobic culture bag at 37 °C. Then 100 μL *P. gingivalis* were taken and the concentration was adjusted to 0.5 Mcfarland Standard. The prepared bacteria were seeded with the Columbia blood agar plate medium evenly. A cylindrical container of 6mm in diameter was placed at the center of the plate. Finally, 100 μL 100 μg/mL drug solution and sterile water are placed into the container. The plates were incubated in anaerobic conditions at 37 °C for three days. Zone of inhibition was measured with vernier caliper three times and the average was reported.

Treatment of Lipopolysaccharide (LPS)-Stimulated HPDLFs with the Drug

After the HPDLFs were stimulated by *E. coli* lipopolysaccharide (LPS) with or without medication, the expression levels of IL-1β, IL-6, TNF-α, RANKL and OPG were assessed by enzyme-linked immunosorbent assay (ELISA) and RT-qPCR Detecting System. Six-well plates were used to seed the HPDLFs of 1×10^6 cells per well followed by 12 hours adhesion. Then they were either given a 100 μg/mL medication solution or were stimulated with *E. coli* LPS (10 μg/mL) for 48 hours. After stimulation, ELISA kits were used to measure the presence of IL-1β (EH001, Excell, Shanghai, China), IL-6 (EH004, Excell, Shanghai, China), TNF-α (EH009, Excell, Shanghai, China) and RANKL and OPG (H5813c, H1341c Elabscience, Wuhan, China) in the cell supernatant. The assay was completed according to the manufacturer's instructions. Three duplicates of each experiment were carried out. Utilizing the Trizol reagent (R411-01/02, Vazyme, Nanjing, China), total RNA was isolated from the cells. Then, cDNA was created using Hiscript III RT SurperMix reagent (R323-01, Vazyme, Nanjing, China).

Real-time polymerase chain reaction (RT-PCR) was carried out utilizing Roche LightCycler 96 RT-PCR System and Taq Pro Universal SYBR Master Mix reagent (Q712-02/03, Vazyme, Nanjing, China). The list of the primers is as follows:

IL-1β F: 5′-ATGGCAGAAGTACCTGAGCTCGC-3′;

R: 5′-ACACAAATTGCATGGTGAAGTCAGTT-3′.

IL-6 F: 5′-GAAAGCAGCAAAGAGGCACT-3′;

R: 5′-TTTCACCAGGCAAGTCTCCT-3′.

TNF-α F: 5′-ATGAGCACTGAAAGCATGATCCGG-3′;

R: 5′-GCAATGATCCCAAAGTAGACCTGCCC-3′.

RANKL F: 5′-ACCAGCATCAAAATCCCAAG-3′;

R: 5′-CCCCAAAGTATGTTGCATCC-3′.

OPG F: 5′-TGTGCGAATGCAAGGAAG-3′;

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

R: 5′-TGTATTTCGCTCTGGGGTTC-3′.
GAPDH F: 5′-CCATGGAGAAGGCTGGGG-3′;
R: 5′-CAAAGTTGTCATGGATGACC-3′.

The typical PCR settings were 30 seconds at 95 °C, followed by 40 cycles of 10 seconds at 95 °C and 30 seconds at 60 °C. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative gene expression. The tests were carried out three times.

In Vivo Study

Delayed Tooth Replantation Rat Model

We bought 18 male wistar rats (Charles River Animal Technology Co., Ltd., Beijing, China) weighing from 250 to 300 g. The animal experiments were conducted in cooperation with Nanjing Agricultural University and carried out in compliance with the guidelines of the Nanjing Agriculture University's Animal Ethics Committee (PZW2020010). The rats were divided into three groups (n = 6) as the following rules: In Group I, calcium hydroxide (CH) paste was injected into the canals; In Group II (the control group), the canals were filled with placebo paste made of methylcellulose; In Group III (the experiment group), the canals were filled with the novel medicament. To simulate dental avulsion, the animals were anesthetized by inhalation with isoflurane (2–2.5%). Subsequently, the PDL (periodontal ligament) fibers were cut and the teeth were shaken loose, then the right central incisors of the maxilla were removed. A retrograde method with an ISO 15 K-File (Dentsply) was used to extract the pulp after 60 minutes. Each canal was cleansed using 2 mL 1% sodium hypochlorite solution and 0.9% sterile saline. The root canals in three groups were then filled with the CH paste, methylcellulose placebo paste or innovative medication, respectively. The root canals were then dried with sterile absorbent paper points and the sockets were flushed with saltwater to get rid of the blood clot and foreign materials. The teeth were subsequently placed back into their proper sockets. The crowns of the replanted teeth were sanded by 2 mm to prevent them from slipping out. 20,000 U.I. Penicillin G benzathine was administered intramuscularly to all animals in one dosage.

Micro-CT (Micro-Computed Tomography)

All animals were put to death by carbon dioxide poisoning 60 days after the replantation. A surgical scissor was used to separate the maxilla, and a #15 scalpel blade was used to remove the replanted incisor from the right side of the bone. The anatomical components were preserved in a 10% formaldehyde solution. Two samples from each group were chosen for micro-CT study. The samples were scanned using a micro-CT scanner with a 9-m resolution (SkyScan 1176, Bruke, Billeric, MA, USA). NRecon software (version 1.6.9.8, Bruker, Billerica, MA, USA) and Data Viewer software (version 1.5.0.2, Bruker, Billerica, MA, USA) respectively showed the generated data as two-dimensional and three-dimensional pictures.

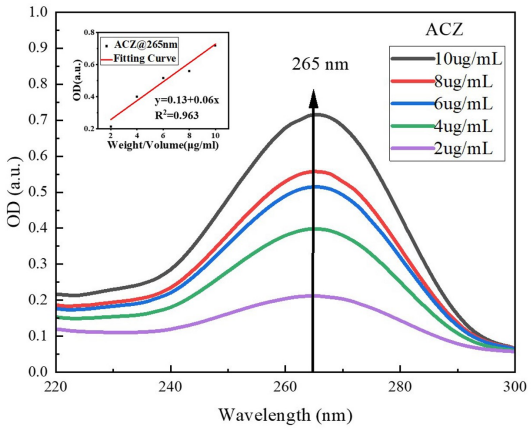

Fig. 1. Detection wavelength and standard curve of acetazolamide (ACZ).

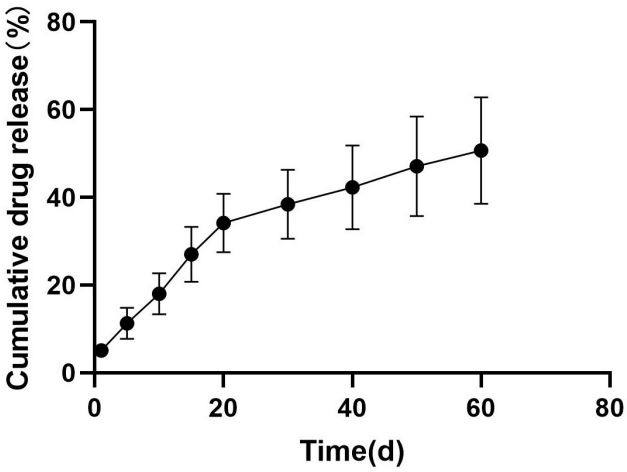

Fig. 2. The proportion of ACZ released at various time points. N = 3 in each group.

Histological and Immunohistochemical Analysis

The anatomical components were preserved in a 10% formaldehyde solution for 48 hours at room temperature. Then samples were placed in a 10% EDTA solution, and the EDTA was replaced every two days until the tissue decalcification process was finished. The samples that had been decalcified underwent many steps of dehydration with ethanol, diaphanization, and paraffin embedding before being cleaned with running water. Within the longitudinal plane of the root, each sample was divided into 40 segments, each measuring 5 m. Hematoxylin-eosin (HE) were used to stain a particular portion. The other part was stained with OPG and RANKL immunohistochemistry. For the immunohistochemical analysis, the samples were exposed to OPG antibody (GB11151-100, Servicebio, Changsha, China) and RANKL antibody (23408-1-AP, Proteintech, Wuhan, China) at the dilution of 1:100, immunostaining with streptavidin-biotin. Pannoramic Desk (2.2.0 soft-

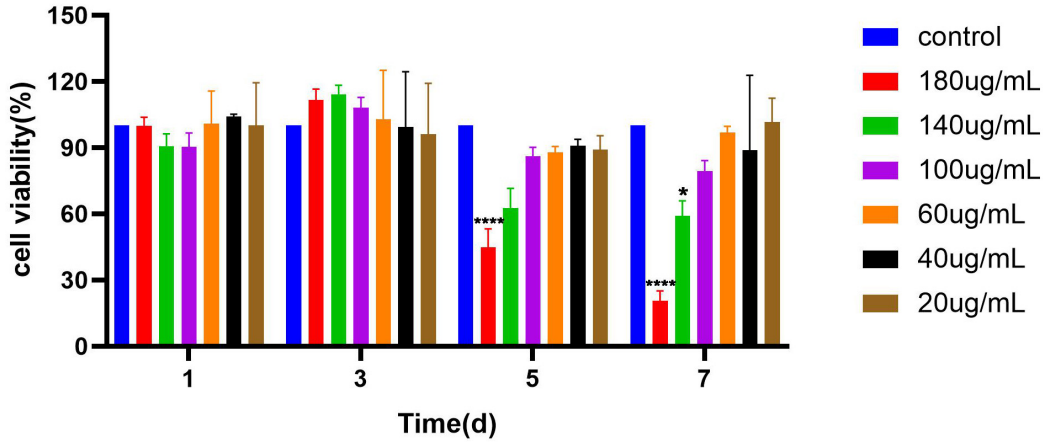

Fig. 3. CCK-8 (cell counting kit-8) tests of HPDLFs (human periodontal ligament fibroblasts) exposed to various test substance quantities (0, 20, 40, 60, 100, 140, 180 µg/mL) on days 1, 3, 5 and 7. The standard deviation of the mean is used to present data. Substantial differences from control are shown by $*p < 0.05$, $****p < 0.0001$. N = 3 in each group.

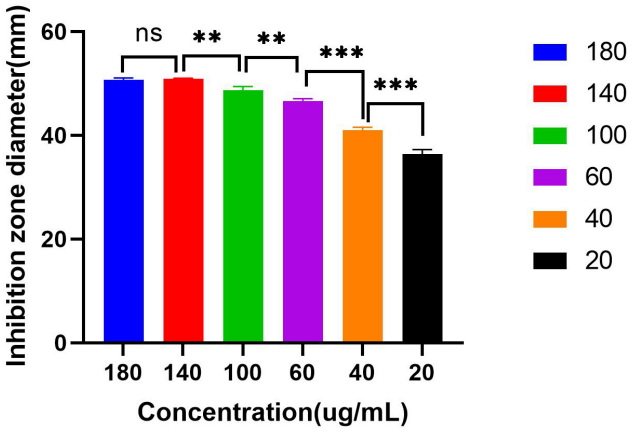

Fig. 4. Zone of inhibition of different concentrations (0, 20, 40, 60, 100, 140, 180 µg/mL) against *Fn* (Fusobacterium nucleatum) in blood agar medium. Bar graphs displaying the sizes of the zones of inhibition brought about by several drugs used to treat *Fn*. Data were shown as mean ± standard deviation (SD), ns, no significance, $**p < 0.01$, $***p < 0.001$. N = 3 in each group.

ware, 3DHISTECH, Budapest, Hungary) and Pannoramic Scanner software (panoramic 250, 3DHISTECH Ltd., Budapest, Hungary) were used to take all of the pictures of the histological sections. The middle third of the palatal root face and its surrounding tissues were the subjects of the analysis since it included cementum and PDL and had not been surgically injured. In order to do a histomorphometric study, Image J software (version 2.1.2, National Institutes of Health, Bethesda, MD, USA) was used to determine the lengths and areas of each kind of root resorption. The measurements were expressed as percentages and were based on the middle third of each root. Image J (1.36 software, NIH Image J system, Bethesda, MD, USA) was used to assess the results of an immunohistochemical investigation to determine the expression of OPG and RANKL.

Statistical Analysis

Standard deviation (SD) was used to represent all data as mean values. The data were examined with one-way analysis of variance (ANOVA) using the SPSS 18.0 software (IBM SPSS Statistics, Chicago, IL, USA). Dunnett's test was used to evaluate comparisons between the groups. Furthermore, statistical significance was determined by $p < 0.05$.

Results

Medicament Release

The maximum absorption wavelength was detected by ultraviolet spectrophotometer at 265 nm. The standard curve equation was drawn with drug concentration as X-axis and peak area as Y-axis: ACZ $y = 0.13 + 0.06x$, $R^2 = 0.963$ (Fig. 1). The data acquired were used to generate an *in vitro* drug release plot of time against the cumulative percentage of drug release, which showed a regulated release over 60 days. The cumulative drug release increases over time (Fig. 2).

Cytotoxicity Test

From 100 µg/mL to 20 µg/mL, the materials exhibited excellent biocompatibility, with cell viability above 90% on days 1, 3, 5 and 7 ($p > 0.05$, Fig. 3), which is above the 70% survival rate advised by global standards for assessing medicinal products (ISO 10993-5). At 140 µg/mL and 180 µg/mL, the materials displayed good biocompatibility on day 1 and 3. On the other hand, on days 5 and 7, a concentration of 140 µg/mL resulted in reductions of 62.7% and 59.1% in cell survival, respectively ($p > 0.05$, Fig. 3). Up to a concentration of 180 µg/mL, the increased cytotoxicity was seen; on day 5, the percent of cell viability dropped by 50% ($*p < 0.05$, Fig. 3). On day 7, a worsening of the drug's impact on cell viability was saw, with

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

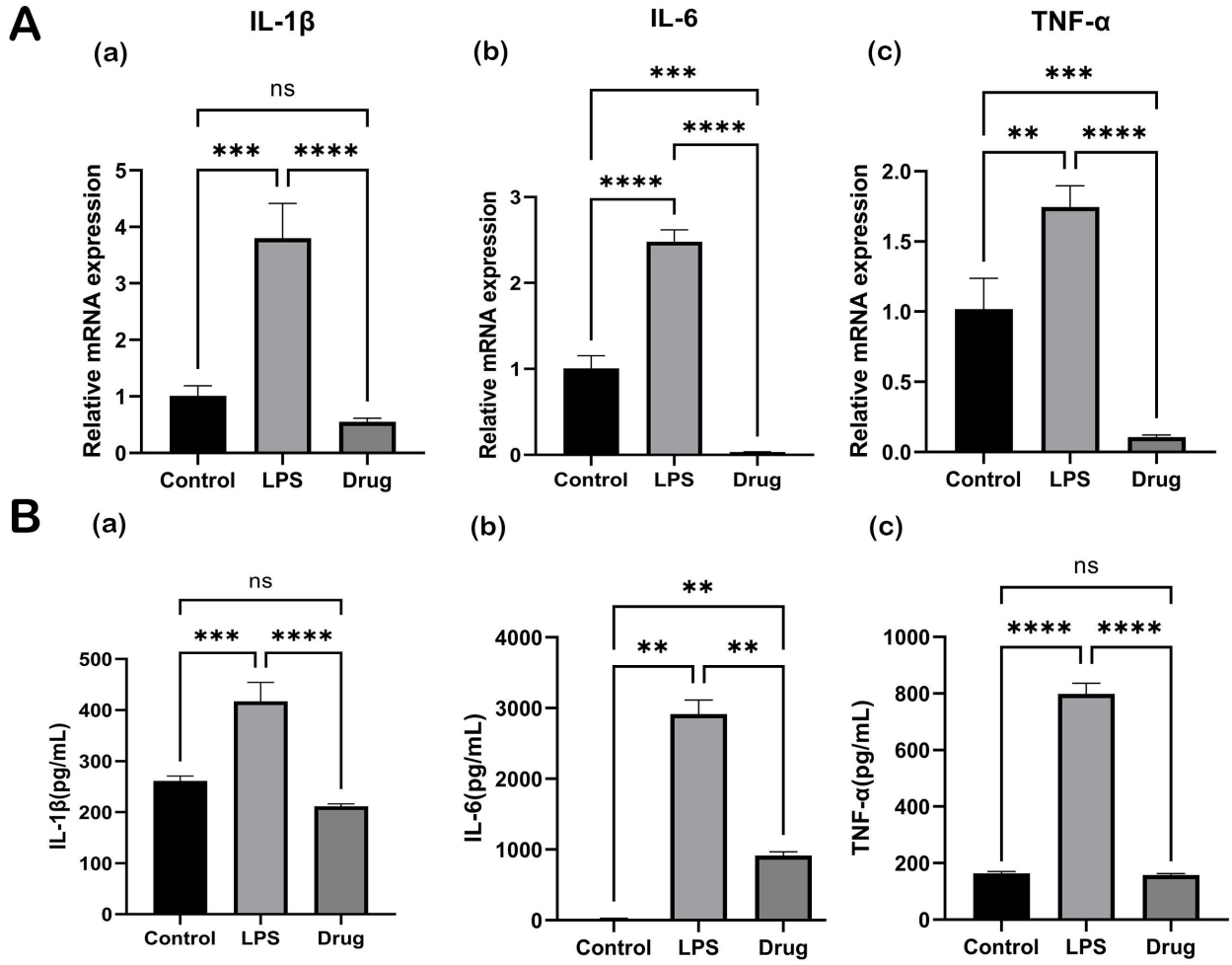

Fig. 5. Impact of medicine on IL-1β, IL-6 and TNF-α expression in HPDLFs cells. (A) Real-time polymerase chain reaction (RT-PCR) was used to analyze the mRNA levels of IL-1β (a), IL-6 (b) and TNF-α (c) after cells were treated with lipopolysaccharide (LPS) (10 μg/mL) with or without medication (100 μg/mL) for 48 hours. (B) ELISA (enzyme-linked immunosorbent assay) was used to quantify IL-1β (a), IL-6 (b) and TNF-α (c) in a conditioned culture medium after 48 hours. The results were displayed as means ± SD (n = 6). ns, no significance, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. N = 3 in each group. Note: IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; HPDLFs, human periodontal ligament fibroblasts; LPS, lipopolysaccharide.

the cell survival rate falling to 20.6%. Therefore, 20–100 μg/mL concentrations of the materials might be suitable for subsequent cell experiments.

Antimicrobial Assay

The minimum inhibition zones (mm^2) against *Fn* (Fusobacterium nucleatum) were measured to assess the antibacterial action of the test drugs at various dosages (Fig. 4). An unmistakable circular band encircling the medications was the inhibitory halo. Drugs in all concentrations showed clear inhibition zones. From 140 μg/mL to 20 μg/mL, the inhibitory zone size significantly increased with higher drug concentration ($p < 0.05$): 50.87 ± 0.15 mm at 140 μg/mL, 48.73 ± 0.70 mm at 100 μg/mL, 46.57 ± 0.49 mm at 60 μg/mL, 41.00 ± 0.56 mm at 40 μg/mL, 36.43 ± 0.84 mm at 20 μg/mL. Nevertheless, there was no statistically noteworthy variation among the 140 μg/mL and 180 μg/mL categories for the diameter of the inhibition zone ($p > 0.05$).

Impact of Medication on HPDLF Cells' Expression of IL-1β, IL-6 and TNF-α after being Exposed to LPS

We measured the levels of IL-1β, IL-6 and TNF-α at both the mRNA and protein levels in HPDLFs cells stimulated by LPS to ascertain the effect of the medication on the production of inflammatory cytokines. We conducted cell cytotoxicity and antibacterial experiments using various concentrations of medicament dilutions and found that a concentration of 100 μg/mL exhibited effective antibacterial properties and satisfactory long-term cell compatibility (Fig. 5). The mRNA levels of IL-1β, IL-6 and TNF-α were shown to have significantly increased after 48-hour expo-

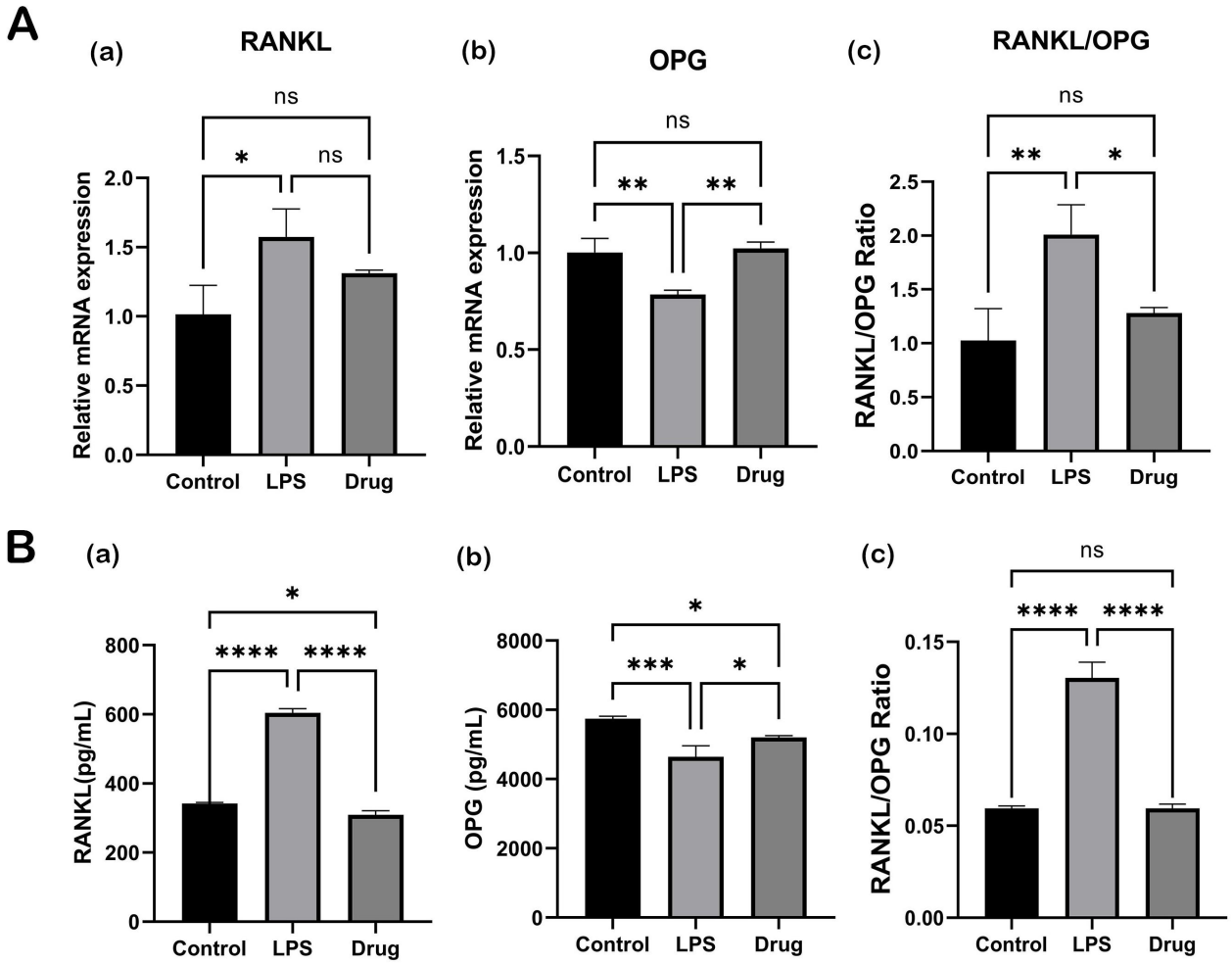

Fig. 6. Effects of medicament on RANKL (Receptor Activator of Nuclear Factor-κB Ligand) and OPG (Osteoprotegerin) expression in HPDLFs cells. (A) RT-PCR was used to analyze the RANKL and OPG mRNA levels after cells were stimulated with LPS (10 μg/mL) with or without medication (100 μg/mL) for 48 hours. (a) RANKL. (b) OPG. (c) RANKL/OPG. (B) At 48 hours, ELISA was used to quantify secreted RANKL and OPG in a conditioned culture medium. (a) RANKL. (b) OPG. (c) RANKL/OPG. The data were shown as means ± SD. ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. N = 3 in each group.

sure to LPS in comparison to the control category, according to the findings of RT-qPCR ($p < 0.01$, Fig. 5). When the medication was added to the culture medium, it prevented LPS from stimulating the release of inflammatory cytokines. The protein levels of IL-1β, IL-6 and TNF-α were considerably higher in the LPS category relative to the control category ($p < 0.01$, Fig. 5), supporting the RT-qPCR findings. When compared to the LPS category, treatment with the medication significantly decreased the levels of the proteins IL-1β, IL-6 and TNF-α ($p < 0.01$, Fig. 5).

Impact of Medicine on LPS-Stimulated RANKL and OPG Expression in HPDLFs Cells

We noticed changes in the expression of RANKL/OPG in HPDLFs cells after LPS stimulation and medication treatment. The mRNA expression of RANKL in the LPS group was significantly higher than in the control group, according to the RT-PCR findings ($p < 0.05$, Fig. 6A). The mRNA expression of OPG, however, was considerably reduced in the LPS cohort ($p < 0.01$, Fig. 6A). These results implied that the RANKL/OPG ratio was upregulated in the LPS group when compared to the control category ($p < 0.01$, Fig. 6A). A small reduction in mRNA expression of RANKL was seen when the medication was added to LPS-stimulated HPDLFs cells, but this difference was not statistically different from the LPS cohort ($p > 0.05$, Fig. 6A). The medication group, in contrast to the LPS group, showed a significantly higher level of OPG expression, which is remarkable ($p < 0.01$, Fig. 6A). The medicine may be able to reduce the RANKL/OPG ratio in the LPS group, according to this evidence ($p < 0.05$, Fig. 6A). Outcome of ELISA experiments were consistent with the findings observed in the RT-PCR analysis. However, treatment with the

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

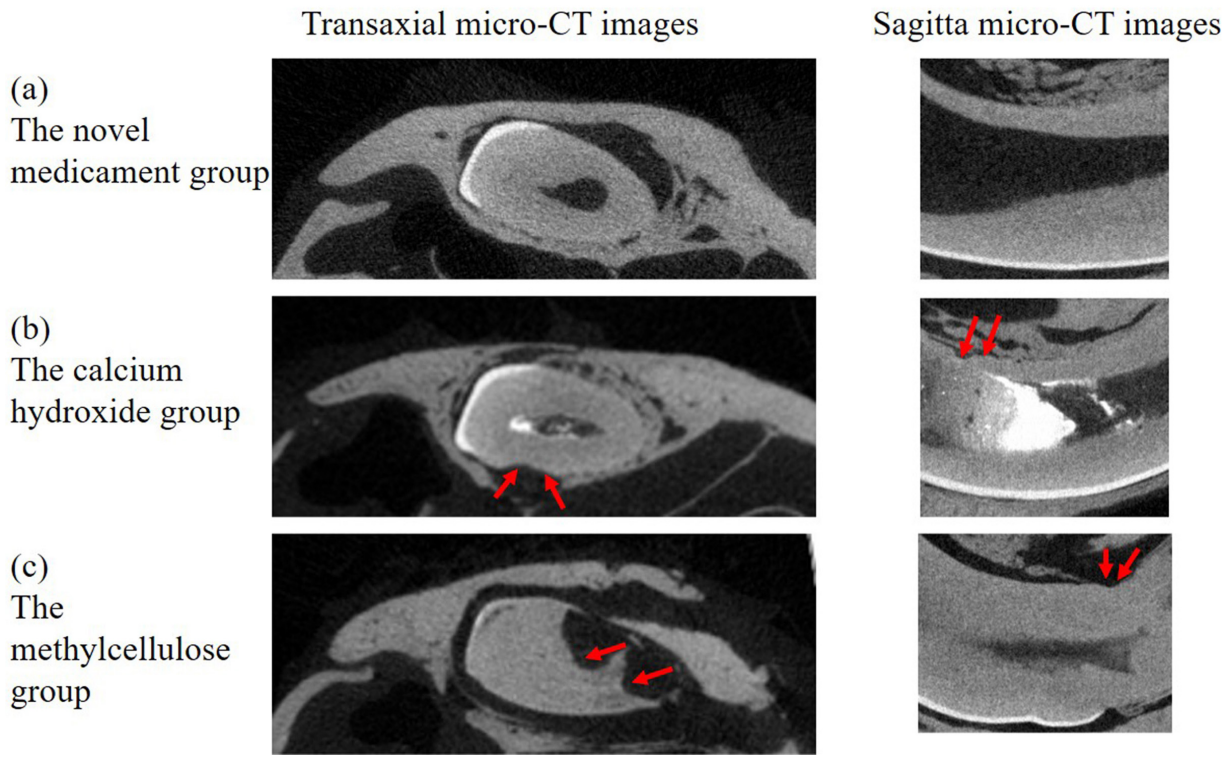

Fig. 7. 60 days following delayed tooth replantation, representative transaxial and sagitta micro-computed tomography images of each group were taken using CTAn software (version 1.17.7.2, Skyscan, Kartuizersweg, Belgium). Red arrows indicate sections showing damage to the root. (a) The novel medicament group. (b) The calcium hydroxide group. (c) The methylcellulose group.

Table 1. Resorption lengths as a percentage of total root length in each experimental group [M (P25, P75), %].

Groups	Inflammatory resorption	Replacement resorption	Surface resorption
Control	11.08 (10.93, 18.55)	70.12 (64.00, 79.15)	10.19 (2.47, 11.02)
CH	$0.02^{\#\#\#\#}$ (0.02, 0.02)	$24.23^{\#}$ (12.82, 35.07)	$5.35^{\#}$ (1.34, 10.62)
Drug	0.01^{****} (0.01, 0.01)	6.13^{****} (0.01, 7.87)	3.01^{*} (1.07, 7.87)

$^{*}p_{\text{(Drug vs. Control)}} < 0.05$, $^{****}p_{\text{(Drug vs. Control)}} < 0.0001$, $^{\#}p_{\text{(CH vs. Control)}} < 0.05$, $^{\#\#\#\#}p_{\text{(CH vs. Control)}} < 0.0001$. N = 6 in each group. CH, calcium hydroxide.

medication effectively reduced RANKL protein levels and increased OPG expression, resulting in a downregulation of the RANKL/OPG ratio (Fig. 6B).

Micro-CT Analysis

The micro-CT examination of the three intracanal medications used to treat the delayed replanted right maxillary incisors. The innovative medication group displayed a reasonably intact root form, with just a small region of root resorption, as shown in Fig. 7a, which displays sample transaxial and sagitta micro-CT images for every group. In contrast, the calcium hydroxide group showed compromised root integrity, with a noticeable amount of absorption observed (Fig. 7b). The methylcellulose group displayed significant damage to the root shape, with a wide range of root resorption observed (Fig. 7c). When compared to the normal control group, all groups demonstrated a certain degree of ankylosis, with the calcium hydroxide group exhibiting the most pronounced degree of ankylosis.

Histological Analysis

All experimental animals tolerated the surgical procedure. However, two teeth in the methylcellulose group were excluded from analysis due to root fracture during exodontia. In the methylcellulose (Control) group, severe root resorption was observed in all specimens (90.08%~91.00%, Table 1, Fig. 8D). Three specimens primarily displayed replacement resorption accompanied by localized inflammation resorption (Fig. 8A). In two of these specimens, a significant presence of active osteoclasts was observed around the areas of bone resorption (Fig. 8A). Additionally, a reduction in the number of periodontal ligament fibers was observed in all samples, with a disorganized pattern (Fig. 8A).

In the calcium hydroxide (CH) group, moderate levels of root resorption were observed (20.26%~52.03%, Table 1, Fig. 8D), with a larger extent of root damage compared to the novel medicament group. All samples exhib-

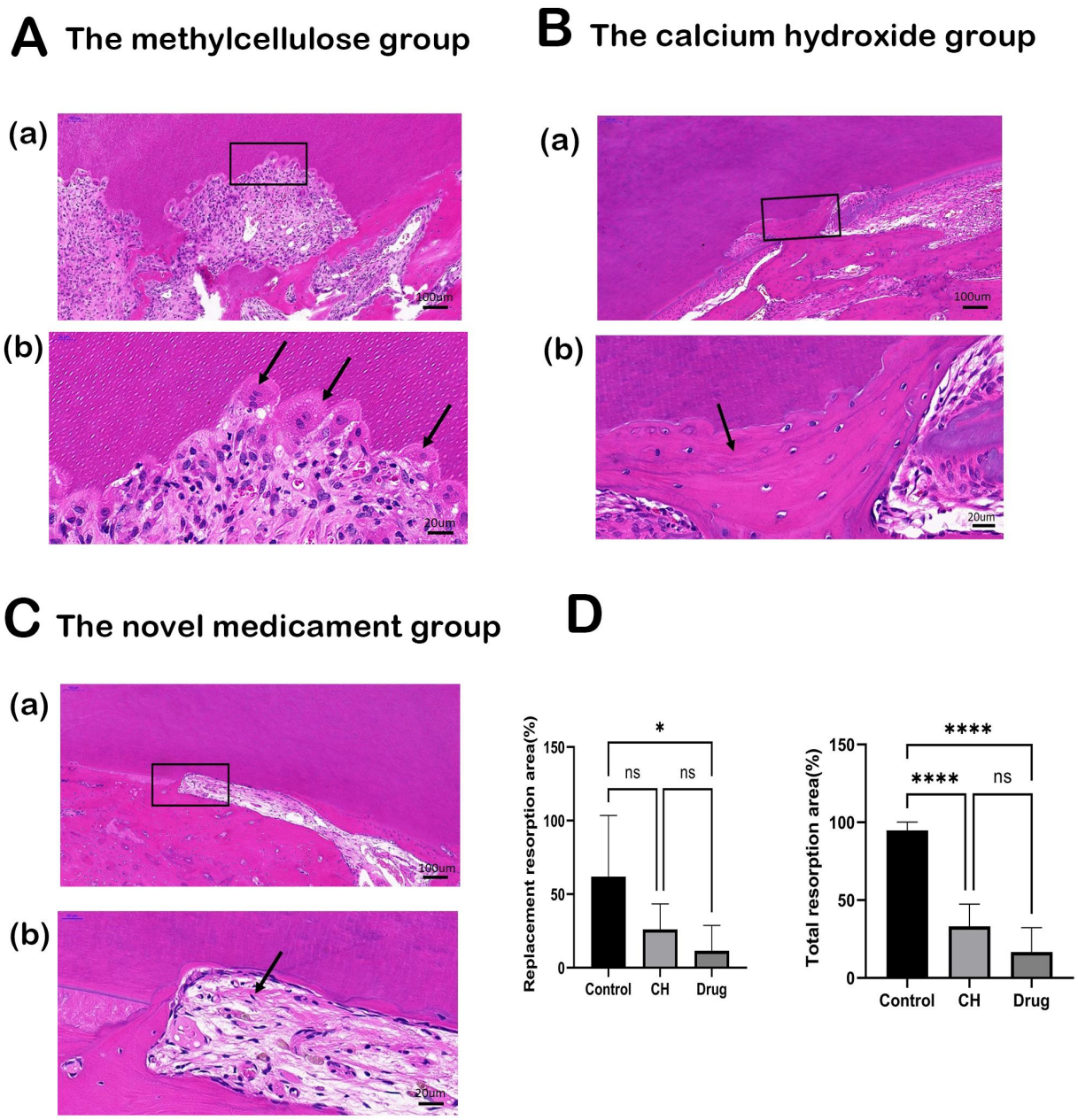

Fig. 8. Representative histological images of the different groups. (A) Inflammatory resorption of the methylcellulose group: (a) Increased dentine depth was compromised by active inflammatory resorption (rectangle). Scale bar: 100 μm. (b) Active clastic cells (arrow) and inflammatory infiltrating in connective tissue. Scale bar: 20 μm. (B) Replacement resorption of the calcium hydroxide group: (a) Extensive ankylosis and scattered disorganized connective tissue (rectangle). Scale bar: 100 μm. (b) Replacement resorption areas (arrow). Scale bar: 20 μm. (C) Periodontal ligament healing of the novel medicament group: (a) Periodontal ligament healing in the resorption area (rectangle). Scale bar: 100 μm. (b) Periodontal fiber junction (arrow). Scale bar: 20 μm. (D) The length percentage of inflammatory absorption and replacement absorption areas in different groups. ns, no significance, *$p < 0.05$, ****$p < 0.0001$. N = 6 in each group.

ited different degrees of replacement resorption, without any signs of inflammatory resorption, among which, four samples showed severe replacement resorption with surface resorption affecting multiple sites (Fig. 8B). In all samples, collagen fibers were disorganized, with some fibrous tissue being replaced by osseous tissue (Fig. 8B). Two categories showed no residual fibrous connective tissue and alveolar bone tissue filled the PDL area instead (Fig. 8B).

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

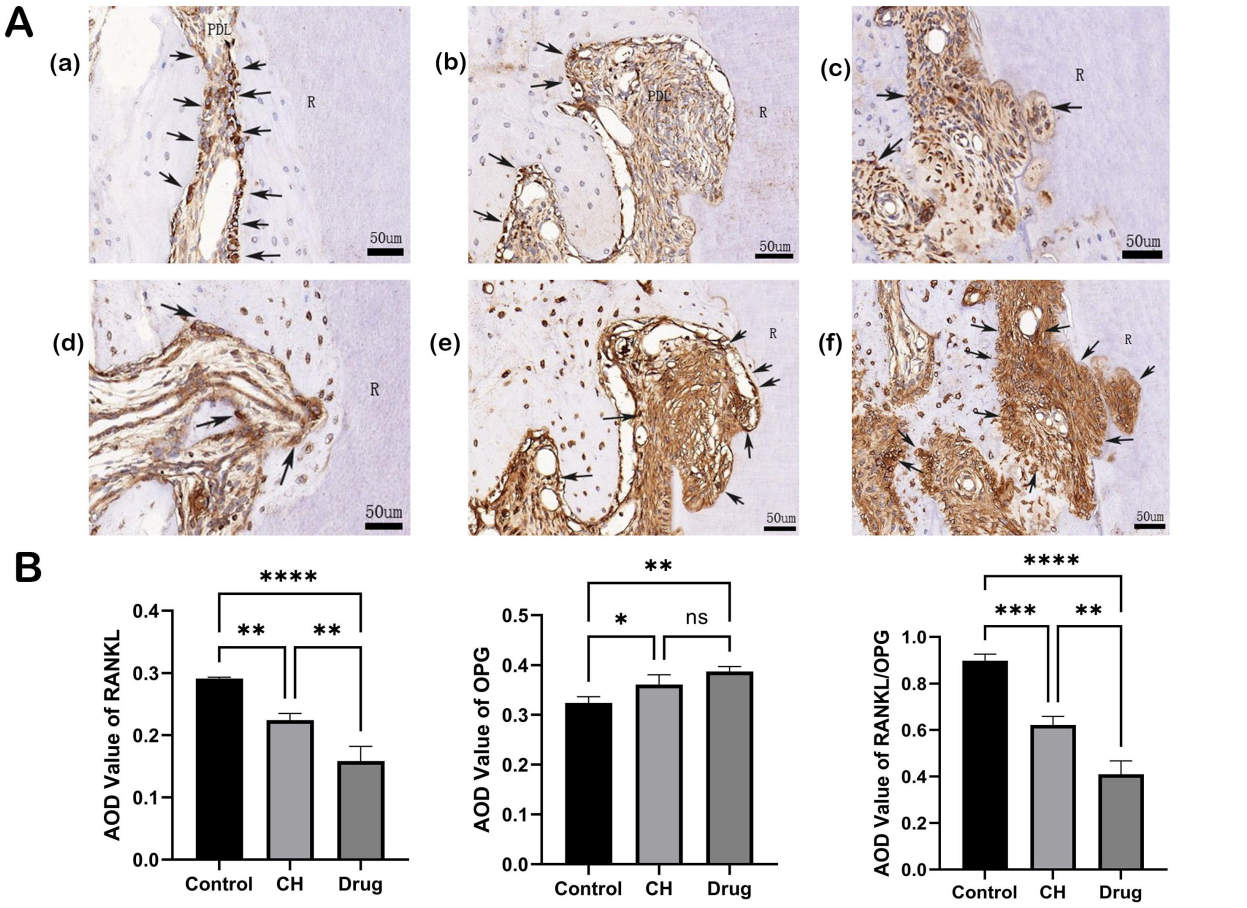

Fig. 9. Effects of medicament on RANKL and OPG expression in delayed tooth replantation rat model. (A) Representative immunohistochemical images of the different groups. (a,b,c) RANKL. (d,e,f) OPG. Immunohistochemically positive cells (arrows); root (R); periodontal ligament (PDL). Scale bar: 50 μm. (B) Mean optical density values with immunohistochemical images and the RANKL/OPG ratio of the different groups. N = 6 in each group. ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

In the novel medicament (Drug) group, the extent of root resorption is the mildest among all the groups (Table 1, Fig. 8D). Only two samples displayed obvious replacement resorption, with resorption length percentages of 49% and 12%, respectively, and the remaining groups showed minor and localized surface resorption and replacement resorption (Fig. 8C). Most of the time, the collagen fibers in the periodontal fibrous tissue were disorganized, but in other spots, they showed a parallel alignment with the root surface (Fig. 8C). In one category, the region of root resorption saw the periodontal ligament reconnect to the root surface (Fig. 8C). There were no instances of root ankylosis or active osteoclasts observed in any of the samples.

Immunohistochemical Analysis

In the root resorption lacunae and disorganised periodontal ligament, immunohistochemical examination showed extensive expression of RANKL and OPG. Further cellular localization studies showed that both PDL fibroblasts and macrophages were capable of secreting RANKL, while osteoblasts and PDL fibroblasts exhibited predominant expression of OPG (Fig. 9A). When the immunostaining intensities of the various categories were evaluated, it was found that CH and Drug groups had significantly lower RANKL immunoreactivity as well as higher OPG staining than the control group ($p < 0.05$, Fig. 9B). As a result, the RANKL/OPG ratio was down-regulated ($p < 0.05$, Fig. 9B).

Discussion

Inflammatory root resorption is widely recognized as the primary cause of treatment failure in delayed tooth replantation. Currently, it is widely accepted that this process involves complex chemical signaling pathways, which stimulate osteoclast-mediated cellular activities including activation/fusion, adhesion, and phagocytosis [15]. Due to its antibacterial characteristics and capacities to induce mineralization, calcium hydroxide (CH) is frequently used in clinical practice to prevent or attenuate root resorption

brought on by bacterial infection. However, CH has limitations including a lack of anti-inflammatory properties, reduced dentin fracture resistance with long-term usage, and the need for multiple medication changes [10]. Therefore, it is crucial to develop more efficacious root canal medicaments to combat tooth root resorption. The objective of this study is to utilize current insights into the pathological mechanisms and design an innovative injectable sustained-release paste. We have found early data suggesting that this new paste affects osteoclast activity through the RANKL/RANK/OPG path through both *in vitro* as well as *in vivo* investigations, thus exerting an inhibitory effect on tooth root resorption.

In vitro, antimicrobial experiments have demonstrated the remarkable antibacterial efficacy of the novel paste within the root canal system. Fouad *et al*. [16] reported that dental pulp or periapical infections arising from dental trauma are primarily caused by facultative and strictly anaerobic bacteria. Additionally, Nagata *et al*. [17] assessed the microbial makeup of developing teeth with damage, revealing the presence of Actinomyces in 33.34% of the root canals. Therefore, in our study, Actinomyces was selected for agar diffusion experiments to verify the antibacterial activity of combining clindamycin with various drugs. The results indicated a concentration-dependent antibacterial effect of the paste within a specific range. Interestingly, elevated concentrations of antibiotic medication beyond this range did not result in stronger antimicrobial activity. It follows that root canal disinfection does not require the use of larger doses of antibiotic medicine. These results are in line with an analysis by Cunha *et al*. [18] that looked at the effectiveness of clindamycin in cleaning up biofilms inside dentinal tubules. The results can be explained by either the saturation of antibiotic-bacterial target binding at maximum bactericidal concentration or reaching the limit of their mechanism of action. Additionally, bacterial peculiarities in terms of drug resistance may enable survival even in the presence of high antibiotic concentrations. Unfortunately, the pharmacokinetics of drugs in the periapical region remain elusive [19], warranting further research to determine whether a concentration of 100 μg/mL clindamycin is sufficient to inhibit bacterial growth in the periapical tissues.

It is critical to analyse the cytotoxicity of intracanal medications in addition to their antibacterial properties. This is because immature permanent teeth with broad apical foramen frequently sustain dental trauma [20]. Facilitating direct contact between the medicament and periapical tissues through the patent apical foramen. Hence, possessing biocompatible properties is important for materials used in replanted teeth to create an optimal microenvironment for healing. The HPDLFs play dual roles in local inflammatory and immunological responses as essential immune regulatory cells and structural components [21]. Furthermore, their functions can be regulated by the microenvironment [22]. According to the study's results, the paste's cytotoxicity was shown to rely on both the level of concentration and length of exposure, with greater concentrations and longer exposure durations resulting in increasing toxicity. Numerous studies have demonstrated that reducing drug concentrations can mitigate cytotoxic effects [23–26]. Ferreira MB, Dubey N and others compared the cytotoxicity of clindamycin with common intracanal antibiotics such as minocycline and metronidazole and found that the clindamycin group exhibited superior cell viability [27,28]. Mori GG and colleagues [29] confirmed the favorable biocompatibility of acetazolamide through subcutaneous tissue injection in rats. Existing research has shown that high doses of corticosteroids in root canals are unlikely to cause systemic side effects [30], indicating good biocompatibility of corticosteroids in root canals [31]. In the field of dentistry, methylcellulose hydrogel has excellent biocompatibility [32] and injectability. They are extensively used for controlled delivery of low-concentration intracanal medications [33]. Despite variations that may occur due to differences in materials and methods [34], the trend is to use lower concentrations of intracanal medicaments while maximizing therapeutic efficacy. This study found that a 100 ug/mL dilution significantly inhibited bacterial growth without causing any toxicity to HPDLFs based on antimicrobial and cytotoxicity assessments. This finding provided support for subsequent foundational experiments.

The resolution of Actinomyces infection does not mean the termination of resorption. Inflammatory root resorption is a process driven by the interaction between bacterial infection, tissue damage, and the host response. Therefore, in addition to employing antibacterial approaches, considering therapeutic strategies that may modulate osteoclast activity is logically sound in anti-resorptive treatment.

To learn more about how the paste affects inflammatory and osteoclastic components in the inflammatory microenvironment, we employed a high concentration of LPS from *Escherichia coli* to stimulate HPDLFs. High doses of LPS have been shown to stimulate osteoclast development and trigger inflammatory responses in several investigations [35–37]. TNF-α, IL-1β and IL-6 expression were all elevated in our research after LPS stimulation, and the RANKL/OPG ratio also increased. The RANKL/OPG ratio was fully reversed after treatment with the paste, which also suppressed the production of inflammatory factors brought on by LPS. Additionally, the new drug's partial potential to prevent osteoclastogenesis is revealed by its suppressive impact on LPS-induced TNF-α, IL-1β and IL-6 production. The results were further supported by *in vivo* animal experiments. The RANKL/OPG ratio significantly decreased in the new paste group, falling below 1, according to immunohistochemical staining of periodontal tissues and teeth collected 60 days after replantation. Furthermore, the presence of sporadic, atypical connective tissue around the

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

teeth suggested a later possibility of new bone growth. Histological examination (HE staining) and micro-CT analysis demonstrated that rats treated with the novel paste displayed improved root integrity without apparent signs of inflammation-induced resorption. Numerous studies have increasingly shown that osteoclast-mediated dental root resorption is regulated by the RANKL/RANK/OPG signaling pathway [38,39]. Particularly, RANK acts as a receptor carried by osteoclasts, whereas RANKL activates osteoclasts by functioning as a ligand for the RANK receptor. On the other hand, OPG, sometimes referred to as osteoprotegerin, inhibits osteoclastogenesis. To manage the resorption process, this route dynamically modulates the ratio of RANKL to OPG [40]. During the host's inflammatory response, TNF-α and IL-6 are potent osteoclastogenic factors [41], inducing osteoclast differentiation by mediating RANKL stimulation through autocrine mechanisms [42]. Studies conducted both *in vitro* as well as *in vivo* have shown that they can encourage the production of osteoclasts, which can enhance root resorption [43,44]. However, only in the presence of IL-1 can osteoclasts induced by TNF-α form resorption lacunae on dentin [44]. In summary, TNF-α, IL-1, and IL-6 work synergistically to increase the ratio of RANKL/OPG and subsequent stimulation of osteoclast differentiation [45]. Treatment with the novel paste suppresses inflammation and the activation of the RANKL/RANK/OPG pathway, thereby reducing inflammation-related root resorption. To clarify the particular chemical pathways at play, more research is necessary.

Additionally, research findings showed that the calcium hydroxide group can also inhibit inflammatory resorption. However, it is associated with a more widespread occurrence of replacement resorption in the root and a higher frequency of ankylosis within 60 days. Although these complications cannot be entirely avoided with the use of novel medication, they can reduce the severity of replacement resorption and delay the progression of ankylosis, thus prolonging the functional lifespan of replanted teeth [46]. Furthermore, *in vitro* simulations of drug release from the apical foramen demonstrated that the paste exhibits excellent sustained-release capabilities. As a result, there is no need to often change medications throughout the 60 days that the International Association of Dental Traumatology (IADT) advises [47] lowering the chance of infection recurrence. A potential medium for intracanal sustained-release therapy, as shown by prior investigations, is the methylcellulose system. Additionally, the apical foramen and dentinal tubules of the tooth itself have anatomical features that impede the quick release of medicament from the canal. However, further clinical trials are required to validate these findings. However, this study is a single center, small sample study, which may limit the generalization of research results and further research is needed to confirm.

Conclusion

In summary, our study developed a novel sustained-release intracanal paste using methylcellulose as a carrier and incorporating clindamycin, acetazolamide, and triamcinolone as constituents, which demonstrated excellent antibacterial activity and biocompatibility *in vitro* and significant anti-inflammatory and anti-resorptive effects both *in vitro* and *in vivo*. Consequently, the novel intracanal paste shows promising therapeutic potential in delayed teeth replantation anti-resorption treatment.

Availability of Data and Materials

The data could be obtained by contacting the corresponding authors.

Author Contributions

YW: contributed to conception, design, data acquisition, analysis, and interpretation, drafted, and critically revised the manuscript. HD: contributed to conception, data acquisition, analysis, and interpretation, drafted, and critically revised the manuscript. YH: contributed to conception, data acquisition and analysis, drafted, and critically revised the manuscript. SHe: contributed to conception, data acquisition and interpretation, and critically revised the manuscript. SHu: contributed to data analysis and interpretation and critically revised the manuscript. XS: contributed to data analysis and interpretation and critically revised the manuscript. BS: contributed to data analysis and interpretation and critically revised the manuscript. NW: contributed to technical and material support and critically revised the manuscript. GC: contributed to conception, design, data analysis and interpretation, drafted, and critically revised the manuscript. TG: contributed to conception, design, data analysis and interpretation, drafted, and critically revised the manuscript. These contributions reflect the collaborative effort of all authors in conducting and presenting this research. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The Affiliated Stomatological Hospital of Nanjing University School of Medicine (Nanjing, China) approved the study (Ethical No. PY2017023). The animal experiments were conducted in cooperation with Nanjing Agricultural University and carried out in compliance with the guidelines of the Nanjing Agriculture University's Animal Ethics Committee (PZW2020010).

Acknowledgment

Not applicable.

Funding

This study was financially supported by the Social Development Foundation of Jiangsu Province (Program No. BE2019623); the "Six Ones Project" of the High-Level Health Talents Top-notch Project of Jiangsu Province (No. LGY2019010); and the Foundation of the Nanjing Commission of Health (No. YKK21184).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Chae YK, Shin SY, Kang SW, Choi SC, Nam OH. Differential gene expression profiles of periodontal soft tissue from rat teeth after immediate and delayed replantation: a pilot study. Journal of Periodontal & Implant Science. 2022; 52: 127–140.

[2] Souza BDM, Dutra KL, Kuntze MM, Bortoluzzi EA, Flores-Mir C, Reyes-Carmona J, *et al*. Incidence of Root Resorption after the Replantation of Avulsed Teeth: A Meta-analysis. Journal of Endodontics. 2018; 44: 1216–1227.

[3] Müller DD, Bissinger R, Reymus M, Bücher K, Hickel R, Kühnisch J. Survival and complication analyses of avulsed and replanted permanent teeth. Scientific Reports. 2020; 10: 2841.

[4] Galler KM, Grätz EM, Widbiller M, Buchalla W, Knüttel H. Pathophysiological mechanisms of root resorption after dental trauma: a systematic scoping review. BMC Oral Health. 2021; 21: 163.

[5] Alhajj MN, Daud F, Al-Maweri SA, Johari Y, Ab-Ghani Z, Jaafar M, *et al*. Effects of calcium hydroxide intracanal medicament on push-out bond strength of endodontic sealers: A systematic review and meta-analysis. Journal of Esthetic and Restorative Dentistry. 2022; 34: 1166–1178.

[6] Shaik I, Dasari B, Kolichala R, Doos M, Qadri F, Arokiyasamy JL, *et al*. Comparison of the Success Rate of Mineral Trioxide Aggregate, Endosequence Bioceramic Root Repair Material, and Calcium Hydroxide for Apexification of Immature Permanent Teeth: Systematic Review and Meta-Analysis. Journal of Pharmacy & Bioallied Sciences. 2021; 13: S43–S47.

[7] Abbott PV. Prevention and management of external inflammatory resorption following trauma to teeth. Australian Dental Journal. 2016; 61 Suppl 1: 82–94.

[8] Vogt BF, Souza CECP, Silva DN, Etges A, Campos MM. Evaluation of two formulations containing mineral trioxide aggregate on delayed tooth replantation: relevance of RANKL/RANK/OPG system. Odontology. 2016; 104: 211–219.

[9] Consolaro A, Miranda DAO, Consolaro RB. Orthodontics and Endodontics: clinical decision-making. Dental Press Journal of Orthodontics. 2020; 25: 20–29.

[10] Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. International Endodontic Journal. 2011; 44: 697–730.

[11] Karczewski A, Feitosa SA, Hamer EI, Pankajakshan D, Gregory RL, Spolnik KJ, *et al*. Clindamycin-modified Triple Antibiotic Nanofibers: A Stain-free Antimicrobial Intracanal Drug Delivery System. Journal of Endodontics. 2018; 44: 155–162.

[12] Zargar N, Rayat Hosein Abadi M, Sabeti M, Yadegari Z, Akbarzadeh Baghban A, Dianat O. Antimicrobial efficacy of clindamycin and triple antibiotic paste as root canal medicaments on tubular infection: An *in vitro* study. Australian Endodontic Journal. 2019; 45: 86–91.

[13] Abbas KF, Tawfik H, Hashem AAR, Ahmed HMA, Abu-Seida AM, Refai HM. Histopathological evaluation of different regenerative protocols using Chitosan-based formulations for management of immature non-vital teeth with apical periodontitis: *In vivo* study. Australian Endodontic Journal. 2020; 46: 405–414.

[14] Perin CP, Papalexiou V, Rodrigues Johann ACB, Ribeiro Mattos NH, Westphalen FH, Ditzel Westphalen VP. Evaluation of intracanal acetazolamide in late reimplanted rat teeth. European Journal of Dentistry. 2017; 11: 417–421.

[15] Iglesias-Linares A, Hartsfield JK, Jr. Cellular and Molecular Pathways Leading to External Root Resorption. Journal of Dental Research. 2017; 96: 145–152.

[16] Fouad AF. Microbiological aspects of traumatic injuries. Dental Traumatology: Official Publication of International Association for Dental Traumatology. 2019; 35: 324–332.

[17] Nagata JY, Soares AJ, Souza-Filho FJ, Zaia AA, Ferraz CCR, Almeida JFA, *et al*. Microbial evaluation of traumatized teeth treated with triple antibiotic paste or calcium hydroxide with 2% chlorhexidine gel in pulp revascularization. Journal of Endodontics. 2014; 40: 778–783.

[18] Cunha Neto MAD, Coêlho JDA, Pinto KP, Cuellar MRC, Marcucci MC, Silva EJNL, *et al*. Antibacterial Efficacy of Triple Antibiotic Medication with Macrogol (3Mix-MP), Traditional Triple Antibiotic Paste, Calcium Hydroxide, and Ethanol Extract of Propolis: An Intratubular Dentin *Ex Vivo* Confocal Laser Scanning Microscopic Study. Journal of Endodontics. 2021; 47: 1609–1616.

[19] Wong M, White A, Jarrett J, Walsh LJ, George R. Evaluation of the diffusion of triamcinolone and demeclocycline through the dentinal tubules and apical foramen: A mass spectrometry study. Journal of Conservative Dentistry. 2022; 25: 420–425.

[20] Osborne R. What is the impact of primary dental avulsion injuries on the permanent dentition Evidence-Based Dentistry. 2021; 22: 136–137.

[21] Nashaat Y, Sabry H, Hassan SA. Evaluation of the Cytotoxicity and apoptotic effect of Nano triple antibiotic paste with Nano anti-inflammatory drug as an intracanal medicament. European Endodontic Journal. 2021; 6: 82–89.

[22] Yadlapati M, Souza LC, Dorn S, Garlet GP, Letra A, Silva RM. Deleterious effect of triple antibiotic paste on human periodontal ligament fibroblasts. International Endodontic Journal. 2014; 47: 769–775.

[23] Althumairy RI, Teixeira FB, Diogenes A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. Journal of Endodontics. 2014; 40: 521–525.

[24] Sabrah AHA, Yassen GH, Liu WC, Goebel WS, Gregory RL, Platt JA. The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established Enterococcus faecalis biofilm. Clinical Oral Investigations. 2015; 19: 2059–2066.

[25] Labban N, Yassen GH, Windsor LJ, Platt JA. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. Dental Traumatology. 2014; 30: 429–434.

[26] Alghilan MA, Windsor LJ, Palasuk J, Yassen GH. Attachment and proliferation of dental pulp stem cells on dentine treated with different regenerative endodontic protocols. International Endodontic Journal. 2017; 50: 667–675.

[27] Ferreira MB, Myiagi S, Nogales CG, Campos MS, Lage-Marques JL. Time- and concentration-dependent cytotoxicity of antibiotics used in endodontic therapy. Journal of Applied Oral Science: Revista FOB. 2010; 18: 259–263.

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

[28] Dubey N, Xu J, Zhang Z, Nör JE, Bottino MC. Comparative Evaluation of the Cytotoxic and Angiogenic Effects of Minocycline and Clindamycin: An *In Vitro* Study. Journal of Endodontics. 2019; 45: 882–889.

[29] Mori GG, Poi WR, Castilho LR. Evaluation of the antiresorptive ability of an experimental acetazolamide paste for the treatment of late replanted teeth: a study in rats. Dental Traumatology. 2013; 29: 34–40.

[30] Khan AA, Diogenes A. Pharmacological Management of Acute Endodontic Pain. Drugs. 2021; 81: 1627–1643.

[31] Kirakozova A, Teixeira FB, Curran AE, Gu F, Tawil PZ, Trope M. Effect of intracanal corticosteroids on healing of replanted dog teeth after extended dry times. Journal of Endodontics. 2009; 35: 663–667.

[32] Cruz EV, Kota K, Huque J, Iwaku M, Hoshino E. Penetration of propylene glycol into dentine. International Endodontic Journal. 2002; 35: 330–336.

[33] McIntyre PW, Wu JL, Kolte R, Zhang R, Gregory RL, Bruzzaniti A, *et al*. The antimicrobial properties, cytotoxicity, and differentiation potential of double antibiotic intracanal medicaments loaded into hydrogel system. Clinical Oral Investigations. 2019; 23: 1051–1059.

[34] Khoshkhounejad M, Sobhi Afshar M, Jabalameli F, Emaneini M, Sharifian M. Cytotoxicity Evaluation of Minimum Antibacterial Values of Different Medicaments Used in Endodontic Regenerative Procedures. European Journal of Dentistry. 2019; 13: 514–520.

[35] Li C, Xiao F, Wen Y, Wu J, Huang N. Krüppel-like factor 5 -mediated Sirtuin6 promotes osteogenic differentiation and inhibits inflammatory injury of lipopolysaccharide-induced periodontal membrane stem cells by inhibiting nuclear factor kappa-B pathway. Bioengineered. 2022; 13: 6966–6977.

[36] Jiang L, Song J, Hu X, Zhang H, Huang E, Zhang Y, *et al*. The Proteasome Inhibitor Bortezomib Inhibits Inflammatory Response of Periodontal Ligament Cells and Ameliorates Experimental Periodontitis in Rats. Journal of Periodontology. 2017; 88: 473–483.

[37] Wei L, Jiang Y, Zhou W, Liu S, Liu Y, Rausch-Fan X, *et al*. Strontium ion attenuates lipopolysaccharide-stimulated proinflammatory cytokine expression and lipopolysaccharide-inhibited early osteogenic differentiation of human periodontal ligament cells. Journal of Periodontal Research. 2018; 53: 999–1008.

[38] Silva I, Branco JC. Rank/Rankl/opg: literature review. Acta Reumatologica Portuguesa. 2011; 36: 209–218.

[39] Gregorczyk-Maga I, Kaszuba M, Olszewska M, Lichołai S, Iwaniec T, Kościelniak D, *et al*. Biomarkers of inflammatory external root resorption as a result of traumatic dental injury to permanent teeth in children. Archives of Oral Biology. 2019; 99: 82–91.

[40] Rani CS, MacDougall M. Dental cells express factors that regulate bone resorption. Molecular Cell Biology Research Communications. 2000; 3: 145–152.

[41] Yu F, Huo F, Li F, Zuo Y, Wang C, Ye L. Aberrant NF-κB activation in odontoblasts orchestrates inflammatory matrix degradation and mineral resorption. International Journal of Oral Science. 2022; 14: 6.

[42] Kwan Tat S, Padrines M, Théoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine & Growth Factor Reviews. 2004; 15: 49–60.

[43] Yokota K, Sato K, Miyazaki T, Kitaura H, Kayama H, Miyoshi F, *et al*. Combination of tumor necrosis factor α and interleukin-6 induces mouse osteoclast-like cells with bone resorption activity both i*n vitro* and *in vivo*. Arthritis & Rheumatology. 2014; 66: 121–129.

[44] Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, *et al*. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. The Journal of Experimental Medicine. 2000; 191: 275–286.

[45] García-López S, Villanueva R, Meikle MC. Alterations in the Synthesis of IL-1β, TNF-α, IL-6, and Their Downstream Targets RANKL and OPG by Mouse Calvarial Osteoblasts *In vitro*: Inhibition of Bone Resorption by Cyclic Mechanical Strain. Frontiers in Endocrinology. 2013; 4: 160.

[46] Marão HF, Panzarini SR, Aranega AM, Sonoda CK, Poi WR, Esteves JC, *et al*. Periapical tissue reactions to calcium hydroxide and MTA after external root resorption as a sequela of delayed tooth replantation. Dental Traumatology. 2012; 28: 306–313.

[47] Fouad AF, Abbott PV, Tsilingaridis G, Cohenca N, Lauridsen E, Bourguignon C, *et al*. International Association of Dental Traumatology guidelines for the management of traumatic dental injuries: 2. Avulsion of permanent teeth. Dental Traumatology. 2020; 36: 331–342.