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Anti-inflammatory and anti-resorptive efficacy of adrenergic blockers on late replanted rat incisors

Abstract

Background/Aim: In addition to their anti-inflammatory and anti-osteoclastogenesis properties, adrenergic blockers may also have promising anti-resorptive effects that can prevent root resorption when teeth are replanted because of avulsion. The aim of this study was to investigate the effects of phentolamine (Ph) and propranolol (Pr) in gels on the repair process of late replanted rat incisors. A further aim was to evaluate the biocompatibility of both drugs to human periodontal ligament fibroblasts (HPDLFs).

Material and methods: Forty-eight maxillary right incisors were extracted from male Wistar rats, stored in paper napkins for 60 minutes and randomly allocated to one of eight groups (n=6). The root canal, root surface and alveolus were treated with 0.75 µg/ml Ph gel (Ph0.75), 10 µg/ml Ph gel (Ph10), 100 µg/ml Ph gel (Ph100), 2.5 µg/ml Pr gel (Pr2.5), 10 µg/ml Pr gel (Pr10), 100 µg/ml Pr gel (Pr100), or sodium carboxymethylcellulose gel (CMC) before replantation. In the control group (CH), only the root canal was treated with calcium hydroxide paste. Thirty days following surgery, the animals were euthanized, and the right hemimaxilla was removed to perform micro-CT and histomorphometric analysis to determine osteoclastic activity. Ethanolic solutions of Ph10 and Pr10 were selected based on the *in vivo* study, and the viability of HPDLFs stimulated with lipopolysaccharide was determined by MTT assays. *Results:* The Micro-CT and histomorphometric analysis revealed no significant differences among the treatments ($P > 0.05$). The presence of active osteoclasts was significantly decreased in the Ph10 and Pr10 groups ($P < 0.05$). Ph10 and Pr10 produced statistically similar cell survival rates compared to the control group ($P > 0.05$). *Conclusions:* Ph10

and Pr10 significantly decreased osteoclastogenesis in delayed replanted rat teeth and were not cytotoxic towards HPDLFs.

Key words: adrenergic blockers; phentolamine; propranolol; root resorption; tooth avulsion; tooth replantation.

Introduction

The maintenance of periodontal ligament (PDL) cell viability and the prevention of root surface contamination are crucial determinants of the success of tooth replantation after avulsion. Replanting the tooth within 15 minutes of the avulsion is believed to be ideal because the chances of PDL regeneration and restoration of the normal function of the tooth are significantly higher, as dehydration of the tissues is prevented and, consequently, necrosis of the PDL and pulp is less likely.¹⁻³ Unfortunately, in most situations involving avulsion, teeth are rarely replanted within this time period, since patients, parents and professionals are not aware of or are unable to perform the replantation immediately after the accident.⁴⁻⁷ If the avulsed tooth is not immediately stored in a suitable medium such as Hank's balanced salt solution (HBSS) or milk, the PDL becomes necrotic and can act as a potent inflammatory stimulus. Therefore, in situations where the dry time is longer than 60 minutes, the PDL will not be expected to heal, and its removal is recommended.¹⁻³

When a tooth with necrotic or absent PDL is replanted, the newly formed bone around the root occupies the PDL space and will fuse with the cementum. Therefore, the hard dental tissues (cementum and dentin) will be incorporated into the continuous physiological remodeling process. Unlike bone tissue that is constantly remodeled and undergoes a process of resorption and deposition, the roots of permanent teeth do not

have this homeostatic mechanism, and if the process of resorption is initiated, it can lead to the total loss of root structure.^{1,8,9} It is estimated that 60 to 85% of replanted teeth are resorbed¹⁰ and lost 4-6 years after injury.¹¹ Such poor success rates have led to attempts to place temporary dressings in the root canal with the aim of preserving the root structure after replantation because immediate root canal filling is not effective for the control of inflammatory resorption.¹²⁻¹⁴

The inhibition of resorption is mainly attributed to the suppression of the recruitment of osteoclasts and the reduction of clastic activity. Osteoclast recruitment is under the control of a system of receptors within the tumor necrosis factor (TNF) superfamily, including osteoprotegerin (OPG) and receptor activator of nuclear factor Kappa-B ligand (RANKL), which are both expressed on the surfaces of osteoblasts.¹⁵⁻¹⁸ Several treatment strategies have already been studied with the aim of preventing or delaying osteoclast-mediated root resorption after replantation, such as the use of fluoride,¹⁹ zoledronic acid,²⁰ acetazolamide,²¹ local or systemic antibiotics,^{8,14} Ledermix,²² indomethacin,²³ bisphosphonates²⁴ and laser therapy.²⁵⁻²⁷ Although some drugs may make the root surface more resistant to resorption and slow down resorptive processes,^{2,14,20,21,24,26,27} to date, none have been able to completely inhibit these complications.

Experimental studies in animal models of periodontal disease have suggested that the sympathetic nervous system (SNS) can influence the regulatory pathways involved in RANKL-mediated osteoclastogenesis through the release of adrenergic neurotransmitters such as epinephrine and norepinephrine in the periodontium in response to stress.²⁸⁻³¹ These neurotransmitters in turn bind to $\alpha 1$ - ($\alpha 1$ -AR) and $\beta 2$ -adrenergic ($\beta 2$ -AR) receptors present on the surfaces of osteoblasts and other PDL cells, which causes an increase in RANKL expression, the stimulation of osteoclastic

differentiation, the regulation of immune cell proliferation and the production of cytokines.^{28–31} On the other hand, the blockade of α 1-AR and β 2-AR in periodontal tissue by phentolamine and propranolol, respectively, have been shown to reduce the levels of inflammatory cytokines, inhibit the formation of osteoclasts, and prevent bone loss in the inflamed periodontium.^{29–32}

The utilization of adrenergic blockers for the control of osteoclastic activity through the adrenergic signaling pathway provides an opportunity to assess therapeutic interventions that modulate these pathways. This work investigated the effects of different doses of phentolamine (nonselective α 1-AR blocker) and propranolol (nonselective β 2-AR blocker) in gels on the repair process in late replanted rat incisors and on osteoclastogenesis. The aim was to evaluate the effectiveness and ideal doses of these adrenergic blocking agents in the control of root resorption by microcomputed tomographic (micro-CT) and histomorphometric analysis in an animal model. In addition, the biocompatibility of the selected ideal doses was assessed in human periodontal ligament fibroblasts (HPDLFs) by MTT assays.

Materials and methods

The methodology is divided into two sections: the anti-inflammatory and anti-resorptive evaluation of late replanted rat incisors and the biocompatibility assessment of human periodontal ligament fibroblasts (HPDLFs).

The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines³³ were used for designing and performing the *in vivo* experiments, and the cytotoxicity testing was performed according to the guidelines of the International Organization of Standardization (ISO) 10993-5:2009.

This was an *in vivo* experimental study conducted in male Wistar rats (*Rattus norvegicus albinus*), which were obtained from the central vivarium of São Paulo State University – Unesp. The protocol for animal experimentation was reviewed and approved by the Animal Research Ethics Committee of the Institute of Science and Technology – Unesp (Process n. 001/2016). The methods were carried out in accordance with all legal and ethical principles of animal experimentation, as provided in the Normative Resolutions of the Brazilian National Council for the Control of Animal Experimentation (CONCEA) and in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

The sample size was calculated based on preliminary data from a pilot study according to the volume of mineralized tissue (TV) obtained by micro-CT analysis. For the pilot study, 15 male Wistar rats were used and randomly divided into three groups ($n = 5$) according to the medication applied inside the root canal: CH – calcium hydroxide P.A. + saline solution (1 g/ml); Ph10 – phentolamine hydrochloride + 2% carboxymethylcellulose (CMC) gel (10 µg/ml); and Pr10 – propranolol hydrochloride + CMC gel (10 µg/ml). The method of preparation of the medications was the same as below. The volume of mineralized tissue was measured in the middle third of the root and adjacent alveolar bone using CTAn software (v1.13.11.0, Bruker microCT, SkyScan) and the same parameters described below. For the sample size calculation, the power calculator for continuous outcomes and equivalence trials on the Sealed Envelope website was used. Based on an equivalence limit of 20%, as determined by Carvalho et al.²⁶ as that at which no significant statistical difference was detected between the groups, a significance level of 5%, a beta power of 80%, a standard deviation of the outcome of 1.2 mm³ and an equivalence limit of 2.16 mm³, a total of six animals per group was determined. The standard deviation value (1.2 mm³) was

calculated using the means of the volume (TV) of mineralized tissue of the CH (10.8 mm³), Ph10 (8.79 mm³) and Pr10 (8.65 mm³) groups. The equivalence limit value (2.16 mm³) was defined as 20%²⁶ of the mean of the control group (CH). Thus, a final study sample of 48 male Wistar rats, weighing 200-250 g each, was used. The animals were acclimatised to the housing conditions at 20-24° C and 50-70% relative humidity with a 12-h light/dark cycle. Food and water were available ad libitum.

Phentolamine hydrochloride (Ph) and propranolol hydrochloride (Pr) (Sigma-Aldrich, St. Louis, MO, EUA) were dissolved in absolute ethanol (Eth) at a concentration of 10 mg/ml according to the manufacturer's recommendations and further diluted in 2% carboxymethylcellulose (CMC) gel (Farmácia de Manipulação Terapêutica, São José dos Campos, SP, Brazil) to obtain the following experimental dosages: Ph 0.75 µg/ml, Ph 10 µg/ml, Ph 100 µg/ml, Pr 2.5 µg/ml, Pr 10 µg/ml, and Pr 100 µg/ml. For each drug, the lowest dosage (Ph 0.75 µg/ml and Pr 2.5 µg/ml) was determined based on the minimal therapeutic plasma concentrations of phentolamine (15 ng/ml) and propranolol (50 ng/ml), respectively, and on the rate of drug release by CMC gel according to previous studies.³⁴⁻³⁶ Dosages of 10 µg/ml and 100 µg/ml were defined as intermediate and high doses for both drugs, since they were approximately 10- and 100-fold higher than the lowest dosages of the drugs (Ph 0.75 µg/ml).

Tooth extraction of the maxillary right incisor from each rat was performed as described by Matos et al.²⁷ First, the rats were anesthetised with ketamine (75 mg/kg; Dopalen[®], AgribRANDS do Brasil Ltda., Paulínia, SP, Brazil) and xylazine (50 mg/kg; Anasedan[®]; Rompun-Bayer, São Paulo, SP, Brazil) by intramuscular injection. Soon after, intraoral antisepsis of the anterior maxillary portion was performed with 0.12% chlorhexidine gluconate (Colgate-Palmolive Industrial Ltda., São Bernardo do Campo, SP, Brazil) followed by detachment of the gingival fibers surrounding the tooth,

luxation, and extraction of the maxillary right incisor of each rat, using a Buser elevator (Trinity Indústria e Comércio Ltda., São Paulo, SP, Brazil) and an adapted forceps #150 (Golgran Ind. Com. Instr. Odontológico Ltda., São Caetano do Sul, SP, Brazil).²⁷

The teeth were stored in paper napkins (Santer[®], Paper Factory Santa Therezinha SA, Bragança Paulista, SP, Brazil) at room temperature for a period of 60 minutes to simulate delayed replantation. The dental papilla and periodontal ligament that adhered to the root surface were removed using a #15 scalpel blade (Suzhou Kyuan Medical Apparatus Co. Ltd., Beiqiao Town, Suzhou City, China) to inhibit the continuing growth of the rat incisor, and the pulp tissue was excised with a #20 Hedström file (Dentsply Maillefer, Ballaigues, Switzerland) through the apical foramen. The root canal and the external root surface of each tooth were irrigated with 5 ml of saline solution (Farmax, Divinópolis, MG, Brazil), and the canals were dried with #20 sterile absorbent paper points (Dentsply Ind. And Com. Ltda., Petrópolis, RJ, Brazil).

Before replantation, antisepsis of the anterior maxilla was again performed with 0.12% chlorhexidine gluconate, and an alveolar lavage was performed with 2 ml of saline solution for clot removal. The teeth were randomly divided into eight groups (n = 6) according to the medication applied inside the root canal, on the root surface and inside the alveolus, except for in the CH group, which only received the medication inside the root canal (Figure 1a-d): CH – calcium hydroxide P.A. + saline solution (1 g/ml) (control); Ph0.75 – phentolamine hydrochloride + CMC gel (0.75 µg/ml); Ph10 – phentolamine hydrochloride + CMC gel (10 µg/ml); Ph100 – phentolamine hydrochloride + CMC gel (100 µg/ml); Pr2.5 – propranolol hydrochloride + CMC gel (2.5 µg/ml); Pr10 – propranolol hydrochloride + CMC gel (10 µg/ml); Pr100 – propranolol hydrochloride + CMC gel (100 µg/ml); CMC – CMC gel (placebo). Each root canal, root surface and alveolus were filled with approximately 15 µL of the

respective medication using a 25 x 0.35 mm capillary tip (UltraCal TM XS, Ultradent Products Inc., South Jordan, UT, USA).

The teeth were replanted in their sockets with slow and delicate movements using tweezers (Golgran Ind. Com. Instr. Odontológico Ltda.) (Figure 1e).²⁷ No splinting was used, as the anatomical form of the tooth itself and of its alveolus was enough to keep it in its original position (Figure 1f).^{27,37} Subsequently, the animals received a single intramuscular injection (0.05 ml/kg) of an antibiotic (Megacilin Super Plus TM; Vansil Ind. Com. e Repr. Ltda., Descalvado, SP, Brazil) to reduce the chance of postoperative infection and subcutaneous injections of buprenorphine (0.05 mg/kg) every 24 h for 3 days to reduce pain.

Thirty days after replantation, all animals were anesthetized with ketamine (75 mg/kg) and xylazine (50 mg/kg) by intramuscular injection and euthanized by transcardial perfusion with 10% formaldehyde and guillotine decapitation. The maxilla was dissected from the cranium, and the right side containing the replanted incisor was removed using a #20 scalpel blade and a Metzenbaum scissor (Golgran Ind. Com. Instr. Odontológico Ltda., São Paulo, SP, Brazil). The anatomic pieces were fixed in 10% formaldehyde solution for 48 hours at room temperature in preparation for micro-CT analysis.

Experimental and control fixed tissue blocks were scanned with a micro-CT scanner (SkyScan 1176 in vivo, Skyscan, Kontich, Belgium) at a resolution of 9 μ m. The resulting images were reconstructed two-dimensionally (2D) with NRecon software (v1.6.9.8, Bruker microCT, SkyScan) and three-dimensionally (3D) with Data Viewer software (v1.5.0.2, Bruker microCT, SkyScan). The volume of interest (VI) chosen for the measurements corresponded to the middle third of the root and adjacent alveolar bone because this region was not damaged by the surgical procedures; 201 transaxial

sections were obtained.^{27,37} The transaxial and 3D images of each sample were quantitatively evaluated with regard to the volume (TV; mm³) and surface (TS; mm²) of the mineralized tissue using CTAn software (v1.13.11.0, Bruker microCT, SkyScan). A rectangular block of 3.5 x 2.1 cm was virtually centered over a particular point of the transaxial image to guarantee a comparative analysis of mineralized tissue within the same region of interest (ROI) for all samples. The dimensions of the rectangle were defined to encompass the entire contour of the root walls, and its major axis was oriented in the bucco-palatal direction from the outermost border of the vestibular wall to the adjacent palatal alveolar bone.

The VI was reconstructed and visualized with a 3D profile in CTVol software (v2.2.3.0, Bruker microCT, SkyScan). Figure 2 shows a representative image of the 2D evaluation of the ROI and a 3D image of the reconstructed VI after quantitative analysis. Normally distributed data (Shapiro-Wilk test) were compared, and significant differences between the groups were determined by analysis of variance (ANOVA) and Tukey tests at a significance level of 5% ($P < 0.05$).

After micro-CT analysis, tissue samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution for 3 months, and the EDTA was replaced every 48 hours. The samples were rinsed, subjected to serial dehydration with increasing concentrations of ethanol, diaphanized and embedded in paraffin. Three semiserial sections (5-μm thick) at 40-μm intervals were obtained from each sample within the longitudinal plane of the root and stained with hematoxylin and eosin (H&E). A total of 144 images of the histological sections were captured using Pannoramic Desk (3DHISTECH Ltd., Budapest, Hungary) and Pannoramic Scanner software (3DHISTECH Ltd., Budapest, Hungary). The histomorphometric analysis was performed on the middle third of the palatal root face and adjacent alveolar bone

because this region was not damaged by the surgical procedures and contained both cementum and PDL.^{27,37} The Pannoramic Viewer software (v1.15.4, 3DHISTECH Ltd., Budapest, Hungary) was used to standardize the area of analysis. A trained blinded examiner used Leica Application Suite software (v4.10; Leica Microsystems Ltd., Heerbrugg, Switzerland) to determine the areas of inflammatory root resorption (IR; dentin resorption areas filled with connective tissue and inflammatory cells), replacement root resorption (RR; dentin resorption areas with bone deposition), ankylosis (AK; direct union of bone tissue with intact cementum), and periodontal repair (PR; intact cementum covered by connective tissue).^{25,27,37} The length of each histomorphometric event quantified was measured in micrometers, transformed into a percentage in relation to the total length analyzed, and classified by using a semiquantitative scoring method adapted from Carvalho et al.²⁶: 1-absent (absence of IR, RR, AK or PR), 2-discrete (presence of IR, RR, AK or PR in <25% of the length observed), 3-moderate (presence of IR, RR, AK or PR in 25-50% of the length observed), and 4-intense (presence of IR, RR, AK or PR in >50% of the length observed). The distribution of the scores was determined by the Shapiro-Wilk test, and the data were compared using the Kruskal-Wallis test and Dunn's test at a significance level of 5% ($P < 0.05$).

In order to evaluate osteoclast activity, three semiserial longitudinal sections from each sample were cut with a thickness of 5 μ m at 40- μ m intervals and stained with a tartrate-resistant acid phosphatase (TRAP) kit (SigmaAldrich®, St Louis, MO, USA) according to the manufacturer's instructions. Briefly, the sections were rehydrated, rinsed, incubated in a solution of naphthol AS-BI phosphoric acid and Fast Garnet GBC for 1 h at 37°C and then stained with hematoxylin. A total of 144 images of the histological sections were captured using Pannoramic Desk and Pannoramic Scanner

software, and the analysis was performed on the alveolar bone adjacent to the palatal face of the middle third of the root. The Pannoramic Viewer software was used to standardize the area of analysis. For each image, TRAP-positive cells containing 2 or more nuclei were identified by a single experienced examiner blinded to the groups using Pannoramic Viewer software. The obtained data were registered and presented as the mean observed in each group. Normally distributed (Shapiro-Wilk test) data were compared, and the significance of the differences among the groups were determined by ANOVA followed by the Tukey test for multiple comparisons at a significance level of 5% ($P < 0.05$).

The protocol used for the cytotoxicity analysis was approved by the Research Ethics Committee of the Institute of Science and Technology – Unesp (Certificate of Presentation for Ethical Consideration: 51548015.4.0000.0077). The biocompatibility assessment was performed with intermediate dosages only (10 µg/ml) of phentolamine hydrochloride (Ph) and propranolol hydrochloride (Pr) (Sigma-Aldrich, St. Louis, MO, EUA) and the corresponding volume of ethanol solvent (1 µl/ml) based on *in vivo* experiments that showed improved overall results at these dosages. Hence, Ph and Pr were dissolved in absolute ethanol at a concentration of 10 mg/ml and further diluted in culture medium to obtain the experimental drug solutions: Ph 10 µg/ml (Ph10 group) and Pr 10 µg/ml (Pr10 group). Absolute ethanol was also diluted in culture medium to obtain the corresponding volume of ethanol: Eth 1 µl/ml (Eth group).

HPDLFs (passage 7) were obtained from the cell bank of the Laboratory of Cell Culture of the Institute of Science and Technology – Unesp and were cultured in 96-well plates (Prolab®, São Paulo, SP, Brazil) at a density of 8×10^3 cells/well. Once the cells were attached, 200 µL of Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Life Technologies, Grand Island, NY, EUA) supplemented with 10% fetal bovine

serum (FBS; Cultilab Ltda., Campinas, SP, Brasil) and 1% penicillin and streptomycin (PenStrep; Sigma-Aldrich, St. Louis, MO, EUA) was added to each well and incubated at 37°C in 5% CO₂ for 24 hours. The culture medium was removed from each well and replaced with 200 µL of an experimental drug solution (Ph 10 µg/ml, Pr 10 µg/ml or Eth 1 µl/ml). A group treated with only normal culture medium (DMEM, 10% FBS and 1% PenStrep) served as a control (DMEM group). To mimic the process of inflammation, the fibroblasts were exposed to 100 µg/ml of lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich, UK).²⁸ The plates were incubated at 37°C in 5% CO₂ for 24 or 48 hours. For each group for each experimental time, 24 wells were used in triplicate.

After culturing the HPDLFs with the tested drugs for 24 and 48 h, the percentage of viable cells in each well was estimated by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Life Technologies, Carlsbad, USA) at a concentration of 5 mg/ml in sterile phosphate-buffered saline (PBS). First, the culture medium from each well was removed, and the cells were gently washed (two times) with 1.0 ml phosphate-buffered saline (PBS) and incubated with 100 µL/well MTT solution at 37°C in 5% CO₂ for 1 h. Then, the MTT solution was removed, and the residue was carefully rinsed out two times with 100 µL/well PBS, after which the blue formazan precipitate was solubilized by adding 100 µL of dimethyl sulfoxide (DMSO; Sigma Aldrich Co., Germany) to each well and shaking the culture plate for 10 minutes at room temperature. The absorbance of the formazan content of each well was measured using a spectrophotometer (Cambrex ELx808cse; Lonza, Basel, Switzerland) at a wavelength of 570 nm. The percentage of cell viability was calculated by dividing the absorbance values of experimental groups by those of the DMEM control group and multiplying by 100. The data had a normal distribution

(Shapiro-Wilk test), and the means for each group were statistically analyzed by ANOVA and Tukey tests for the determination of the 5% significance level ($P < 0.05$).

Results

The micro-CT analysis of the delayed replanted maxillary right incisors treated with different therapeutic regimens revealed no significant statistical differences in the comparisons of the volume (TV) and surface (TS) of the mineralized tissue in the middle third of the root and adjacent alveolar bone (VI) ($P > 0.05$) (Figure 3). Figure 4 shows representative transaxial micro-CT images for each group, in which it is possible to observe that all groups exhibited areas of dentin and bone resorption.

Inflammatory resorption (IR) and periodontal repair (PR) occurred in all experimental groups (Figure 5). Replacement resorption (RR) and ankylosis (AK) were considered to be absent in all groups except the CH group (Figure 5). No significant statistical difference was observed among the groups in relation to the IR, RR, AK and PR scores ($P > 0.05$) when the data were compared (Figure 5). Figure 6 shows representative images of each group.

The application of adrenergic blockers in the replanted teeth suppressed osteoclast formation by significantly decreasing the number of TRAP-positive multinuclear cells in a dose-dependent manner ($P < 0.05$) (Figure 7a). Treatment with Ph10 and Pr10 significantly decreased the number of TRAP-positive multinuclear cells compared to treatment with other medications ($P < 0.05$) (Figure 7a). The number of TRAP-positive multinuclear cells was significantly higher in the CH, Ph100, Pr100, and CMC groups than in the Ph0.75 and Pr2.5 groups ($P < 0.05$) (Figure 7a), which presented statistically similar results ($P > 0.05$) (Figure 7a). Representative images of each group showing the decrease in the number of TRAP-positive multinuclear cells

resulting from treatment with low (Ph 0.75 µg/ml, Pr 2.5 µg/ml) and intermediate dosages (Ph 10 µg/ml, Pr 10 µg/ml) of both adrenergic blocker drugs are presented in Figure 7b.

The results of the cell viability assay (Figure 8) showed that Ph10, Pr10 and Eth produced statistically similar cell survival rates compared to that of the control group (DMEM), regardless of the experimental time ($P > 0.05$). There was no statistically significant difference between Ph10 and Eth for both experimental times ($P > 0.05$), but Pr10 produced higher cell viability than Ph10 and Eth ($P < 0.05$) (Figure 8).

Discussion

Although the primary function of the SNS is to stimulate the body's fight-or-flight response, it is also responsible for other homeostatic mechanisms. The innervation of the SNS has been shown to modulate osteoclastic activity in periodontal tissues through the activation of α 1-AR and β 2-AR expressed by PDL cells, especially osteoblasts/osteoclasts, and the pharmacological blockade of these receptors can prevent alveolar bone loss induced by periodontitis.^{28–32} The present study hypothesized that local adrenergic blockade with phentolamine (nonselective α 1-AR blocker) and propranolol (nonselective β 2-AR blocker) in gels used to treat the external surfaces of the roots would have beneficial effects on the regulation of bone and root resorption in rat teeth after late replantation following simulated avulsion. To check these assumptions, micro-CT was used in combination with histological analysis due to the limitation of 2D methods.³⁸ Although the drugs are not antimicrobial, which is thought to be an important property of an agent used to treat an avulsed tooth, both drugs were chosen as therapeutic agents because of their well-known preventive effects on periodontal degradation by inhibiting osteoclastogenesis and reducing the levels of

inflammatory cytokines.^{29–32} The authors proposed that the local application of these agents should be made due to the cardiovascular side effects caused by their systemic administration. Clearly, such side effects are not desirable, especially because avulsed teeth occur most frequently in children aged 7 to 12 years.³⁹ In this study, CMC gel was used as a vehicle for drug delivery because it is a highly viscous mucoadhesive gel composed of hydrophilic polymers that is commonly used for the local delivery of drugs to diseased mucosa. It is minimally irritating and adaptable towards biological surfaces, thus allowing the release of drugs close to the site of action.⁴⁰ Although the medications were designed to treat the root surfaces, they were also inserted into the root canals because the use of calcium hydroxide as an intracanal medication could interfere with the study results as it can diffuse into the periodontal ligament space through the apical foramen.

Rats are a commonly used animal model for reproducing tooth avulsion and replantation,^{12,16,19,21,25–27,37} and a 30-day experimental time that was chosen to evaluate the *in vivo* effects of the tested medications is sufficient to observe the inflammatory and repair processes.¹⁶ The findings of the present study suggest that the application of adrenergic blockers to the root surface may influence the process of the periodontal destruction of replanted teeth by reducing osteoclastogenesis. The enzymatic histochemical analysis used in this animal model showed that both low and intermediate dosages of phentolamine (Ph0.75, Ph10) and propranolol (Pr2.5, Pr10) significantly decreased the number of TRAP-positive cells in a dose-dependent manner. These findings agree with those in previous reports that suggest that the systemic use of these drugs in rats reduces osteoclastic activity in periodontal disease.^{29,30,32} TRAP is a resorptive enzyme found in the ruffled border of the osteoclast membrane and is considered a specific marker of osteoclast differentiation.⁴¹ The suppression of

osteoclastic formation is reported to be a result of the pharmacological blockade of $\alpha 1$ -AR and $\beta 2$ -AR on the surfaces of osteoblasts, which prevents their binding to adrenergic neurotransmitters and reduces the expression of RANKL, the main inducer of osteoclastogenesis and osteoclastic activity.^{15,28,30–32} Similar effects were found by Okada et al.²⁹ and Rodrigues et al.³⁰, who reported a reduction in the number of osteoclasts and the prevention of alveolar bone loss in rats with experimental periodontitis after the blockade of β -receptors by the intraperitoneal or oral administration of propranolol. In addition, these drugs have been shown to reduce the levels of IL-1 β (Ph and Pr), IL-6 (Ph), IL-8 (Ph), TNF- α (Pr) and RANKL (Pr) in gingival tissues and increase the expression of OPG (Pr).^{30,31} It is noteworthy, however, that the beneficial effects observed at these dosages on osteoclastogenesis in this study were not enough to produce a significant improvement in periodontal repair or the formation and resorption of bone, cementum and dentin.

Both the micro-CT and histomorphometric analyses were limited to the middle third of the root to avoid the operation-related risk of bias, since the cervical and apical third of the root suffered variable damage resulting from the surgical procedures due to the use of forceps during extraction and a scalpel blade during the removal of the dental papilla.^{27,37} Micro-CT analysis of the middle third of the replanted incisors and surrounding tissues showed no significant differences among the control group (CH), the experimental groups (low, intermediate and high dosages of Ph and Pr) and the placebo group (CMC) in matters of the 2D and 3D parameters quantified. Calcium hydroxide is the most commonly used root canal dressing for late replantation, and it is considered to be the medication of choice due to its alkaline pH and bactericidal effects, which may slow or limit inflammatory and replacement resorption.⁴² To observe these effects, a long application time with successive changes is required, which has been

associated with a detrimental effect on the fracture resistance of root dentin.^{13,14,43,44} Additionally, as observed in this research, studies in animal models have demonstrated that calcium hydroxide is ineffective in preventing inflammatory and replacement resorption following tooth replantation.^{4,8,12,13,21,25,27} It is also possible that calcium hydroxide may diffuse into the periodontium through the thin dentinal walls via dentinal tubules or the apical foramen and cause coagulation necrosis of the periodontal tissue, triggering the release of proresorptive inflammatory factors.^{4,12} This could explain the increased osteoclast formation found in the CH group, which was similar to that induced by high doses of the tested medications (Ph100, Pr100) or in the placebo group (CMC). However, CH induced histologically discreet occurrence of replacement resorption and ankylosis probably by the activation of alkaline phosphatase, an enzyme with important effects on the mineralized tissue formation process, and by the release of calcium ions that react with carbon dioxide in the tissues to form granulation consisting of calcium carbonate.⁴²

Based on the histological assessment, no significant statistical difference was observed among the treatments in relation to the occurrence of inflammatory resorption, replacement resorption, ankylosis, and periodontal repair. This result is in accordance with microcomputed tomographic findings and may be related to the drug release and absorption mechanisms in the target tissue or to the observation period, which may not have been enough to evidence the beneficial effects of the local adrenergic blockade on the periodontal repair, specifically in the groups treated with Ph10 and Pr10 that significantly reduced osteoclastogenesis. Although the dosages resulting from the local delivery of adrenergic drugs were not determined as part of this study, it is known that local release in the 50-100 ng/ml range for propranolol is necessary to induce beta-receptor blockade.³⁵ Thus, the drug release at lower dosages may not have been

sufficient to be absorbed at a level in the target tissue to induce a potent adrenergic blockade, which would have resulted in more evident histological effects. At the other extreme, high dosages of nonselective adrenergic blockers such as phentolamine and propranolol can suppress both $\alpha 1/ \alpha 2$ and $\beta 1/ \beta 2$ adrenergic receptors, thereby reducing their beneficial effects via $\alpha 1$ -AR and $\beta 2$ -AR.^{30,31}

HPDLFs are the most abundant and important periodontal ligament cells. They can repair the periodontal ligament collagen and aid in recovery from periodontal disease. When stimulated, HPDLFs can also activate intracellular cascades that lead to the production of inflammatory and immunomodulatory chemokines and cytokines.^{31,45,46} LPS is a primary cell wall component in gram-negative bacteria and a potent inflammatory inducer.^{28,31,41} Thus, LPS was used to simulate the inflammatory environment to explore the effects of ideal doses of adrenergic blockers (Ph10 and Pr10) on the viability and proliferation of HPDLFs *in vitro*. The biocompatibility assessment showed that Ph10, Pr10 and a corresponding volume of solvent ethanol (Eth) were not cytotoxic. In addition, Pr10 increased the viability of cells exposed to LPS compared to Ph10 and Eth, which would probably favor periodontal repair *in vivo*. Lu et al.³¹ also investigated the proliferation of HPDLFs stimulated with LPS and observed that the pretreatment with phentolamine dramatically ameliorated cell proliferation, but these effects were not apparent in the propranolol-treated group. These results were different from the current study probably due to the methodological differences regarding the dosage of the drugs to which the fibroblasts were exposed. The cytotoxicity assay is considered one of the prerequisites for validating the clinical application of biomaterials tested on animals and, as Ph10 and Pr10 did not impair cell survival, they could be safely clinically tested. However, new experiments are suggested to fill this gap and bring more clarity to the topic.

The beneficial effects of sympathetic receptor blockade on bone metabolism were first demonstrated in a surgically fractured rat model, which showed an increase in the bone formation rate after 19 consecutive days of treatment with intraperitoneal injections of propranolol (0,1 mg/kg/day).⁴⁷ Since then, several studies have demonstrated the sympathomimetic effects on clastic cells with adrenergic receptors and their effects on the local regulation of osteoclastogenesis in physiological and pathological conditions.^{15,30,31} The first evidence that the SNS was involved in the pathogenesis of periodontal disease was reported in 2005, when it was demonstrated that rats with peripheral chemical sympathectomy exhibited a significant reduction in alveolar bone loss and the inhibition of periodontitis progression.²⁸ To the best of the authors' knowledge, this is the first evidence of the effects of adrenergic blockers on tooth replantation, which may provide a novel therapeutic strategy for managing avulsed teeth by limiting osteoclastogenesis and subsequent inflammatory root resorption and ankylosis. However, the findings of the present study must be interpreted as preliminary results and as a guide to plan further *in vitro* and *in vivo* studies with new methodological approaches. The main limitation relates to the experimental animal model, which limits extrapolating the results directly to humans, although it is considered an essential step when a new material or drug has unknown human health risks.⁴⁸ Further studies are also necessary to clarify whether the anti-osteoclastogenesis effect observed in this study is indeed due to α 1-AR and β 2-AR blockade or due to a general anti-inflammatory effect (e.g., the suppression of inflammatory cytokines).

In conclusion, the current data reveal that replanted teeth treated with Ph10 and Pr10 produced a significant reduction in the number of osteoclasts detected by TRAP staining. None of the tested medications significantly improved the repair process of late replanted rat incisors when assessed histologically and by micro-CT. Additionally,

it was verified that Ph10 and Pr10 do not exhibit a cytotoxic effect in HPDLFs and that Pr10 increases the viability of fibroblasts exposed to LPS.

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Figure Legends

Figure 1 – Treatment of the exposed roots: (a) Root canal filling with calcium hydroxide paste. (b) Root canal filling with CMC gel. (c) Application of CMC gel on root surface. (d) Application of CMC gel inside the alveolus. (e) Tooth replantation. (f) Maxillary right incisor replanted after root treatment.

Figure 2 – (a) Transaxial image showing 2D evaluation of the ROI with a rectangular geometric figure that was 3.5 cm wide and 2.1 cm high (CTAn software). (b) 3D image of the reconstructed VI after quantitative analysis (CTVol software).

Figure 3 – Mean and standard deviation of the volume (TV) and surface (TS) of mineralized tissue within the volume of interest (VI) for each group, as detected by micro-CT analysis at 30 days after delayed tooth replantation. ANOVA and Tukey tests showed no statistically significant differences among the groups for all quantified 2D and 3D parameters ($P > 0.05$).

Figure 4 – Representative transaxial micro-CT images of each group obtained with CTAn software 30 days after delayed tooth replantation.

Figure 5 – Median and interquartile ranges of the histomorphometric scores for each

group at 30 days after delayed tooth replantation. The Kruskal-Wallis test and Dunn's test showed no statistically significant differences among the groups for all histomorphometric events investigated ($P > 0.05$). Inflammatory root resorption (IR), replacement root resorption (RR), ankylosis (AK), periodontal repair (PR). Semiquantitative scores: 1-absent, 2-discrete, 3-moderate, 4-intense.

Figure 6 – Representative images of each group showing areas of inflammatory root resorption (IR = red arrows), replacement root resorption (RR = yellow arrows), ankylosis (AK = blue arrows), and periodontal repair (PR = green curly brackets). Dentin (D), cementum (C), periodontal ligament (PDL), bone tissue (BT). Hematoxylin and eosin stain, scale bar = 500 μ m in the top left-hand corner of each image.

Figure 7 – (a) Mean and standard deviation of the number of TRAP-positive cells in each group at 30 days after delayed tooth replantation. Bars with different letters indicate significant differences (ANOVA followed by the Tukey test, $P < 0.05$). (b) Representative images of each group showing TRAP-positive multinuclear cells (red arrowheads). Tartrate-resistant acid phosphatase stain, scale bar = 50 μ m in the top left-hand corner of each image.

Figure 8 – Mean and standard deviation of the percentage of cell viability of each group (Ph10, Pr10 and Eth) relative to the mean of the control group (DMEM) at 24 and 48 hours. Bars with different letters indicate significant differences (ANOVA followed by the Tukey test, $P < 0.05$).