# Mediators involved in tooth resorption following delayed replantation: an experimental study

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# Abstract

Inflammatory and replacement tooth resorption are common outcomes following tooth replantation, biological mediators involved in these processes are widely unknown. The aim study was to investigate molecules involved in tooth resorption following permanent tooth avulsion and delayed replantation. Dog premolars were extracted and kept dry for 20, 60 and 90 minutes (n= 30). The teeth were replanted, splinted. After 120 days, the animals were euthanized and tissues were removed for histological processing. Slides were stained for microscopic analysis, submitted to tartrate resistant acid phosphatase (TRAP) histoenzymology and immunostained for RANK, RANKL, OPG, alkaline phosphatase and periostin. Data obtained were submitted to statistical analysis using the chi-square, Fisher and one-way ANOVA tests (alpha= 0.05). In inflammatory resorption areas, TRAP + and RANK + osteoclasts surrounding the replanted teeth were identified, regardless of the extra-alveolar time. RANKL synthesis in this region was higher in longer extra-alveolar times and was more intense after keeping the tooth dry for 90 minutes compared to other periods. In the replacement reabsorption area, there was lower synthesis of periostin and higher alkaline phosphatase production. Inflammatory resorption was characterized by osteoclast recruitment and RANKL synthesis and replacement resorption was characterized by inhibition of periostin and alkaline phosphatase syntheses.


# Introduction

Avulsion is a traumatic dental lesion where complete alveolar tooth exarticulation occurs, exposing it to the external environment with rupture of the fibers of the periodontal ligament and rupture of the apical vascular-nervous bundle, which can lead to pulp necrosis. According to the International Association of Dental Traumatology (IADT) [1], avulsion of permanent teeth represents 0.5 - 16% of all dental injuries. Treatment for dental avulsions is replantation, and the faster and more suitable it is, the greater the chance of success [2,3].

Trauma to the periodontal ligament can trigger external root resorption, so the correct handling of an avulsed tooth before replantation is of paramount importance [4]. External root resorption is a strictly local phenomenon and is more frequent in replanted teeth [5]. They can be classified as inflammatory resorption or can be related to infection and replacement resorption or ankylosis [1]. Even though it is common, there are few studies [6, 7] on its determinants and the biochemical mediators involved in the process.

Replanted teeth have varying degrees of resorption, are generally asymptomatic and are often not diagnosed [4]. These complications are related to the involvement of the healing of the pulp and the periodontal ligament [3]. When the extra alveolar time is long and the tooth is kept in a dry environment, root resorption by replacement is the most common complication after the replantation of an avulsed tooth [6]. This pathology occurs due to necrosis of the tooth periodontal ligament and cementoblasts [5]. Thus, the extra-alveolar period and storage medium in which the tooth was kept before replantation are the critical factors that most affect the survival and regeneration of the damaged periodontium [3].

Mediators that regulate osteogenesis and osteoclastogenesis may be involved in tooth root resorption. The RANK-RANKL-OPG canonical system, discovered in the late 1990s, is considered an important regulator of bone metabolism [8]. The receptor activator of nuclear factor kappa B (RANK) and its ligand (RANKL) are responsible for the formation and activity of osteoclasts, while osteoprotegerin (OPG) protects bone from resorption, acting as a decoy receptor that, binding to RANKL, prevents it from connecting to the RANK [9,10]. This system plays an important role in the remodeling of mineralized tissues and any imbalance between these components can lead to bone and dental resorption [11]. A qualitative analysis of the results of a study with replanted rat teeth demonstrated that the OPG, RANK and RANKL system showed evident marking in the late replantation, which demonstrates the real participation of the process of root resorption [12]. Another study demonstrated increased expression of RANKL and low levels of OPG in the cells of the periodontal ligament of human primary teeth undergoing resorption. On the contrary, abundant expression of OPG and no expression of RANKL in permanent or deciduous teeth were found before the exfoliation process [13].

Periostin is expressed by cells of the periodontal ligament [14] and is a regulator of osteogenesis [15, 16]. Periostin is an extracellular matrix protein essential in the processes of tooth eruption, homeostasis and periodontal integrity [17]. It is involved in wound repair and tissue healing [18, 19]. Periostin in the periodontal ligament can play a fundamental role in the migration of cells of the periodontal ligament that is necessary for remodeling and structural maintenance [17, 20]. By promoting the migration of fibroblasts [21] and osteoblasts [22], periostin helps in the remodeling of the periodontal ligament and surrounding bone [23]. Alkaline phosphatase (ALP) is also a marker of bone formation [16] and is highly expressed in mineralized tissue cells and plays an important role in the formation of hard tissue. ALP increases local rates of inorganic phosphate, facilitates mineralization and reduces the concentration of extracellular pyrophosphate, an inhibitor of mineral formation [24]. The expression of these mediators in late dental replantation is extremely important for improving this treatment.

Therefore, the aim of the present study was to investigate the molecules involved in the processes of inflammatory tooth resorption and replacement after exarticulation and the maintenance of teeth for different periods in a dry environment. Understand the molecular and microscopic mechanisms in tooth resorption following delayed replantation, thus allowing the development of more effective treatments.

# Methods

The slides used in this section were obtained from the specimen database at the Department, of Pediatric Dentistry of the School of Dentistry of Ribeirão Preto, University of São Paulo (FORP–USP) and the material and methods were detailed in a previous study [6].

Briefly, after approval by the Animal Welfare Committee of the University of Washington, 4 beagle dogs (aged 12 to 18 months and average weight of 15 kg) were used. Thirty premolar roots (15 teeth) of the second and third upper premolars and of the second, third and fourth lower premolars were used. Each

premolar with two roots was hemisected and each root was considered a specimen. Atraumatic root extraction was performed with number 150 forceps (Quinelato, Rio Claro, SP, Brazil) and the canals were instrumented and filled with gutta-percha cones (Dentsply-Herpo, Teresópolis, RJ, Brazil) and AH Plus cement (Dentsply-DeTrey, Konstanz, Germany) by the lateral condensation technique. The access cavities were restored with amalgam.

The groups were divided according to the period that the teeth were kept in an extraoral dry medium before replantation as follows: Group1: the roots were replanted after a 20 minutes extraoral time in a dry medium; Group 2: the roots were replanted after a 60 minutes extraoral time in a dry medium; Group 3: the roots were replanted after 90 minutes the extraoral time in a dry medium; Group 4 (negative control): healthy teeth, not extracted. The teeth were splinted with passive containment using a 0.4 mm nickel-titanium (Ni-Ti) wire and Z 250 composite (3M ESPE, St. Paul, MN, USA) for 10 days. The dogs were maintained on a canned food diet after the surgical procedure, and radiographic monitoring was performed monthly for 120 days. After 120 days, the dogs were killed by an overdose of 6% sodium pentobarbital solution administered intravenously.

### Microscopic evaluation

Maxillary and mandibular blocks containing the roots and the surrounding alveolar bone were prepared and fixed in 10% formalin, decalcified in Formical solution (Decal Chemical Corporation, Congers, NY, USA) for 1 week followed by Immunocal solution (Decal Chemical Corporation, Tallman, NY, USA) for 2 months. Paraffin embedded sections were cut parallel to the long axis of the roots with a thickness of 5 µm at 90 µm intervals and stained with hematoxylin-eosin.

The frequency of inflammatory root resorption, replacement root resorption and periapical bone resorption in different groups was compared using the Fisher exact test ($\alpha = 0.05$).

### Determination of the presence of osteoclasts by histoenzymology analyses for TRAP

Deparaffinized tissue sections were incubated in a solution containing 8 mg of naphthol AS-MX di-sodium phosphate (Sigma-Aldrich) in 500 µL of NN-dimethylformamide followed by the addition of 50 mL of a 0.2 mol buffer solution/L sodium acetate (pH 5.0) containing 70 mg of Fast Red ITR (Sigma-Aldrich). Subsequently, the sodium tartrate substrate dihydrate (50 mmol/L) was added to the solution and incubated at 37°C for 12 h. Subsequently, the sections were washed in distilled water and counterstained with Fast Green. Quantitative analysis of the number of osteoclasts that were positive for the TRAP enzyme was performed taking into consideration the total number of osteoclasts per dental root. The groups were compared by means of one-way ANOVA followed by the Tukey's test ($\alpha = 0.05$).

### Immunohistochemistry

The slides were deparaffinized, hydrated in a decreasing series of ethanol, and kept in phosphate-buffered saline (PBS). Next, tissue sections were microwaved (7 x 12 seconds at 2-minute intervals) with sodium citrate buffer (pH = 6.0) for antigen retrieval. After temperature stabilization, the slides were washed with PBS (3x) for 5 minutes, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 40 minutes. Slides were further washed with PBS (3x) for 5 min and non-specific binding sites were blocked with 5% bovine serum albumin (Sigma-Aldrich) for 60 min. The tissues were then incubated with primary antibodies for RANK (sc-7626; Santa Cruz Biotechnology, Dallas, TX, USA), RANKL (sc-7628; Santa Cruz Biotechnology), OPG (sc-8468; Santa Cruz Biotechnology), alkaline phosphatase (ab54778; Abcam, Cambridge, MA, USA) and periostin (sc-67233; Santa Cruz Biotechnology Inc.) at 4 °C overnight. Next, the slides were washed and incubated with donkey anti-goat, goat anti-rabbit and rabbit anti-mouse biotinylated secondary antibody (Biocare Medical, Concord, CA, USA) for 1 h, washed in PBS, and incubated with streptavidin conjugated with horseradish peroxidase (HRP) for 20 min. The 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) was used as the enzyme substrate for 5 min; the slides were washed with PBS, counterstained with haematoxylin for 15 s, washed with distilled water, dehydrated in increasing ethanol concentrations, and mounted in Entellan® (Merck, Darmstadt, Germany). Control slides in which the primary antibody was omitted were used to test the specificity of immunostaining. For this analysis, all slides were prepared in the same batch to obtain a standardized staining and were evaluated by an experienced blind examiner. Slides were scored according to the intensity of staining as (1) mild, (2) moderate, and (3) intense. The groups were compared using the Kruskal Wallis followed by the Dunn's tests ($\alpha = 0.05$).

# Results

Inflammatory root resorption occurred in 100% of the groups of teeth maintained for 90 minutes in dry extra-alveolar medium before replantation. In 20 and 60 minutes, the occurrence was less than 50% of the cases (Figure 1). There was a significant difference between extra-alveolar times, that is, time interferes with the presence or absence of inflammatory root resorption ($p = 0.0002$).

Replacement root resorption occurred in 100% of the cases kept in a dry medium for 60 and 90 minutes while, in the extra alveolar time of 20 minutes, replacement root resorption occurred in 66% (Figure 1) ($p < 0.0001$).

Regarding the number of TRAP + osteoclasts, in the 60 and 90 minutes extra alveolar time, the numbers were similar, while for the 20 minutes extra alveolar time, it was lower (Figure 2) ($p = 0.00078$). Those cells were positively stained to RANK. The presence of RANKL was moderate to intense in 90 minutes, and in 20 minutes, this presence was less than 50%. OPG synthesis was detected in a low percentage of specimens solely at 90 minutes dry time ($p < 0.05$) (Figure 3).

Periostin was found only in the group of healthy teeth and synthesis was abrogated in the periodontal ligament of teeth maintained in a dry environment or in the process of resorption by replacement ($p < 0.05$). The synthesis of alkaline phosphatase was moderate in the groups of healthy teeth and more intense in the groups maintained in an extra alveolar dry medium, regardless of time teeth were kept outside of the alveolar socket (Figure 4).

# Discussion

According to some studies [6,30], the degree of root formation and the condition of the periodontal ligament cells are factors that influence the choice of treatment after tooth avulsion [25]. The condition of the cells of the periodontal ligament is directly dependent on the storage medium and the extra alveolar time [6], with 60 minutes been currently set as the threshold in which ankylosis and root resorption are expected [1].

The most serious complication after tooth avulsion and replantation is ankylosis, which occurs with the increase of extra alveolar time. Teeth with an open apex have a lower risk of developing ankylosis [3]. The storage medium also influences the occurrence of ankylosis. Previous studies have shown that the risk of ankylosis of human teeth replanted after an extended period of dry storage is greater [26, 27]. Thus, in order to try to reduce these damages, it is necessary to evaluate and understand the microscopic and molecular aspects after replantation.

The present study evaluated the molecular biology involved in the process of root resorption after avulsion and replantation of teeth kept in a dry medium after 20, 60 and 90 minutes. These molecules are part of the TNF-alpha (tumor necrosis factor) superfamily, and are regulators of bone remodeling and are essential for osteoclast activation, known as the RANK, RANKL and OPG systems [28, 29]. In the periods of 60 and 90 minutes, ankylosis occurred followed by root resorption by replacement in 100% of the examined teeth. RANKL was also observed in all slides in the 60 and 90 minutes and slightly more than 30% in the slides in 20 minutes. When RANKL predominates, cell signaling occurs for reabsorption of the periodontal tissues. As in a study comparing replanted teeth immediately with replanted teeth after 60 minutes, there was a predominance of immunostaining for OPG in the cementum, periodontal ligament and alveolar bone in the immediately replanted group. While the synthesis RANKL was weaker, the opposite occurred in the late replantation group, which seems to indicate the beginning of the root resorption process [12].

According to the IADT Guidelines [1], after an extra alveolar time of 30 minutes exposed to the dry or in a time greater than 60 minutes, regardless of whether the tooth was kept or not in an adequate storage medium, the cells of the periodontal ligament may not be viable. This maintenance time in a dry environment differs from the literature and some studies mention the time greater than 60 minutes for the cells of the periodontal ligament to become unviable [2, 31]. However, in the present study, it was shown that the extra-alveolar time of 20 minutes was as damaging to the cells of the periodontal ligament as the times of 60 and 90 minutes. In fact, when we investigated the molecules that regulate bone remodeling

and the periodontal ligament, we observed that at all times evaluated, periostin was absent and ALP was present, which indicates that even in the time of 20 minutes in the dry medium, the cells of the ligament were damaged and a process of bone neoformation was installed in the region that was occupied by the connective tissue of the periodontal ligament. Because periostin is essential in the development and processes of tooth eruption, homeostasis and periodontal integrity [17], it is also involved in the repair of wounds and tissue healing [18, 19].

All teeth replanted after a greater extra-alveolar time may develop ankylosis and replacement resorption over time [32]. However, replantation has advantageous for the patient, as it maintains the aesthetics, function and alveolar bone [33]. The progression of replacement resorption depends on age and systemic factors. Thus, in adults, ankylosing teeth remain functional longer than in children [30, 34]. Due to the faster pace of bone remodeling, infra-occlusion of reimplanted teeth is the main clinical problem in growing individuals [35,36]. Decoronation is one of the therapeutic indications for cases of infraocclusion [37], with the survival rate of reimplanted permanent teeth after traumatic avulsion being 50% after 5.5 years [38].

# Conclusion

Delayed tooth replantation led to inflammatory and replacement dental resorption, the first being characterized by recruitment of osteoclasts and RANKL synthesis and the second by inhibition of periostin and increased synthesis of ALP.

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# Declarations

### Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by J V B B, L A B S, R A B S and N C. The first draft of the manuscript was written by F M M P C O, F W P S e R A B S. All authors made contribution on the others versions of the manuscript. All authors read and approved the final manuscript.

### Additional Information

### Competing Interests

The authors declare no competing interests.

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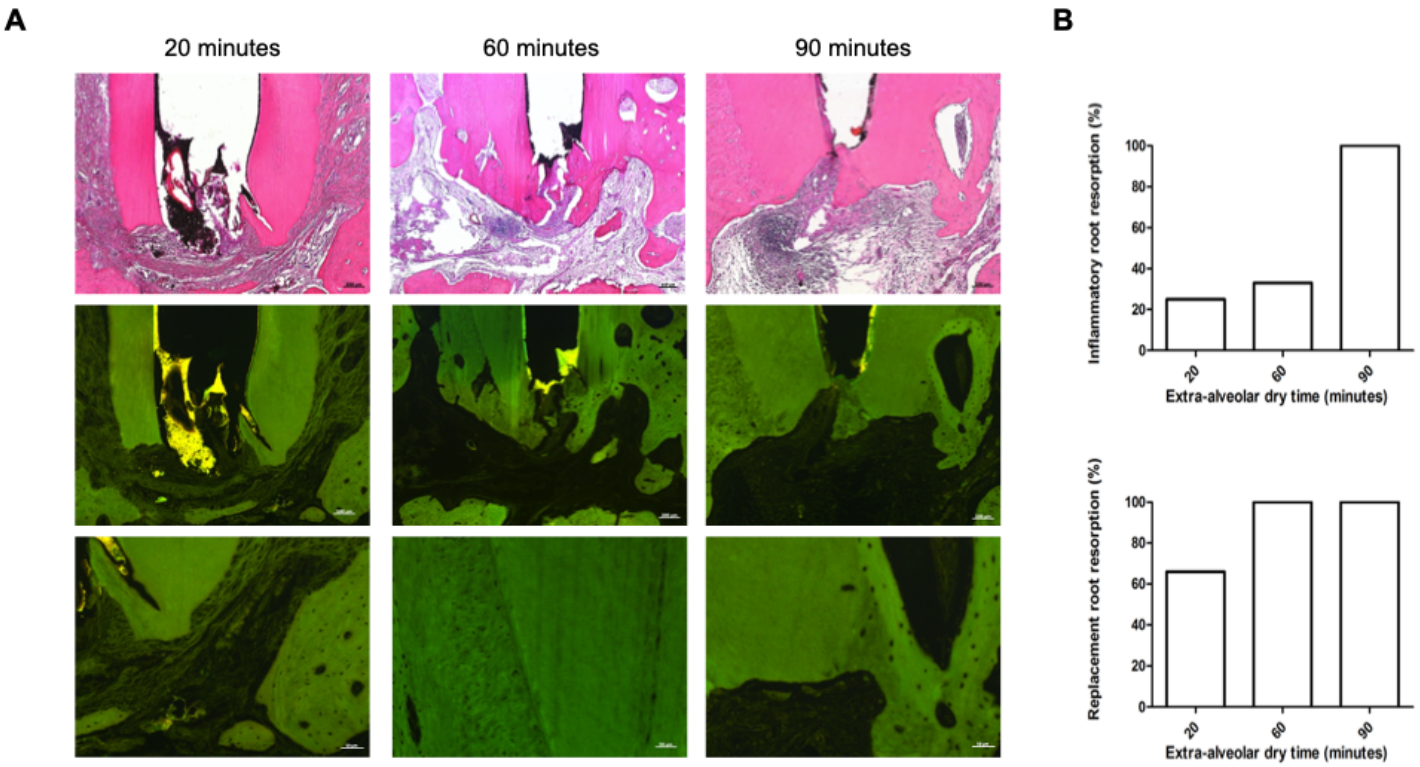
### Ethical approval:

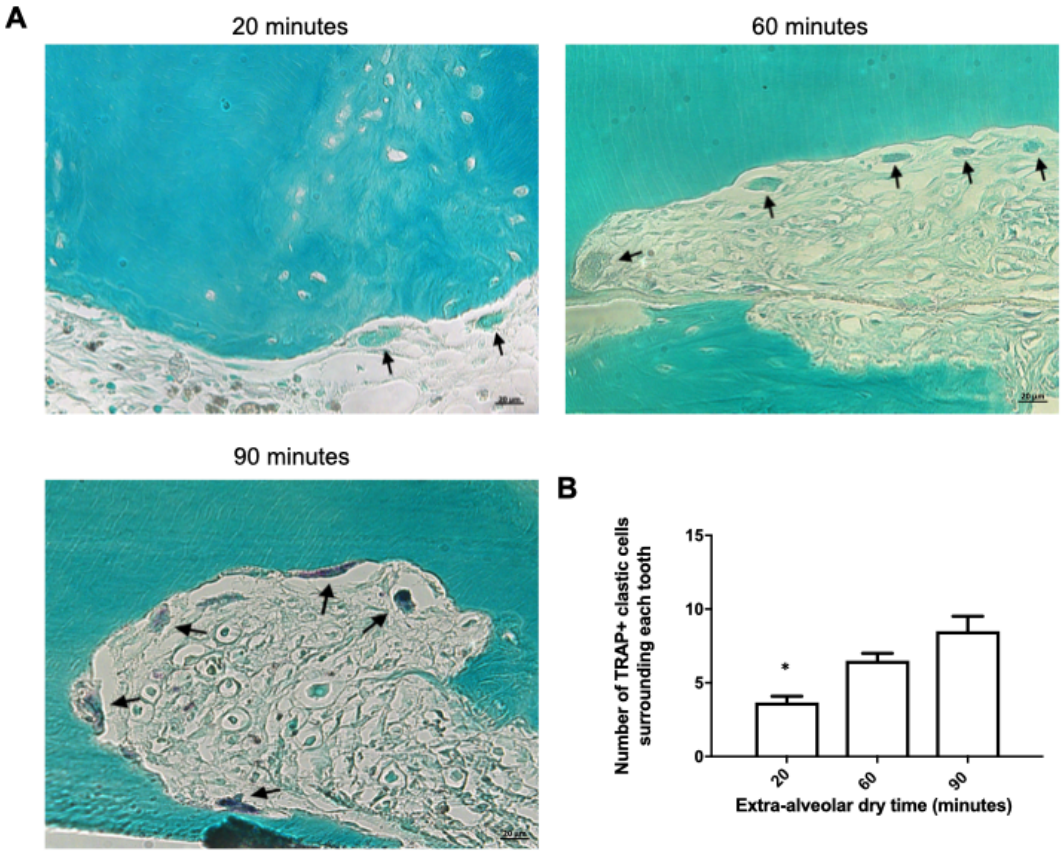
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was approved by the Institutional Animal Research Ethics Committee from University of Washington.

# Figures



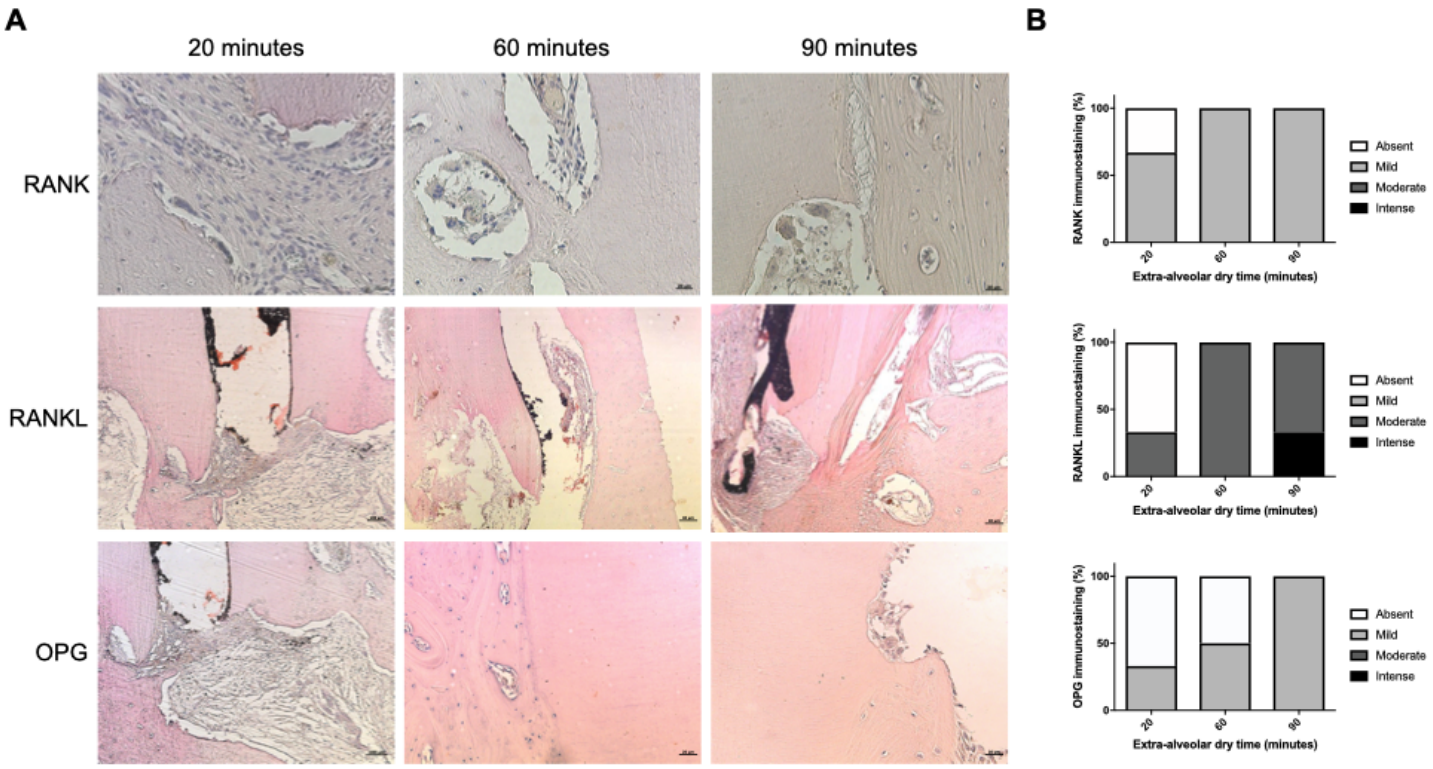

### Figure 1

Inflammatory and replacement root resorption in 20, 60 and 90 minutes in dry extra alveolar medium before replantation



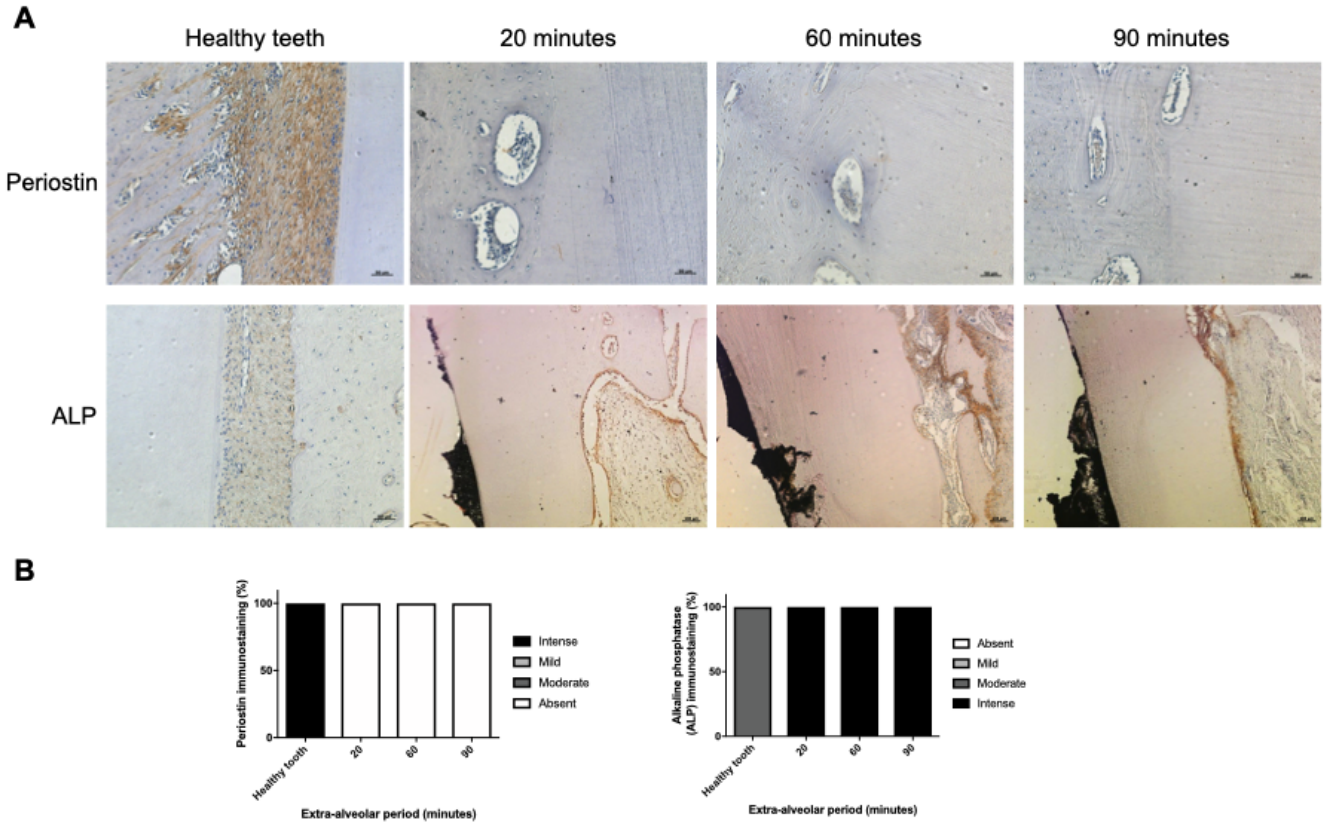

## Figure 2

Number of osteoclasts TRAP + and RANK + around the root surface in teeth after 20, 60 and 90 minutes extra-alveolar time




## Figure 3

Immunostaining for RANK, RANKL and OPG in teeth maintained for 20, 60 and 90 minutes of extra-alveolar time




## Figure 4

Immunostaining for periostin and alkaline phosphatase in the periodontal ligament of healthy teeth and in teeth after 20, 60 and 90 minutes extra-alveolar time