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Delayed Tooth Replantation after Root Surface Treatment with Papain and Sodium Fluoride in Rats: A Histological and Histomorphometrical Evaluation

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ABSTRACT

Introduction: The aim of this study was to examine the histological effect of papain and sodium fluoride in delayed replantation of rat incisor teeth on the repair process. **Methods and Materials**: Forty upper incisors of Wistar rats were randomly assigned to four groups (n=10). In group I, the dental papilla and the pulp tissue of extracted teeth were removed before immediate replantation in their sockets. In the other groups, the extracted teeth were maintained in dry storage for 60 min and subjected to different root surface treatments. In group II, the teeth were immersed in 10% papain for 20 min, scrubbed with gauze soaked in saline for 1 min, and immersed in a 2% acidulated-phosphate sodium fluoride solution for 20 min. In group III, the teeth were immersed in saline for 20 min, scrubbed with gauze soaked in saline for 1 min, and immersed in a 2% acidulated-phosphate sodium fluoride solution for 20 min. In group IV, root surface treatment was not applied. The root canals were treated and filled with a calcium hydroxide paste and the teeth were replanted. The animals were euthanized after 60 days and anatomic specimens containing the teeth were subjected to routine histochemical processing and staining with hematoxylin and eosin. The Kruskal-Wallis test was used, followed by the Dunn's test for multiple comparisons. **Results**: Groups I and II had less inflammatory root resorption and total area of root resorption (P<0.05) than groups III and IV respectively. **Conclusions:** Based on this animal study, root surface treatment with papain and sodium fluoride in delayed tooth replantation showed greater efficacy in controlling inflammatory root resorption and may be a viable option for clinical application.

Keywords: Avulsion; Papain; Sodium Fluoride, Tooth Replantation

Introduction

Avulsion of permanent teeth is one the most serious forms of dental trauma, because the tooth is completely displaced from its alveolar socket. This causes rupture of the gingival epithelium and periodontal ligament (PDL), damage to the cementum and alveolar bone, and destruction of the neurovascular bundle of the dental pulp [1]. Avulsion is prevalent in children due to sports, falls, and fights. It can result in tooth loss leading to impairment of aesthetics and function [2]. The healing of replanted teeth is a complex process that depends on the potential recovery of the several cell types involved and the proportion of healing in each of them [1]. The chances of successful tooth replantation procedures primarily depend on the maintenance of viable cemental PDL cells and cementoblasts [3, 4]. Therefore, shortening the time between trauma and replantation, and keeping the avulsed tooth in an appropriate storage medium until the moment of replantation may attenuate the deleterious effects of extra-alveolar dry time on the root surface and improve prognosis [4]. However, clinical experience has shown that immediate replantation is rare and most of the avulsed teeth, are replanted after a long extra-alveolar time in dry or inadequate wet storage, which may lead to failure of replantation and tooth loss [1]. Teeth replanted with non-vital PDL cells will invariably present with partial or total root resorption, failure of replantation and consequently, tooth loss [5-7].

The main etiologic agent in inflammatory root resorption is pulp necrosis associated with PDL damage and contamination. Inflammatory resorption is characterized by the presence of multinucleated resorption cells adjacent to the root surface and inflammatory cells in the adjacent periodontal tissue. The loss of vitality in the PDL and cementoblasts also leads to replacement resorption, characterized by the presence of the alveolar bone in contact with a resorbed root surface with or without inflammatory cells in the adjacent connective tissue [8] .

In order to increase the survival of the replanted tooth, several factors that influence the prognosis were studied [9-13]. Endodontic treatment and systemic antibiotic therapy are recommended for controlling bacterial contamination and prevention and limiting the progression of inflammatory root resorption [10, 11]. The treatment of the root surface with fluoride is also suggested in an attempt to increase its resistance to the resorptive process [12]. Removal of surface-adhered non-vital PDL cells have also been considered as they may act as stimuli for inflammatory root resorption [5, 13]. Scraping of root surfaces with a scalpel blade is one of the options suggested for mechanical PDL removal. However, the use of rubber cups at low-speed with a pumice-water slurry can be an alternative due to the non-homogeneous distribution of necrotic PDL remnants on root surfaces and the possibility of damage to the cementum layer by excessive force during scraping [14]. Preservation of the cementum layer is important because it is less susceptible to replacement resorption than dentin [13].

Removal of surface-adhered PDL cells before replantation with chemical substances such as sodium hypochlorite (NaOCl), has also been investigated. NaOCl has excellent antimicrobial action and capacity to dissolve organic materials and necrotic tissue remnants, which is an advantage over mechanical methods [5, 13]. While a reduction of root resorption rates after surface treatment with NaOCl has been demonstrated [5, 13], it has also been reported that the stability of NaOCl-treated replanted teeth is compromised by the formation of a fibrous connective tissue capsule involving the root, even when the chemical is used at low concentrations [9].

The guidelines of the International Association of Dental Traumatology for the management of avulsion of the permanent teeth currently suggests that devitalized PDL can be removed by cleaning with gauze [9]. According to the authors, the best way to this has not yet been decided and needs further studies [9]. Therefore, we believe that it would be interesting to find a biocompatible substance that could disorganize necrotic organic matter without causing damage to the cement layer, and have additional advantages such as disinfection. Since papain possesses some of these properties, it proved to be an option.

Papain is a purified protein obtained from the latex extracted from the leaves and fruits of adult green papaya (*Carica papaya*) [15-17]. This proteolytic enzyme is widely used in the food, cosmetics and pharmaceutical industries [15], and is able to displace necrotic tissue and prevent infection. It also possesses antioxidant and antimicrobial properties [16]. Depending on the characteristics of the wound, it is used at different concentrations ranging from 2% to 4% for dry wounds or granulation tissue. In the presence of purulent exudate and infection, concentrations ranging from 4% to 6% can be used, with a concentration of 10% recommended if abundant necrotic tissue is present [17]. In dentistry, papain is used for pulp tissue digestion and root canal irrigation during endodontic therapy and as a chemomechanical caries removal agent [18, 19].

The aim of this study was to histologically examine the repair process in delayed replantation of rat incisor teeth after root surface treatment with a papain (*Carica papaya*) solution followed by an acidulated-phosphate sodium fluoride solution. We hypothesize that papain would provide greater efficiency in the removal of necrotic PDL and contribute to the reduction of root resorption.

Materials and Methods

The methodology used in this study is based on previous tooth replantation studies in rats [5, 12]. The research protocol was approved by the Animal Care and Use Committee of the Araçatuba Dental School, UNESP (protocol number: 2009-008871). The animals included in the experiment were obtained from the bioterium of the Araçatuba Dental School, UNESP. Adults male Wistar rats (*Rattus norvegicus albinus*) weighing between 250 and 300 grams, with healthy periodontium and intact upper incisors, were selected. Animals with periodontitis, fractured crowns, and signs of weakness (low weight, coat deficiency) were excluded.

Forty animals were randomly assigned to four groups of 10 animals each. The animals were housed in plastic cages under climate-controlled conditions (12 h light/12 h dark; thermostatically regulated room temperature), were fed a standard solid chow, and given water *ad libitum* except during the 12 pre-operative h.

For the surgical procedure, the animals received an intramuscular injection of xylazine hydrochloride (Dopaser, Laboratório Calier do Brazil Ltda., Osasco, SP, Brazil; 10 mg/kg body weight) to attain muscular relaxation, and were anesthetized with ketamine hydrochloride (Dopalen; AgriBrands do Brazil Ltda., Paulínia, SP, Brasil; 80 mg/kg body weight).

Asepsis of the maxilla was performed before non-traumatic extraction of the maxillary right incisor of all animals. In group I, the dental papilla and the enamel organ of each tooth were removed with a #15 scalpel blade, and the pulp tissue was extirpated in a retrograde manner *via* a slightly curved size 15 K-file (Sybron Kerr Corporation, Orange, CA, USA). The canals were filled with saline, and the teeth were replanted in their respective sockets 7 min after the extraction. In the other three groups, the teeth were maintained in a dry storage at room temperature for 60 min and received the same endodontic procedure described for group I. The teeth of these groups were then subjected to different root surface treatments depending on the group.

In group II, the teeth were immersed in 10 mL of 10% papain solution (Farmácia Aphoticário, Araçatuba, SP, Brazil) for 20 min. The immersion time, volume, and concentration of the solution were established in a previous pilot study. The roots were then scrubbed with gauze soaked in saline for one min [5] and immersed in a 2% acidulated-phosphate sodium fluoride solution (Farmácia Aphoticário, Araçatuba, SP, Brasil), pH 5, for 20 min [5, 12]. In group III, the teeth underwent the exact same procedure described for group II, except the teeth were immersed in saline rather than papain for 20 min. In group IV, no root surface treatment was made. Before replantation, the root canals were irrigated with saline, dried with absorbent paper points, and filled with a calcium hydroxide (Calcium Hydroxid Für Analyse, Criedel, De Rainag Seelge, Hannover, Germany) and propylene glycol paste [20]. The paste was packed in a cartridge syringe with a needle and inserted into the canals in retrograde manner. The sockets were gently irrigated with saline for removal of clots, and the teeth were replanted. No splinting was placed. All animals received a single intraperitoneal dose of 20,000 IU benzathine penicillin G [5] (Eurofarma Laboratório, Itapevi, SP, Brazil).

Sixty days after replantation [21, 22], the animals were euthanized by anesthetic overdose. The anatomic pieces containing the replanted teeth were removed, fixed in 10% formalin for 24 h, and decalcified in a 4.13% ethylenediaminetetraacetic acid (EDTA) solution pH 7.0, for 60 days [23]. After decalcification, the specimens were embedded in paraffin from which semi-serial longitudinal 6-µm-thick sections were cut using a microtome and stained with hematoxylin and eosin for histological and histomorphometrical analyses under light microscopy. Only the palatal surface of the roots were examined because in rats, the PDL fibers attach only to this region of the root [5, 6].

Histological analysis

Images of the longitudinal root sections were first separated into three thirds (cervical, middle, and apical) using a compass, a ruler, and a fine pen. The region corresponding to the middle root third was analyzed with a light microscope (Axiolab; Zeiss, Oberkochen, Germany), and the characteristics of the PDL, cementum, dentin, and alveolar bone, as well as the occurrence of inflammatory and replacement root resorption, and extension of preserved cementum layer, were analyzed.

Histomorphometrical analysis

For the histomorphometrical measurements of the resorbed root surface area, sections of the four groups obtained at the 60-day period were analyzed. For each tooth, the image of the middle third of the root was captured using a digital video camera (JVC TK-1270

Table 1. Distribution of the scores attributed in the groups for replacement resorption, inflammatory resorption, total root resorption, and area of preserved cementum

	Replacement Resorption				Inflammatory Resorption				Total Root Resorption				Area of Preserved Cementum			
Score	GI	GII	GIII	GIV	GI	GII	GIII	GIV	GI	GII	GIII	GIV	GI	GII	GIII	GIV
1	6	1	1	2	8	7	5	1	6	1	-	-	6	2	1	-
2	1	7	6	6	-	1	3	3	1	6	4	2	2	1	1	-
3	1	-	2	1	-	-	1	1	1	1	4	2	-	4	2	2
4	-	-	-	1	-	-	-	4	-	-	1	4	-	1	3	6
5	-	-	-	-	-	-	-	1	-	-	-	2	-	-	2	2
N	8	8	9	10	8	8	9	10	8	8	9	10	8	8	9	10

GI (immediate replantation); GII (Papain and fluoride); GIII (Fluoride); GIV (No treatment); N (number of specimens). Scores: 1=no resorption; 2=0.1% to 25% of the area with resorption; 3=25.01% to 50% of the area with resorption; 4=50.01% to 75% of the area with resorption; and 5=75.01% or more of the area with resorption

Table 2. Dunn's test for inter-group comparisons with respect to inflammatory resorption, total area of root resorption and area of preserved cementum

	P-value		
Inter-group Comparison	**Inflammatory Resorption**	**Total Area of Root Resorption**	**Area of Preserved Cementum**
Group I×Group II	0.05	0.05	0.05
Group I×Group III	0.05	0.05*	0.05*
Group I×Group IV	0.001*	0.001*	0.001*
Group II×Group III	0.05	0.05	0.05
Group II×Group IV	0.05*	0.05*	0.05
Group III×Group IV	0.05	0.05	0.05

**statistically significant difference*

Color Video Camera, Tokyo, Japan) coupled to the light microscope and connected to a computer using Microsoft VidCap video capture software (Microsoft Corp., Redmond, WA, USA). Captured images (720×480 pixel) covered the entire middle third and were saved as individual TIFF files. Care was taken to standardize the size and position of the areas examined in all sections. ImageLab® 2001 image-analysis software (Diracom Bio Informática, Vargem Grande do Sul, SP, Brazil) was used for delimitation of the areas of root resorption. The area of resorbed root dentin in each specimen was demarcated (in pixels) in each image. The percentage of resorbed dentin was calculated from the dentin area delimited along the entire third and classified according to each type of root resorption. Inflammatory resorption was considered when multinucleated resorption cells were found on the root surface and inflammatory cells were found in the adjacent periodontal tissue. Replacement resorption was considered when the alveolar bone was in contact with a resorbed root surface, with or without inflammatory cells in the adjacent connective tissue. The total resorption was measured using the sum of the two types of resorption [8]. For analysis of the areas of preserved cementum, the root surface perimeter that presented an intact cementum layer in relation to the perimeter of the entire third was computed. Data were organized and statistical calculations were performed.

As resorption was not observed in several specimens, the value that could not be used for statistical calculations, the absolute values were transformed into percentages, and a 5-point scoring system was used as follows [6]: score 1=no resorption; score 2=0.1% to 25% of the area with resorption; score 3=25.01% to 50% of the area with resorption; score 4=50.01% to 75% of the area with resorption; and score 5=75.01% or more of the area with resorption. A score of 1 was given when the cementum layer was preserved in more than 75% of root surface, while a score of 5 was given when no cementum was present on root surface. The intermediate scores were given according to the proportion of cementum preservation. The distribution of the scores for replacement root resorption, inflammatory root resorption, total root resorption, and area of preserved cementum is presented in Table 1.

Statistical analysis

Since the morphometric parameters showed great variability, nonparametrical tests were used for statistical analysis of the areas of root resorption and preserved cementum. The Kruskal-Wallis test was used, followed by the Dunn's test for multiple comparisons. A significance level of 5% was set for all analyses.

Results

The animals adequately tolerated all experimental procedures. One animal from group III and two animals from groups I and II were removed from the study due to root fracture during tooth extraction.

Group I (immediate replantation; *n*=8): The main characteristic of this group was the integrity of root surface found in majority of specimens (mean=91.6%). The cementum layer was preserved, exhibiting a connective tissue rich in collagen fibers, fibroblasts and blood vessels. In most cases the collagen fibers presented an oblique arrangement suggestive of reinsertion (Figure 1). This group was least affected by root resorption, and only replacement resorption was found (mean=6.47%). The alveolar bone tissue was preserved throughout its extension. In all specimens, acute inflammatory infiltrates were seen in the apical foramen region.

Group II (papain and sodium fluoride; *n*=8): Unlike group I, fewer areas of fibrous connective tissue were observed on root surfaces. The collagen fibers did not exhibit a well-defined organization (Figure 2). In majority of cases, the PDL was replaced by alveolar bone tissue characterizing dentoalveolar ankylosis (Figure 3). Root surface was less affected by resorption than in groups III and IV (mean=13.6%). Replacement resorption occurred in 12.1% of the examined areas and inflammatory root resorption was observed in only 1.5%.

Group III (sodium fluoride; *n*=9): The histological features of PDL in this group were similar to those of group II. The collagen fibers did not exhibit a well-defined organization. Areas of preserved cementum represented 27.1% of the root surface. In majority of cases, the PDL was replaced by alveolar bone tissue. Compared to groups I and II, the incidence of root resorption was greater in this group (mean=19.3%). Replacement resorption (mean=12.4%), occurred in all specimens (Figure 4) and inflammatory root resorption was observed in four specimens (mean=6.8%). In some areas, clastic cells were found close to the resorbed dentin (Figure 5).

Group IV (no root surface treatment; *n*=10): Some areas of fibrous connective tissue were found on root surface. In most cases, they did not exhibit a well-defined organization, and presented with mild inflammatory infiltrates (Figure 6). This group was the most affected by root resorption (mean = 50%) with few areas of reserved cementum (mean=11.3%). Most parts were affected by inflammatory root resorption (mean=33.5%), being present in nine specimens. Replacement resorption was observed in eight specimens, corresponding to the highest incidence of this type of resorption among the groups (mean=16.5%). Multiple areas of active resorption were observed (Figure 7). Osteoclasts were also found on the alveolar bone wall in several areas. Statistically significant differences ($P<0.05$) among the groups were found with respect to inflammatory root resorption, total area of root resorption and area of preserved cementum. Table 2 summarizes the inter-group comparisons.

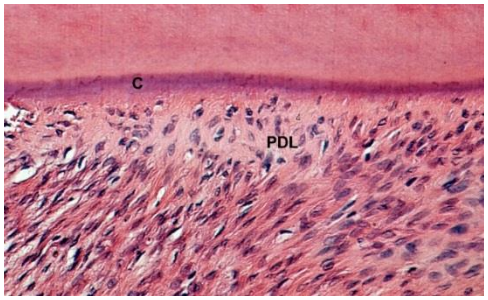

Figure 1. Group I (Immediate replantation): Root surface with preserved cementum layer (C) and reinsertion of PDL fibers can be seen. (Hematoxylin-eosin, original magnification ×160)

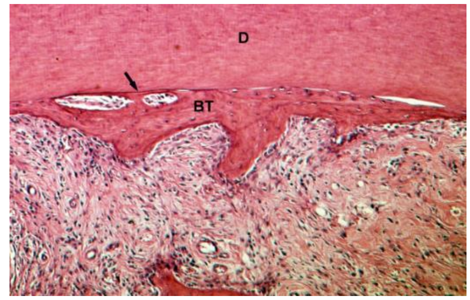

Figure 3. Group II (Papain and sodium fluoride): Bone tissue (BT) adjacent to the intact cement (arrow) and dentine (D), forming ankylosis. (Hematoxylin-eosin, original magnification ×63)

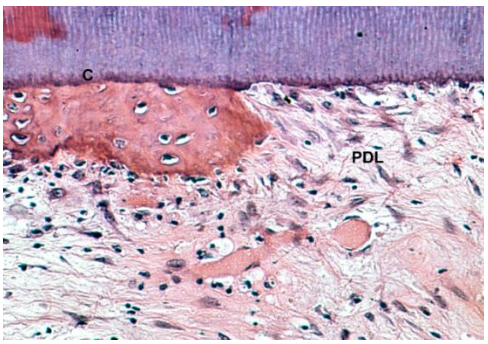

Figure 2. Group II (Papain and sodium fluoride): Root surface with preserved cementum layer (C), reinsertion of PDL fibers and bone tissue (BT) adjacent to the intact cement in the left side can be seen. (Hematoxylin-eosin, original magnification ×160)

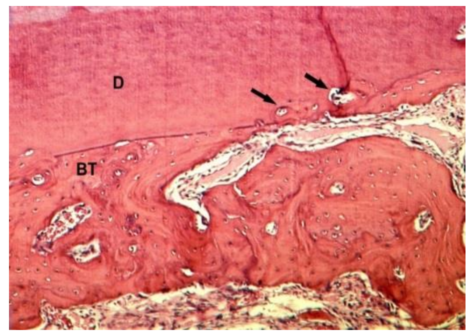

Figure 4. Group III (Fluoride): Alveolar bone tissue (BT) occupying the PDL space. Areas of active replacement resorption (arrows) compromising the superficial dentin layer (D). (Hematoxylin-eosin, original magnification ×63)

Discussion

In the present study, the low incidence of root resorption observed in group I demonstrates that the vitality of PDL cells and cementoblasts was preserved [4, 6, 24], the adopted extraction and replantation procedures were adequate, and that these procedures did not cause additional trauma that could have affected the results. If the extra-oral dry time is short, the influence of root handling prevails. In the other groups, the extracted teeth were maintained in dry storage for a longer time to simulate a common clinical condition in cases of dental avulsion due to trauma [5, 24]. In this case, PDL and pulp tissue necrosis is expected, acting as a source of bacterial contamination for the development of inflammatory root resorption [10, 11, 25]. This type of resorption was found in all groups with delayed replantation and was more accentuated in group IV. The incidence of inflammatory root resorption in groups II and III were not high and were in accordance with a previous study [25].

Tooth replantation studies with rat incisors have been performed by several authors [5, 6, 26, 27]. The rat incisor is a continuously erupting tooth, such that when the dental papilla and the pulp tissue are removed, continuous growth of the root is ceased [28]. This procedure was performed in this study to favor comparison with human teeth.

Only the middle third of the lingual surface of the roots were examined histologically and histometrically because this region is not damaged by surgical procedures. The cervical and apical thirds may be damaged by the grasping action of the forceps during tooth extraction and the cutting action of the scalpel blade during dental papilla removal, respectively.

The endodontic treatment and systemic antibiotic therapy given had an important role in subduing bacterial contamination and controlling the occurrence or progression of root resorption [10]. Intracanal therapy with calcium hydroxide has a direct action against residual bacteria and their toxins present inside the dentinal tubules [29]. Systemic antibiotic therapy acts against the infection on root surfaces and inside the alveolar sockets, which could be caused by contact with saliva, especially in cases of long extra-alveolar periods [11]. Despite not using calcium hydroxide as intracanal therapy, the absence of inflammatory resorption in group I may have been due to the preservation of the cementum layer by the presence of viable PDL cells and cementoblasts, which prevented bacteria and their byproducts from connecting with the connective tissue on the root surfaces [10, 12].

Removal of surface-adhered non-vital PDL cells is of equal importance for controlling the progression of inflammatory root resorption, which may culminate in the loss of the replanted tooth

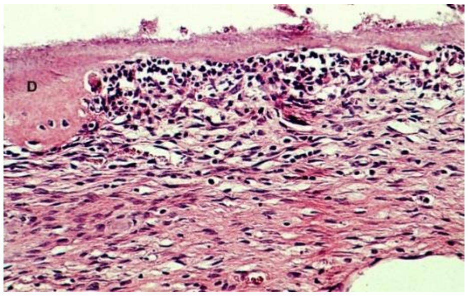

Figure 5. Group III (Fluoride): Inflammatory root resorption affecting dentin (D) more deeply and inflammatory infiltrate in the connective tissue close to the resorbed area has been shown. (Hematoxylin-eosin, original magnification ×160)

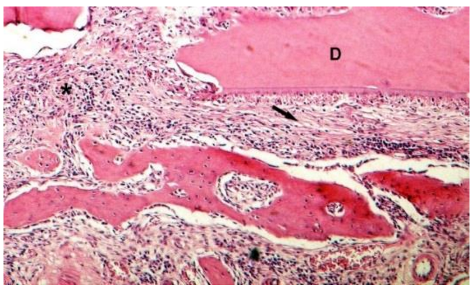

Figure 6. Group IV (No treatment): Areas of root resorption affecting great part of the dentin and inflammatory infiltrate in great part of the fibrous connective tissue (asterisk) has been shown. PDL remnants and absence of reinsertion of collagen fibers (arrow) can be seen. (Hematoxylin-eosin, original magnification ×63)

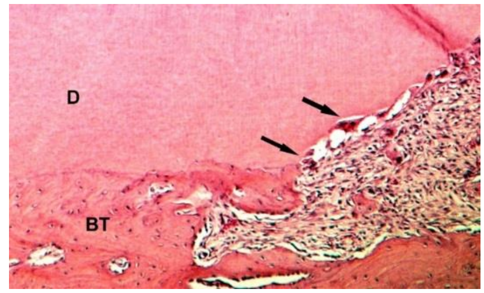

Figure 7. Group IV (No treatment): We can see area of replacement resorption affecting dentin (D) more deeply in the left side and areas of active root resorption with presence of clasts close to dentin (arrows). (Hematoxylin-eosin, original magnification ×63)

[1, 4-6, 28]. The maintenance of necrotic PDL remnants on root surfaces leads to the release of enzymes and inflammatory chemical mediators resulting from their degradation during the repair process [5, 13, 30]. Group IV exhibited this condition and was affected more than other groups by inflammatory root resorption, demonstrating the need of PDL removal as part of the root surface treatment that should precede tooth replantation. In the present study, scrubbing with gauze was the method used [5, 9]. In addition to inflammatory resorption, teeth replanted under these conditions were also affected by replacement resorption [27, 30, 31]. In order to avoid root resorption, treatment of root surface with fluoride has been recommended [5, 12, 26]. The aim is to increase dentin resistance and inhibit clastic activity by the formation of fluorapatite crystals [12]. The combination of these procedures contributed to the observations seen on the root surfaces of groups II and III. However, the surface treatment used in group II resulted in lesser root resorption than group III, which led us to infer that the use of papain contributed to this result.

It is described in the literature that papain is an enzyme similar to human pepsin. It has great efficiency in debriding necrotic tissues without causing damage to healthy tissues [32]. The existence of a plasma anti-protease in normal tissues, α-1-antitrypsin, prevents the proteolytic action of papain [33]. The absence of this anti-protease in necrotic tissues permits this action and causes rupture of partially degraded collagen molecules [33]. It has also been demonstrated that papain has antimicrobial activity, being capable of inhibiting the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These bacteria are commonly found in acute and chronic wounds [15]. Bactericidal and bacteriostatic actions of papain with inhibition of the growth of gram-positive and gram-negative bacteria, have also been demonstrated by Dawkins *et al.* [33]. These properties could have led to more efficient removal of necrotic PDL and eradication of bacteria, which are important factors in the development and persistence of inflammatory root resorption. As the inflammatory resorption manifests in the very early stages of the repair process and progresses rapidly, controlling this type of resorption is a very important step for preserving the cementum layer on teeth to be replanted [30, 31]. Even without the protection of PDL and cementoblasts, the cementum is less susceptible to replacement resorption than dentin, which makes its preservation advantageous even if ankylosis occurs [13, 14]. Papain is commercially available in lyophilized form. However, this form is inactivated when in contact with oxidant agents and after long periods of exposure to the air. Nevertheless, papain has few side effects, is affordable, and easy to handle, thus making it an interesting option for root surface treatment of avulsed teeth prior to replantation [17].

Conclusion

Based on this animal study, the use of papain in combination with sodium fluoride solution for root surface treatment in delayed replantation of teeth with non-vital PDL showed great efficacy in controlling inflammatory root resorption progression. Therefore, it can be considered as a viable option for these cases.

Conflict of Interest: 'None declared'.

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