Original Article

Does periodontal ligament removal using natural proteolytic enzymes alter the surface structure and microhardness of cementum of avulsed tooth? An *in vitro* analysis

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Abstract

Introduction: Surface treatment of avulsed tooth must be directed towards the debridement of necrotic periodontal fibers while having no effect on the cementum microhardness. The role of proteolytic enzymes on the periodontal ligament (PDL) surface and hardness of cementum has not been investigated in the literature.

Aim: The present study aimed to evaluate the effect of 5.25% sodium hypochlorite (NaOCl), 10% bromelain, 10% papain and 10% panzyme on root surface topography and microhardness of avulsed teeth.

Materials and Methods: Forty healthy premolars were freshly extracted and randomly allocated into five treatment groups based on the surface agent employed; Group 1: 50 ml saline, Group 2:20 ml of 5.25% NaOCl, Group 3: 20 ml of 10% bromelain enzyme, Group 4: 20 ml of 10% papain enzyme, and Group 5: 20 ml of 10% mixed panzyme enzyme. The specimens were then subjected to confocal laser scanning microscopy and scanning electron microscopic examination. Supplemental set of freshly extracted forty premolars were decoronated longitudinally and one-half from each specimen was submitted for Vickers microhardness testing before and after application of the above-mentioned agents.

Statistical Analysis: Intragroup comparison of mean percentage hardness reduction was assessed using paired *t*-test.

Results: Treatment with NaOCl led to complete removal of PDL fibers with the presence of cracks and crazes on the root surface along with a significant reduction in microhardness. Bromelain and papain revealed regular and even distribution, while few PDL fibers were evident with papain. Panzyme revealed a shiny surface with minute cracks and a significant reduction in microhardness.

Conclusion: Ten percentage bromelain for 10 min was effective in the removal of necrotic PDL fibers and preserved the cementum integrity better.

Keywords: Avulsion, cementum, microhardness, periodontal ligament, proteolytic enzymes

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Submission: 28-04-22 **Revision:** 06-06-22 **Acceptance:** 10-06-22 **Web Publication:** 11-01-23

Access this article online	
Quick Response Code:	Website: www.saudiendodj.com
	DOI: 10.4103/sej.sej_80_22

How to cite this article: Taneja S, Dudeja C, Bhalla VK, Taneja P. Does periodontal ligament removal using natural proteolytic enzymes alter the surface structure and microhardness of cementum of avulsed tooth? An *in vitro* analysis. Saudi Endod J 2023;13:57-62.

INTRODUCTION

Immediate replantation of avulsed tooth is recognized as the most determinant factor that significantly contributes to enhanced survival of periodontal ligament (PDL) cells.[1] However, in conditions of delayed replantation, the supporting periodontal tissues may lose their viability and the presence of necrotic remnants may provoke external root resorption which may negatively impact the survival rate of the replanted tooth.[2,3] As a result, special surface treatment needs to be directed on the surface of the periodontium to improve the prognosis in these cases.

Acids, enzymes, and fluoride solutions have been advocated for the removal of devitalized PDL fibers from an avulsed tooth.[4] Lindskog *et al*. suggested removing necrotic PDL from the surface of the root with the use sodium hypochlorite (NaOCl).[5] However, studies by Becking and Kaufman and Keila reported that NaOCl affected the physical properties of tooth such as reduction in hardness apart from other harmful effects such as tissue irritation and allergenic potential.[6,7] This risk of tissue damage was proportional to the increase in the concentration of the solution.[8] This fact indicates the need to search for other alternative substances that are as effective as NaOCl but do not possess such caustic adverse effects.

Another alternative for the removal of necrotic PDL is the use of proteolytic agents such as bromelain and papain.[9] Bromelain belongs to a group of cysteine protein-digesting enzymes extracted commercially from the stem of pineapple fruit. Bromelain is composed of a sulfhydryl group, peroxidase, acid phosphatase, glycosidases along with several protease inhibitors It also possesses bacteriostatic and anti-characteristics.[10,11]

Papain is a cysteine endopeptidase derived from the latex of the unripen fruit of green papaya, *Carica papaya*. It has bactericidal, bacteriostatic, and anti-inflammatory characteristics.[12] It is nontoxic and naturally occurring enzyme and extensively used in the fields of medicine and could be used as a potential agent for dental applications for surface management of avulsed teeth.[13] Panzyme is a mixture of plant-derived enzymes constituting ginger (31%), bromelain (6%), and papain (2%). These properties instigate the need for future research on the use of these proteolytic agents before replantation of avulsed teeth.

No study in the literature has evaluated the surface structure and microhardness of avulsed teeth with the use of these proteolytic agents. Therefore, the present study was conducted to analyze and compare the surface structure and microhardness of cementum of freshly extracted teeth after necrotic PDL removal after treatment with NaOCl and natural proteolytic enzymes.

MATERIALS AND METHODS

The sample size was calculated based on the results of the pilot study with effect size as 0.6, 80% power and 5% α error. The final sample size was estimated to be 40 using G*power software, version 3.1.9.7 (Franz Faul Universität, Kiel, Germany).

Ethical clearance for the study was obtained from Institutional Ethical Committee Review Board Under Protocol Number ITSCDSR/IIEC/RP/2017/001. Forty freshly extracted sound maxillary premolars extracted for orthodontic reasons from adolescents of age group ranging between 14 and 16 years were included. The teeth were then allowed to dry at room temperature (26°C–28°C) for 60 min to simulate delayed replantation and further rehydrated by immersion in 50 ml saline for 10 min. Teeth were viewed under an operating microscope (LaboMed Prima DNT, New York) and those with cracks, fractures, or hypoplastic defects were excluded from the study.[14] In this study, as no teeth were excluded, the roots of 40 teeth were gently wiped with a gauze piece to detach the PDL fibers and then randomly allocated into five groups of eight teeth each depending on the agent used for surface treatment:

1. Group 1 (control) (n = 8): Teeth were immersed in 50 ml saline (Lifusion, India)
2. Group 2 (n = 8): Teeth were immersed in 20 ml of 5.25% NaOCl (Samagra Lifecare, India)
3. Group 3 (n = 8): Teeth were immersed in 20 ml of 10% bromelain enzyme (Bromelain 020-04262, FUJIFILM WAKO pure Chemical Corporation, Osaka, Japan)
4. Group 4 (n = 8): Teeth were immersed in 20 ml of 10% papain enzyme (Papain 164–00172, FUJIFILM WAKO pure Chemical Corporation, Osaka, Japan)
5. Group 5 (n = 8): Teeth were immersed in 20 ml of 10% mixed Panzyme solution.

The 30 capsules of Veg Panzyme Plus (Optim Health Plus, Twilight Litaka Pharma Limited, India) weighing 10 g were grinded to a fine powder and mixed with 100 ml of water to obtain a 10% panzyme solution.

After treatment with different agents for 10 min at room temperature, samples were rinsed for 30 s with saline and further submerged for another 10 min in 50 ml saline solution. All the crowns were decoronated using a high-speed diamond disc (Kerr Dental, USA) under

constant water irrigation. Four roots from each group were subjected to fluorescence staining and observed under confocal laser scanning microscopy (CLSM), while the remaining four roots were used for scanning electron microscopic examination (SEM).

Confocal laser scanning microscopy

For CLSM analysis, a total of 20 samples (4 from each group) were cut along the longitudinal axis in half using a diamond saw under constant water cooling. Thereafter, all the 40 specimens were immersed in a 50% methanol solution with 0.5 mg rhodamine solution (0.5 mg in 50 mL) for 5 min and then washed in deionized water for 3–5 min. The middle third area of each specimen was examined using CLSM and rhodamine was detected (Em 525/50 nm band pass filter).

Scanning electron microscopy

The remaining 20 samples were taken up for SEM analysis and fixed in 2.5% glutaraldehyde solution at 4°C for 12 h and then sectioned longitudinally in half using a diamond disc under copious water cooling. The 40 specimens obtained were washed in phosphate-buffered saline for 20 min and further immersed in the same solution for additional 12 h and finally dehydrated in a graded manner with ethanol.

Double-faced adhesive tapes were used to fix the specimens on metallic stubs which were then sputter-coated with platinum, vacuumized, and further observed with SEM (Carl Zeiss, Evo Ls 10, Germany). The middle third portion of each specimen was observed at ×4000–8000 and surface images of cementum were captured.

Vickers microhardness indentation test

Another set of 40 freshly atraumatically extracted healthy intact premolars from patients with age group 14–16 years with single mature roots were selected for microhardness testing. Teeth with cracks or hypoplastic defects visible under an operating microscope were eliminated and the selected samples were stored in distilled water under room temperature. The crowns were decoronated and then sectioned in half longitudinally using a double-sided diamond disc to obtain a total of 80 specimens. One-half from each specimen was embedded in polymerizing cold cure acrylic resin with the outer exposed cementum surface ground polished using a water-cooled carborundum paper and further subjected to Vickers hardness testing (Banbros, India) to get the pretreatment (Baseline) values.

Vickers test was done using a diamond indenter applied with a loading force of 0.1 kg for 10 s on a minimum of three similar positions. The average of the three values was calculated for each specimen. Samples were divided into four groups ($n = 10$) depending on the agent used for surface treatment of exposed cementum:

1. Group A: The samples were immersed in 20 ml of 5%NaOCl solution at room temperature for 10 min. After treatment, the samples were rinsed with saline and dried
2. Group B: Similar procedure was repeated using 20 mL of 10% bromelain enzyme for 10 min
3. Group C: Similar procedure was repeated using 20 mL of 10% papain enzyme for 10 min
4. Group D: Similar procedure was repeated using 20 ml of 10% mixed panzyme enzyme for 10 min.

Vickers microhardness indentation tests were done again to obtain the posttreatment values and the mean difference in microhardness was calculated.

Statistical analysis

Mean percentage reduction in hardness values pre- and post-treatment in different groups was evaluated using paired *t*-test.

RESULTS

CLSM images of the control group showed PDL remnants in each of the root sections and layer of thin fibrous tissue covering the roots [Figure 1a]. CLSM images revealed complete removal of PDL fibers after treatment with NaOCl and 10% bromelain [Figure 1b and c],while few PDL remnants were seen with 10% papain [Figure 1d].The root surfaces of panzyme treated solutions revealed a shiny surface with a complete absence of necrotic PDL fibers [Figure 1e].

SEM results of NaOCl treated samples revealed cracks and crazes on the surface of the root [Figure 2a]. The SEM images of Bromelain treated samples revealed regular and even distribution of the mineralized fibers of cementum. At 4000 × SEM image magnification, cementum lacunae were clear with no evidence of cracks or defects [Figure 2b]. A preserved cementum integrity with a mineralized structure was seen in Papain-treated samples. It was distributed evenly with no cracks or defects on the root surface [Figure 2c]. Panzyme-treated samples showed minute visual cracks and defects on the middle third section of the root surface with the mineralized fibers of cementum exhibiting an irregular and uneven distribution [Figure 2d].

There were statistically significant differences ($P < 0.05$) between the pre- and post-operative hardness for all

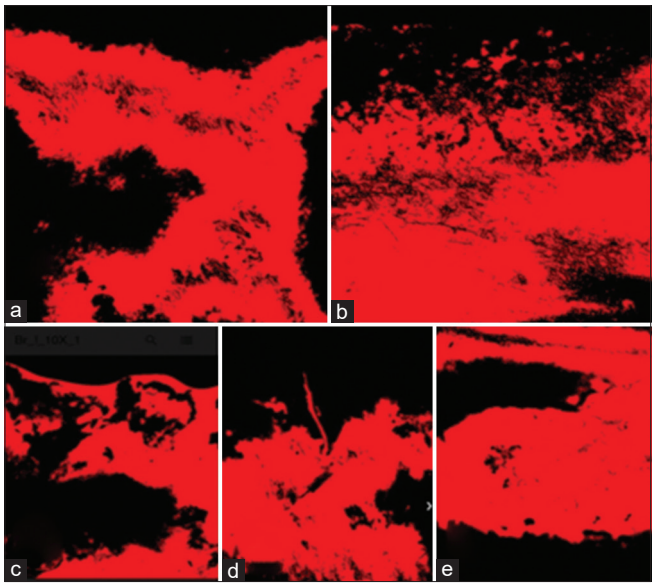


Figure 1: Confocal laser microscopy images: (a) In Group 1 (control), PDL remnants and thin fibrous tissue was seen on root surface. (b,c) Group 2 (NaOCl) and Group 3 (Bromelain) showed complete removal of PDL fibers. (d) Group 4 (Papain) showed few PDL remnants on root surface, (e) Group 5 (Panzyme) showed shiny surface with absence of necrotic PDL fibers. PDL: Periodontal ligament, NaOCl: Sodium hypochlorite

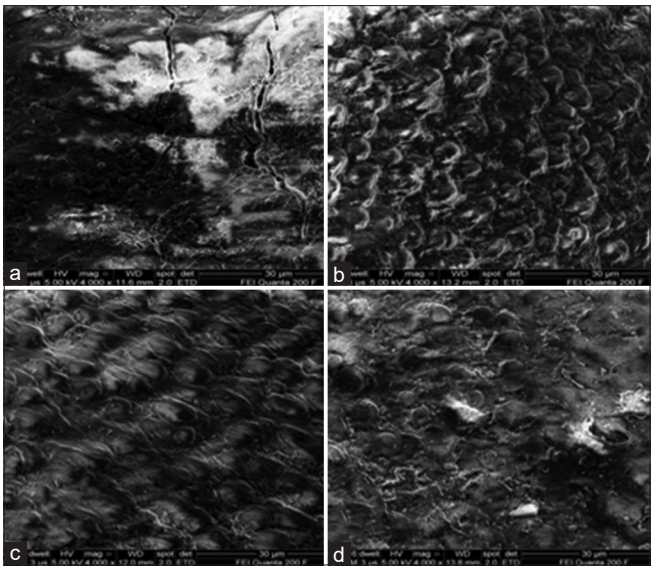


Figure 2: Scanning electron microscopy (×4000) (a) In Group 2 (NaOCl), cracks (depicted in arrows) and crazes could be seen. (b) In Group 3 (Bromelain), the mineralized fibers of cementum were arranged regularly and distributed evenly, lacunae were clearly seen while no cracks were visualized (c) In Group 4 (Papain), mineral structure was distributed evenly and no cracks were observed. (d) In Group 5 (Panzyme), irregular distribution of fibers was seen with minute cracks on the middle third. NaOCl: Sodium hypochlorite

the groups and followed the order: NaOCl > mixed Panzyme > Bromelain > Papain [Graph 1].

DISCUSSION

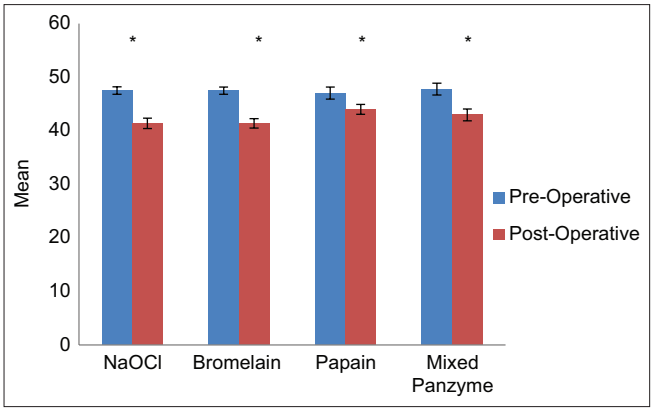
Root resorption is a frequent sequelae that follows when avulsed teeth are replanted after prolonged extra alveolar dry time and are not subjected to ideal physiological conditions.[15] In the present study, the clinical scenario of delayed tooth replantation with necrotic PDL fibers was simulated and natural surface agents aimed at chemical modification of the root surface were evaluated.[15] Mineral gain or loss within the dental hard tissues after treatment with natural proteolytic enzymes can be indirectly evaluated using microhardness testing for which the Vickers microhardness test was employed as it provides a suitable and practical approach for assessment of surface changes.[16]

The findings of this study confirm that when the surface of the root was wiped using a wet gauze piece, the cemental layer was well protected with intact PDL remnants and fibers on the surface of the root, further reinforcing the need for more effective surface treatment agents to achieve complete periodontal debridement. The present study highlighted the efficacy of NaOCl with bromelain, papain, and mixed panzyme in removing necrotic PDL remnants as well as their effect on microhardness of the root surface.

In the present study, 5.25% NaOCl treated root surfaces exhibited complete removal of PDL fibers and a statistically significant reduction in hardness values. This may be due to the detrimental effect of NaOCl on the organic content of cementum as seen with dentin.[17] The organic content of dentin is 20% while that of cementum is much higher accounting for 50%–55% indicating the enhanced deleterious effect of hypochlorite on cementum. The results of this study are in accordance with Goldberg and Bai *et al*., who reported that 5% NaOCl was effective in the removal of necrotic PDL but decreased the microhardness of the root surface.[17,18]

Treatment with 10% bromelain solution also exhibited removal of PDL fibers with a significant reduction in hardness values. This removal of fibers is because of the proteolytic action or protease activity of the reactive sulfydryl group present in bromelain which acts by the formation of amino acid residues after the breakdown of proteins through a hydrolytic reaction.[19-21] The proteolytic activity of bromelain has been emphasized in the study by Hale *et al*.[22] However, its effect on the surface topography of the cemental surface has never been investigated in previous researches.

When the root surfaces were treated with 10% papain solution, it showed few remnants of PDL fibers with a significant reduction in microhardness. SEM showed



Graph 1: Mean percentage reduction in hardness values pre and post treatment in different groups using Paired *t*-test. *Suggests statistically significant differences between the preoperative and postoperative values in all the groups. ($P < 0.05$ was considered statistically significant). NaOCl: Sodium hypochlorite

well-preserved cementum. The proteolytic action of papain is by cleaving collagen molecules and the ability to digest dead cells. It has been investigated to aid in the process of wound healing due to its ability to promote the debridement of devitalized tissue.[22] Peptide bonds involving basic amino acids are preferentially cleaved to form amino acid residues and weakening the collagen cross-linkages.[23,24] The results can be correlated to the study by Flind.[25] Cracks and fractures were minimal after use of 10% papain which is in accordance with the study by Pithon *et al*.[26]

The root surfaces of panzyme treated samples showed a shiny surface with a complete absence of fibrous layers. SEM confirmed cementum integrity with minute visual cracks and defects with unevenly and irregularly arranged cemental fibers when compared to images of bromelain and papain-treated root surfaces. The possible explanation is that it possesses a synergistic proteolytic activity of all proteases together. It exhibits not only bromelain and papain protease activity but is also composed with a major percentage of ginger. Proteases in ginger rhizome are a part of the cysteine enzyme family, which possessed a very strong catalytic action.[27] This is in congruence with the study by Kim *et al*., where the collagenolytic activity of ginger proteases was found to be superior to the other plant cysteine proteases such as papain and bromelain.[28]

Considering the limitations of the study, it may be inferred that a 10 min application of hypochlorite solution effectively removed devitalized PDL from delayed replanted teeth but with an unfavorable effect on the structural integrity of cementum. Ten percentage bromelain for 10 min was effective in the removal of necrotic PDL fibers and preserved the cementum integrity better. Further clinical studies are required to validate the findings of this study.

CONCLUSION

5.25% NaOCl solution was found to be effective in the removal of necrotic PDL while negatively affecting the cementum microhardness. Ten percentage papain did not negatively affect the surface topography of cementum but left remnants of PDL fibers on its surface. The use of 10% bromelain may be considered a potential alternative agent for surface treatment of avulsed teeth.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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