The effect of local propolis irrigation as an adjunct to scaling and root planning on alveolar bone loss in experimental periodontitis in rats (A biochemical and histological study)

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Background and objectives: The present study aimed to investigate the efficacy of local use of propolis (PRO) as adjunctive to scaling and root planing (SRP) in the treatment of alveolar bone loss in rats with ligature induced periodontitis (LIP).

Material and methods: 60 male rats were divided into three groups of 20 rats: the control group (C) with no experimental periodontitis or treatment (gp1); ligature-induced periodontitis treated by SRP with a vehicle irrigation (dimethyl sulfoxide + physiological saline solution) group (gp2); and ligature-induced periodontitis treated by SRP with 10% PRO extract irrigation group (gp3).

The irrigation materials were locally applied three times daily for 11 days After removal of the ligature, SRP was performed in gp2 and gp3. Five rats from each group were euthanized on days 0, 7, 14, and 21 after local treatment. Intracardiac blood samples and mandible were obtained for biochemical and histopatholical analysis.

Results: Rats that received local treatment by SRP with propolis irrigation in gp3 revealed features of acceleration of the periodontal tissue-repair process, alveolar bone formation, and significantly higher alkaline phosphatase serum levels (ALP) than gp2 were treated by SRP with vehicle irrigation.

Conclusion:

Local use of propolis as an adjunct therapy to SRP can be effective in the treatment of alveolar bone loss in rat model of ligature-induced periodontitis.

Key-words:

Alveolar bone resorption, Alkaline phosphatase enzyme, Ligature induced periodontitis, Propolis.

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Introduction

Periodontitis is a chronic inflammatory disease associated with dysbiotic plaque biofilms resulting in progressive destruction of the periodontal structures[1,2] characterized by the inflammation of the periodontal connective tissue with influx of inflammatory cells.[3] The resultant influx of inflammatory cells produces a host of cytokines that promote bone resorption through osteoclasts, the primary bone resorbing cell.[4]

Therapeutic approaches for periodontal diseases include mechanical scaling and root planing (SRP) is the most commonly therapy used for the treatment of periodontitis, and remains the "gold standard" for the non-surgical management of chronic periodontitis.[5] Nevertheless, these procedures are not always satisfactory. Thus, adjunctive therapies may be necessary including antibiotics and non-steroidal anti-inflammatory drugs.

The major disadvantage of these agents is the development of bacterial resistance and gastric/renal toxicity. Thus, the search for newer and safer therapeutic agents continues to overcome the adverse effects of both anti-

microbial and anti-inflammatory agents as an adjunct to conventional mechanical therapy.[6] Propolis has attracted researchers' interest in the last decades because it possesses a broad spectrum of biological and pharmacological properties, such as immunomodulatory, anti-inflammatory, and antibacterial.[7,8] In periodontal disease treatment, propolis was able to prevent alveolar bone loss and promote bone formation.[9,10] Since the ligature- induced periodontitis (LIP) is characterized by an inflammatory alveolar bone resorption, in light of the above information on propolis, it could be advantageous to use propolis in the treatment of periodontal disease.

Consequently, the aim of the present study was to evaluate in vivo the therapeutic effects of local use of propolis as a monotherapy or an adjunctive therapy with scaling and root planing over inflammatory response and alveolar bone loss on rats with ligature-induced periodontitis.

Methods

Settings and Design:

The present study was carried out at Hawler Medical University, College of Dentistry, and the animals used in the study were rats that were housed in an animal facility at the College of Medicine. The experimental part of the study was carried between December 2016 and August 2017.

Animals

Experimental study was conducted on 60 adult male Wister rats weighing 250-300 grams were used. Five rats were allocated to each stainless steel cage and maintained under a 12-hour light/dark cycle at temperature of 24± 2°C and a relative humidity of 20%-30% with access to standard rat chow pellets and water ad libitum. All experimental protocols of the present study were approved by the animal ethics committee of Hawler Medical University/College of Dentistry on 14 January 2016.

Induction of experimental periodontitis:

Experimental periodontitis was induced by silk ligation under general anesthesia (administered by intramuscular injection of 0.1 ml of ketamine hydrochloride and 0.05 ml of xylazine hydrochloride per 100 g of body weight) by placing 3.0 sterile black braided silk threads around the cervix of the mandibular left incisor teeth. The ligature was kept in place for 4 weeks. In intact control rats, ligatures were not placed.

Experimental design:

Animals were equally assigned into three experimental groups of 20 animals each:

Group 1: Control group.

Group 2: LIP +SRP +irrigation with a vehicle treatment group,

Group 3: LIP + SRP + irrigation with PRO treatment group.

Propolis Collection

Propolis was collected by beekeepers who manually scraped off the frames of beehives located in Haj Umran city, Erbil governorate, Iraq. The propolis sample was cleaned and frozen at -20°C

Extraction of 10% Propolis Solution

While still frozen, propolis samples were ground into a powder using a precooled mortar and pestle. The propolis extract was prepared according to a method presented by AL-Ani *et al.,*2018.[11] they recommended 10 grams of propolis powder were mixed with 100 ml of 70% v/v ethyl alcohol in hermetically sealed glass vessels at a ratio of 1 gram of propolis to 10 ml of ethanol for 24 hours at room temperature in the dark with constant agitation by a magnetic stirrer. The resulting solutions were clarified by centrifugation at 26,000 g for 30 minutes, and the supernatants were collected, filtered through Whatman #4 filter paper to remove waxes and relatively insoluble substances and evaporated in a rotary evaporator (Heidolph, Germany) under reduced pressure at 50°C to remove the solvent and obtain a brown semisolid residue referred to as ethanol extract propolis (EEP). The extracts were re-dissolved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany) to obtain an EEP solution at a concentration of 10%. The prepared solution was diluted with saline, and the final concentration of DMSO did not exceed 1%, which is nonlethal for microorganisms

Local Treatment:

Ligatures were removed after 4 weeks. On this day (day 0), local treatment by SRP with vehicle irrigation performed for gp2, and SRP with PRO irrigation for gp3 animals. Irrigation was locally applied three times daily (7 a.m.,1 p.m., and 8p.m.) for 11 days according to previous studies,[9,12,13] us-

ing 0.5 ml of irrigating material administered with an insulin syringe. The mandibular left incisor was subjected to scaling and root planing using manual #1–2 mini-five Gracey curettes.

Five animals from each experimental group were euthanized on days 0, 7, 14, and 21 after local treatment, and 5 mL of blood was collected from each animal via cardiac puncture. The collected blood samples were centrifuged at 1500 g for 10 minutes within 1 hour of collection, aliquoted into Eppendorf tubes and kept frozen at −20°C. The level of ALP was determined using diagnostic kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols, and the results are expressed as the mean (U/L) ±SD.

The animals were scarified using overdose anesthesia, and then the mandible was removed and fixed in 10% of formalin for 48 hours at room temperature for histological analysis. Following 48 hours of fixation the specimens were decalcified using decalcifying solution (24.4% formic acid and 0.5 N sodium hydroxide) and were subjected to conventional histological processing, including paraffin embedding. Serial paraffin sections were obtained in longitudinal (labiolingual) direction then stained with H&E for descriptive histology and ranked using a score of 0 to 3 according to Park *et al.*, (2016),[14] considering the influx of inflammatory cells, as well the integrity of the alveolar bone and cementum (Table 1), and with Masson's trichrome stain for the determination of new collagen fibre deposition. Sections of 5 µm thickness were evaluated under light microscope using different magnifications (X40, X100, and X400).

Statistical analysis

All data are presented as the mean ± standard deviation (SD) of the experimental values. Comparisons of data among groups and periods were performed with analysis of variance (ANOVA) to evaluate the effect of different local treatments and experimental periods on the means of serum ALP, When ANOVA detected a significant difference, multiple comparisons were assessed by Tukey's test, and a p-value < 0.01 was considered statistically significant**.**

Results

Biochemical Analysis

Alkaline phosphatase activity

Ligature induced periodontitis resulted in a marked decrease in the value of serum alkaline phosphatase in periodontitis groups at 0 day when compared to control group. At day 14, and 21 post periodontitis treatment, the decreased serum ALP level raised in treated groups. Treatment with propolis as adjunctive to SRP (gp3), revealed a significantly higher value at days 14, and 21 (Table 2).

Histological analysis

Control group (gp1)

Histological study of the mandibular left central incisor's periodontium of the control group revealed normal histological features receiving score 0 (Figure 1; A-C).

Ligature induced Periodontitis gps. (gp2, gp3)

Induced periodontitis caused many histological changes characterized by intense infiltration of inflammatory cells in both gingival and periodontal tissues, destruction of connective tissue attachment together with sever alveolar bone resorption with necrotic spicules of bone covered by inflammatory cells, receiving score 3 (Figure

Table 1: The histological Scores used in this study

Scores	Remarks
0	Absence or only a discrete cellular infiltration (inflammatory cell infiltration is sparse and restricted to the region of the marginal gingival), preserved alveolar process and cementum.
1	Moderate cellular infiltration (inflammatory cellular infiltration present all over the insert gingival), some but minor alveolar process resorption and Intact cementum
2	Accentuated cellular infiltration (inflammatory cellular infiltration present in both gingival and periodontal ligament), accentuated degradation of the alveolar process and partial destruction of cementum.
3	Accentuated cellular infiltrate, complete resorption of the alveolar process and severe destruction of cementum.

Table 2: Mean concentration and standard deviation (M±SD) of ALP level (U/L) in control and in ligature induced periodontitis groups.

Treatment groups	ALP level (U/L)			
	Periods			
	0 day	7days	14days	21days
C (gp1)	249.60±9.5[a]	255.60±9.3[a]	248.00±2.5[a]	249.80±4.2[a]
SRP+ vehicle (gp2)	102.00±2.0[b]	105.00±3.5[b]	139.80±2.3[b]	150.20±2.4[b]
SRP+PRO(gp3)	101.60±2.1[b]	107.00±4.9[b]	149.40±2.4[c]	176.80±1.9[c]

The results are expressed as Means ±standard deviation. [a-c] The footnote letters in the same column indicates significant differences between treatment groups (ANOVA and Tukey; p <0.01).

1; D-F).

SRP with vehicle irrigation treatment group (gp2)

Intense inflammatory reaction was seen in the periodontal tissue at 7 days' post periodontal treatment, the collagen fibres were greatly destroyed with sign of necrosis. The alveolar crest became irregular that devoid from osteoblasts, receiving a mean score (2.8). Masson's trichrome stained section showed scanty delicate disoriented newly formed collagen fibrous tissue (Figure 2; A-C).

At 14 days, there was remarkable reduction in the inflammatory process and newly formed collagen fibres bundles with active fibroblasts can be seen. Bone trabeculae surrounded by numerous active osteoblasts and, contained osteocytes, receiving a mean score (1.6). Masson trichrome stained sections revealed dense and mature bundle of newly formed collagen fibres (Figure 3; A-C). At 21 days, both gingival connective tissue and periodontal ligament showed few inflammatory cells and blood vessels within dense and organized collagen fibres interposed by many active fibroblasts, receiving a mean score (0.8). Masson's trichrome stained section revealed a large amount of thick well oriented newly formed collagen fibres.

Alveolar bone surface appeared smooth and regular, and the active osteoblasts arranged as continuous cuboidal chain adjacent to osteoid deposition. (Figure 4; A-C).

SRP with propolis extract irrigation treatment group (gp3)

At 7 days following SRP and local irrigation with propolis extract, severe infiltration of inflammatory cells in both gingival connective tissue and periodontal ligament. The alveolar bone trabeculae had irregular contour, absent osteoblasts, receiving a mean score (2.6). Masson's trichrome stained section revealed loos to moderately thin newly formed collagen fibre (Figure 2; D-F).

At 14 days, well organized collagen fibre was distinguished in the gingival tissue and in the periodontal ligament. The alveolar bone appeared matured, and a rime of osteoid tissue superimposed by active osteoblasts receiving a mean score (1.2) (Figure 3; D-F). At 21 days, the principal fibres were well and highly organized. The alveolar bone became lamellated with continuous osteoid deposition and prominent chain of active osteoblasts. The newly formed blood vessels were well recognized toward the bone side, receiving a mean score (0.2). Trichrome stain revealed a large amount of

dense well-oriented of collage fibres with newly formed bone (Figure 4; D-F).

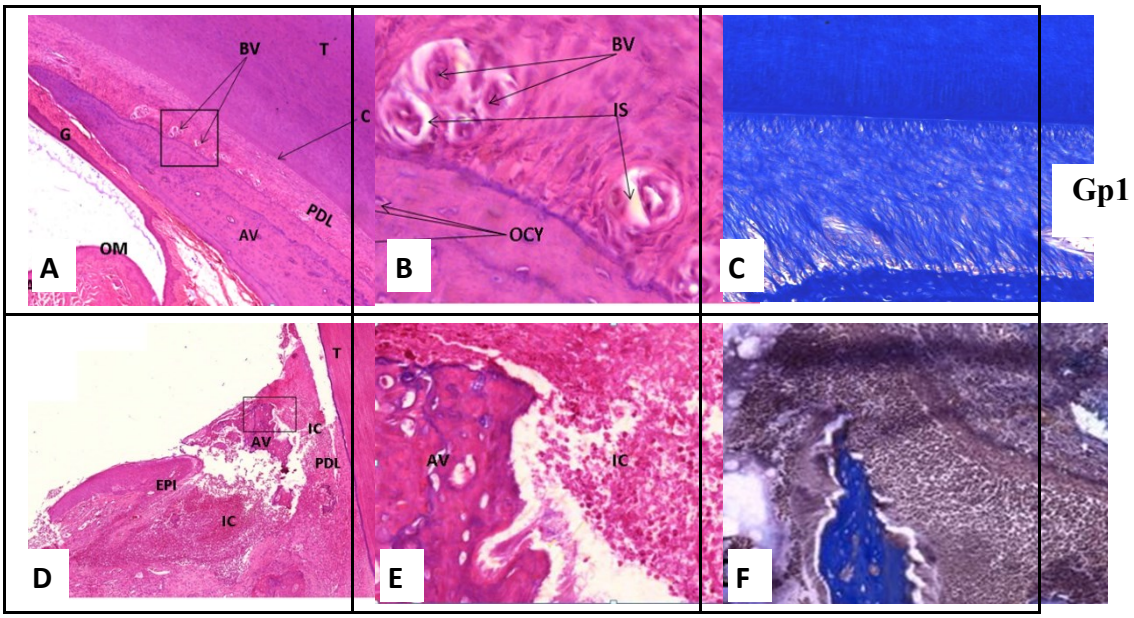

Figure 1. Rat's periodontium in the control (gp1) (A, B, C), and ligature induced periodontitis (gp2, gp3) (D, E, and F) groups at ligature removal (0 day). AV: alveolar bone, IC: inflammatory Cells, PDL: periodontal ligament, T: tooth. H&E staining. Original magnification: Aand D: 40 X; B and E: X400). Masson's trichrome staining. Original magnification: C and F: X100).

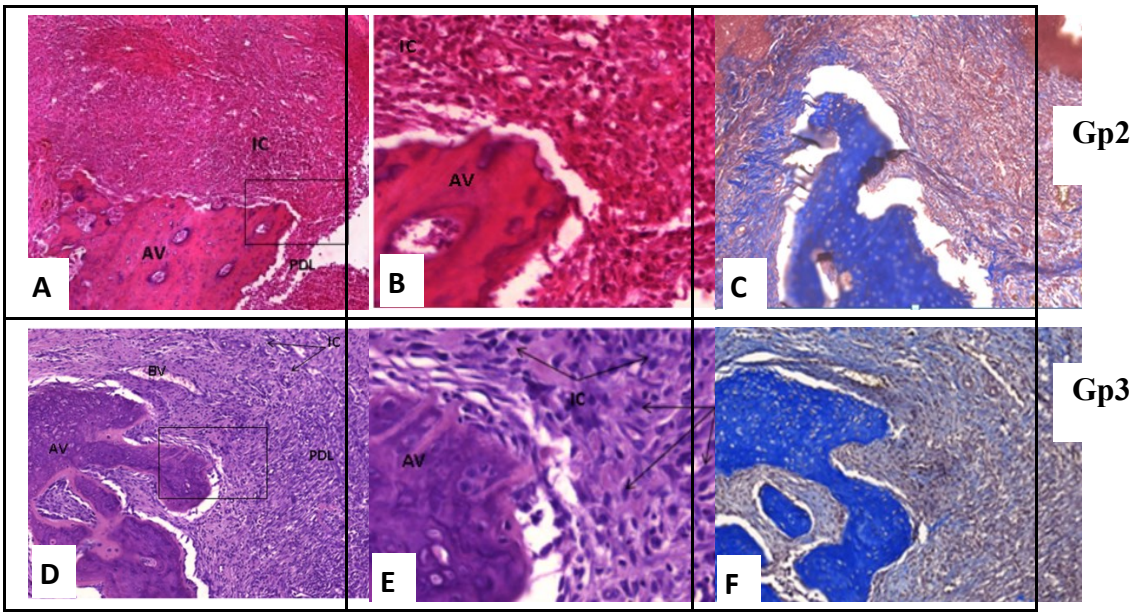

Figure 2. Rat's periodontium in the different treatment groups at 7 days' post periodontitis treatment in SRP with vehicle irrigation (gp2) (A, B, C), and SRP with propolis irrigation (gp3) (D, E, F) groups. AV: alveolar bone, IC: inflammatory Cells, OB: osteoblast, PDL: periodontal ligament. H&E staining Original magnification: Aand D:X100; B and E:X400). Masson's trichrome staining. Original magnification: C, F, I, and L, X 100).

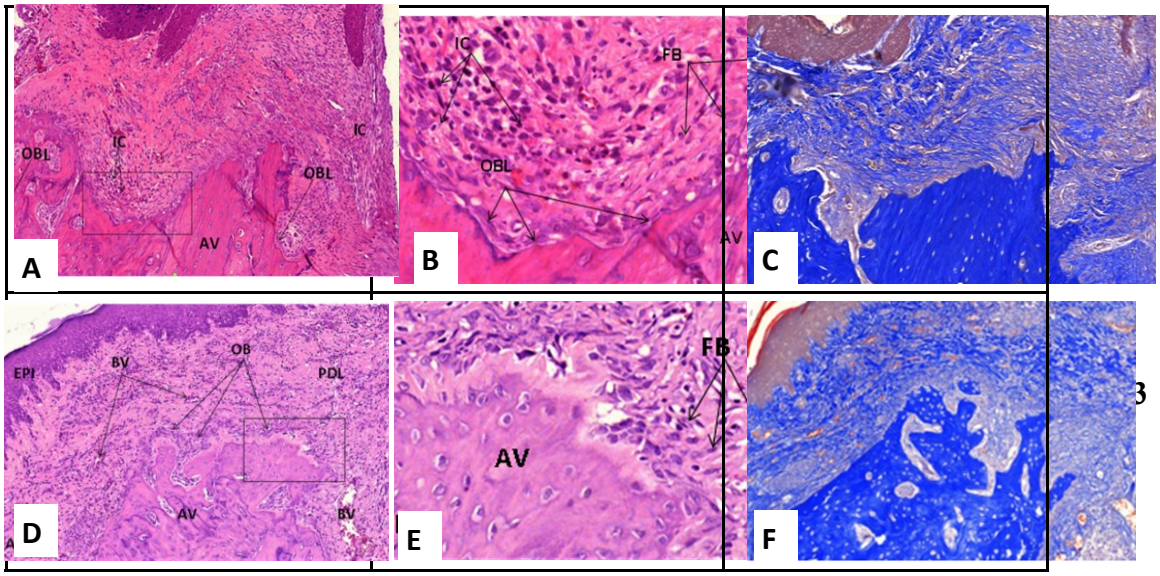

Figure 3. Rat's periodontium in the different treatment groups at 14 days' post periodontitis treatment SRP with vehicle irrigation (gp2) (A, B, C), and SRP with propolis irrigation (gp3) (D, E, F). AV: alveolar bone, IC: inflammatory Cells, OB: osteoblast, PDL: periodontal ligament. H&E staining. Original magnification: A and D: X100; B and E: X400). Masson's trichrome staining. Original magnification: C and F:X100)

Discussion

The use of ligature in rats as an experimental model of periodontitis was realized in the current study, as it has been accepted as a useful experimental model of periodontitis with destruction of the tooth-supporting tissues similar to what has been observed in human periodontitis cases.[15]

In the present study, placement of ligature around rat's mandibular incisor tooth was efficient in the induction of periodontitis with marked plaque formation around ligated teeth accompanied by clinical signs of gingival inflammation and attachment loss, these macroscopic findings were corroborated by histological analysis which revealed a greater magnitude of the inflammatory response with sever periodontal tissue breakdown which resulted in decreased collagen occupied region, as well as intense alveolar bone destruction as a result of osteoclastic activity when compared to ligation free gp1 which showed histopathological appearance of normal periodontium. These findings confirms the report of park et al. (2016)[14] and Lima et al. (2017),[16] where they reported that ligation installation around rat incisors produces sever inflammatory cell infiltrations, collagen fibre destruction and sever alveolar bone loss.

Regarding the effect of different local periodontal treatment used in this study; although SRP exhibited satisfactory result in gp2, as SRP remove the main etiological factor of periodontal disease, and this corroborating the consensus in the literature that SRP treatment is effective in Periodontitis remission,[17] this approach is frequently insufficient because it cannot completely eliminate microbial factors and does not have a direct effect on the host response.[18] Considering the important roles of immune inflammatory response in periodontal disease, it seems interesting to use pharmacological agents that exert an anti-inflammatory and immunomodulatory effect. Hence, propolis was used in the current study as adjunct to mechanical therapy sinceit possesses a broad spectrum of biological activities, including anti-inflammatory and immunomodulatory properties,[8] as well as the ability of enhancing new collage fibre and bone formation.[19,20]

In the present study, the animals that received local treatment by SRP with propolis irrigation in gp3 revealed better periodontal tissue healing, since the Haematoxylin & Eosinand Masson's trichrome stained sections showed marked deposition of thick well-oriented newly formed collagen fibres with formation of osteoid tissue covered by active osteoblasts at both evaluation periods

of 14 and 21 days' post periodontitis treatment with less histological scoreswhen compared with rats that were treated by SRP with vehicle irrigation (gp2).In the same line Pereira et al., in 2018 [21] reported an acceleration of alveolar bone formation in experimental induced periodontitis in rats when treated with local propolis.

On the other hand, De Freitas et al. (2016),[22] and Nakajima et al. (2016)[23] demonstrated that administering propolis had no effects on periodontal tissue and alveolar bone resorption. This discrepancy in the result may be due to the different methods for inducing experimental periodontitis and rout of propolis administration or may be due to the fact that the chemical composition of propolis is complex and depends on the flora where it was collected.

A possible explanation of propolis accelerates periodontal tissue repair could be related to it is chemical constituents which contains a great amount of polyphenols including flavonoids, phenolic acids and their esters, and terpenoids, since they process anti-inflammatory activity[24,25] as well as their abilities to improve and maintain bone health, thereby stimulant effects on osteoblast proliferation and differentiation, besides inhibitory effect on osteoclasts.[26,27] Histological findings of this study were confirmed by biochemical analysis which examined biomarker of bone turnover. In the current study, alkaline phosphatase activity was chosen, since it considered an early marker of osteoblast cell differentiation and bone formation and plays a critical role in the mineralization of bone matrix.[28]

In the present study, serum ALP value in rats subjected to ligature experimental periodontitis was significantly lower compared to that of ligation free gp1. These results are in consistent with the previous studies that demonstrated a significant reduction in the ALP values in serum.[29,30] The significant reduction in ALP levels indicates that osteoblastic activity decreased in periodontal tissue.

Concerning the different periodontal treatment used in this study, the animals that received local treatment by SRP with propolis irrigation gp3 showed significantly higher serum values of ALP activity than those received local treatment by SRP with vehicle irrigation gp2 at both 14 and 21 days' post periodontitis treatment. The highest level of serum ALP observed in group 3 can be justified as histological evaluation of this group which revealed an obvious new bone formation with active osteoblasts, since ALP is produced by osteogenic cells and the increase in ALP activity can indicate the start of new bone formation by these cells.[31]

Conclusion

It can be concluded that the local use of propolis as an adjunct therapy to SRP can be effective in the treatment of alveolar bone loss in rat model of ligature-induced periodontitis.

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