Effect of Local Propolis Irrigation in Experimental Periodontitis in Rats on Inflammatory Markers (IL-1β and TNF-α) and Oxidative Stress

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Abstract

Aims: The present study aimed to investigate the efficacy of local use of propolis (PRO) as a monotherapy or adjunctive therapy to scaling and root planning (SRP) in the treatment of ligature-induced periodontitis (LIP) in rats by evaluating inflammatory markers and oxidative stress. **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. **Subjects and Methods:** In total, 125 rats were equally divided into 5 groups of 25 animals: the control group (C) with no experimental periodontitis or treatment (gp1), LIP treated by local irrigation with a vehicle (dimethyl sulfoxide + physiological saline solution) group (gp2); LIP treated by SRP with vehicle irrigation group (gp3); LIP treated by local irrigation with 10% PRO extract group (gp4); and LIP treated by SRP with 10% PRO extract irrigation group (gp5). Experimental periodontitis was induced by placing a silk ligature around the cervix of the mandibular incisor for 4 weeks. After removal of the ligature, SRP was performed in gp3 and gp5. Five rats from each group were euthanized on days 0, 3, 7, 14, and 21 after local treatment. Intracardiac blood samples were obtained for analysis of the inflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) and oxidative stress, which were represented by the expression of serum malondialdehyde (MDA). **Statistical Analysis Used:** Data were analyzed statistically by analysis of variance followed by Tukey's test ($P < 0.05$). **Results:** Gp5 demonstrated significantly lower IL-1β, TNF-α, and MDA serum levels than the other treatment groups at all experimental time points ($P < 0.05$). No significant differences were found when PRO was used as a monotherapy ($P > 0.05$). **Conclusions:** Local use of a PRO extract was effective as an adjunctive treatment with SRP in a rat model of experimental periodontitis via anti-inflammatory and antioxidative activities.

Keywords: *Experimental periodontitis, inflammatory markers, oxidative stress, propolis*

Introduction

Periodontitis is an immune-mediated inflammatory disease characterized by the destruction of the alveolar bone as a result of the host response to the presence of polymicrobial subgingival biofilms.[1] In turn, bacterial pathogens in the dental plaque stimulate host cells to release various proinflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), which attract polymorphonucleocytes (PMNs) to the site of infection.[2] Activated PMNs produce a large amount of reactive oxygen species, which lead to local oxidative stress and mediate periodontal tissue destruction.[3,4] Elevated malondialdehyde levels indicate an increase in oxidative stress.[5] Although scaling and root planning (SRP) promotes an improvement in clinical periodontal parameters in the majority of cases, it is frequently insufficient to change the bacterial profile associated with periodontitis because of a high probability of pocket reinfection resulting from residual biofilms.[6,7] Thus, to achieve the maximum effect, some patients may need drugs or certain agents with inhibitory properties such as antimicrobial, anti-inflammatory, or antioxidant properties as an adjunct to SRP.[8] Among various natural products, propolis (PRO) has attracted researchers' interest because it possesses a broad spectrum of biological and pharmacological properties, such as immunomodulatory, anti-inflammatory, antioxidant, and antibacterial activities,[9,10] especially effective against periodontopathic

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bacteria.[11,12] PRO is a plant product collected by honeybees as a resinous mixture created from various plants that is mixed with beeswax and other bee secretions. Although the chemical composition of PRO depends on its location of origin, it basically contains beneficial substances, such as phenolic acids, flavonoids, and vitamins.[13,14] Since ligature-induced periodontitis (LIP) is characterized by inflammatory alveolar bone resorption, in light of the above information on PRO, it could be advantageous to use PRO 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 PRO as a monotherapy or an adjunctive therapy with SRP on rats with LIP in relation to levels of proinflammatory cytokines and oxidative stress.

Subjects and Methods

Animals

A total of 125 adult male Wister rats weighing 250–300 g were used. Five rats were allocated to each stainless steel cage and maintained under a 12-h light/dark cycle at a 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 (50 mg/ml, Romvac Company, Romania) and 0.05 ml of xylazine hydrochloride (2 g/100 ml, Alfasan Company, Holland) per 100 g of body weight by placing 3.0 sterile black braided silk threads around the cervix of the mandibular 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 five experimental groups of 25 animals each:

- Group 1: Control group,
- Group 2: LIP + irrigation with a vehicle treatment group,
- Group 3: LIP + SRP + irrigation with a vehicle treatment group,
- Group 4: LIP + irrigation with PRO treatment group, and
- Group 5: LIP + SRP + irrigation with PRO treatment group.

Propolis collection and extraction

Propolis collection

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

Extraction of 10% propolis solution

While still frozen, PRO samples were ground into a powder using a precooled mortar and pestle. The PRO extract was prepared according to a method presented by Thirugnanasampandan *et al*.[15] and AL-Ani *et al*.;[16] they recommended that 10 g of PRO powder was mixed with 100 ml of 70% v/v ethyl alcohol in hermetically sealed glass vessels at a ratio of 1 g of PRO to 10 ml of ethanol for 24 h 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 min, 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 redissolved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany) to obtain an EEP solution at a concentration of 10%.[17] The prepared solution was diluted with saline, and the final concentration of DMSO did not exceed 1%, which is nonlethal for microorganisms.[16]

Local treatment

Ligatures were removed after 4 weeks. On this day (day 0), local treatment with SRP was performed for gp3 and gp5 animals, and rats were irrigated with the vehicle (gp2 and gp3) or PRO extract (gp4 and gp5). Irrigation was locally applied three times daily (7 a.m., 1 p.m., and 8 p.m.) for 11 days using 0.5 ml of irrigating material administered with an insulin syringe (BD Microfine 1 ml 0.30 mm (30 G) × 8 mm, Becton Dickinson, USA). The mandibular incisor was subjected to SRP using manual 1–2 mini-five Gracey curettes (Hu-Friedy Company, USA).

Five animals from each experimental group were euthanized on days 0, 3, 7, 14, and 21 after local treatment, and 8 ml of blood was collected from each animal via cardiac puncture. The collected blood samples were centrifuged at 1500*g* for 10 min within 1 h of collection, aliquoted into Eppendorf tubes, and kept frozen at −20°C. The levels of IL-Iβ and TNF-α were determined by enzyme-linked immunosorbent assay (ELISA; Diaclone SAS, Besançon Cedex, France) according to the manufacturer's protocols, and the results are expressed as the mean (pg/ml) ± SD of the concentration of each factor in the serum. The MDA concentration was determined with an ELISA kit specific for rat MDA (SunLong Biotech Company, China) according to the manufacturer's protocols, and the results are expressed as ng/ml.

Statistical analysis

All data are presented as the mean ± standard deviation 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 IL-1β, TNF-α, and MDA values. When ANOVA detected a significant difference, multiple comparisons were assessed by Tukey's test, and a *P* value <0.05 was considered statistically significant.

Results

In the periodontitis groups, the baseline value of serum IL-1β was nearly five times higher than that in the normal group. On days 3, 7, 14, and 21 postperiodontitis treatment, the elevated serum IL-1β level was reduced in the treated groups. Gp5 and gp3 animals revealed significant reductions in serum IL-1β levels compared to gp2 and gp4 animals. Interestingly, treatment with PRO as an adjunctive therapy to SRP (gp5) produced significantly lower values at days 7, 14, and 21 than the gp3 treatment. Compared with the treatment of gp2 animals, treatment with PRO as a monotherapy (gp4) did not affect serum IL-1β values significantly over any of the time intervals [Table 1].

The periodontitis groups showed significantly higher levels of TNF-α at baseline. On days 3, 7, 14, and 21 postperiodontal treatments, there were reductions in the serum TNF-α value in the treated groups, and gp5 exhibited a significantly lower serum level of TNF-α than gp3 and the groups that received irrigation only (gp2 and gp4). However, there were no statistically significant differences observed between gp4 and gp2 over any of the time intervals [Table 2].

Progressive decreases in the serum MDA concentration were found with regard to the treatments and time intervals. The effect of SRP with PRO irrigation in gp5 caused significant reductions in the serum MDA level at all-time points compared to the other treatments. No significant differences were recorded between gp4 and gp2 at any of the time points [Table 3].

Discussion

In the present study, local PRO irrigation was used with and without SRP for the treatment of LIP. The ligature was kept on rat tooth surfaces for 4 weeks in accordance with previous studies,[18-20] and this model was efficient in the induction of periodontitis with marked plaque formation and a local inflammatory response, which was characterized by clinical signs of gingival inflammation and significantly elevated serum levels of TNF-α, IL-1β, and MDA in LIP groups compared with the nonligated control group. Several studies have shown that this model is able to reproduce the main characteristics seen in human periodontitis, such as bone loss, destruction of the periodontal ligament,[21,22] an increase in oxidative stress,[23] and exacerbation of the inflammatory process.[22,24] However, Miyajima *et al*.[25] showed that there were no differences in the serum concentrations of proinflammatory cytokines between control and experimental periodontitis rats.

Table 1: Mean concentration and standard deviation (M±SD) of the serum IL-1β level (pg/ml) in control and ligature-induced periodontitis groups according to treatment group and time point

Treatment groups	IL-1β level (pg/ml) Time points				
	0 days	3 days	7 days	14 days	21 days
C (gp1)	25.33±1.31[a]	25.75±1.56	25.94±0.51	25.15±0.7	25.67±0.51
Vehicle (gp2)	133.22±7.8[b]	105.68±1.54[a]	101.52±1.71[a]	88.72±1.42[a]	84.01±1.43[a]
SRP + vehicle (gp3)	135.79±1.48[b]	85.79±0.88[b]	81.44±0.67[b]	68.49±2.28[b]	61.58±0.96[b]
PRO (gp4)	134.48±1.47[b]	101.18±9.54[a]	98.77±8.06[a]	85.76±3.26[a]	82.60±5.54[a]
SRP + PRO (gp5)	136.34±2.01[b]	80.83±1.55[b]	72.53±1.96[c]	50.9±2.03[c]	38.54±1.53[c]

The results are expressed as the mean±standard deviation. [a-c]The footnote letters in the same column indicate significant differences among the treatment groups (ANOVA and Tukey's test; *P*<0.05)

Table 2: Mean concentration and standard deviation (M±SD) of the serum TNF-α level (pg/ml) in control and ligature-induced periodontitis groups according to treatment group and time point

Treatments groups	TNF-α level (pg/ml) Time points				
	0 days	3 days	7 days	14 days	21 days
C (gp1)	4.67±0.71[a]	4.07±0.32	4.07±0.32	4.52±0.36	4.75±0.22
Vehicle (gp2)	50.45±3.08[b]	33.26±1.28[a]	31.52±1.47[a]	25.28±0.61[a]	21.15±1.64[a]
SRP + vehicle (gp3)	48.48±1.62[b]	24.84±0.53[b]	22.11±1.08[b]	17.39±1.20[b]	13.83±1.36[b]
PRO (gp4)	47.21±2.09[b]	32.74±1.19[a]	30.87±0.68[a]	24.66±1.09[a]	19.49±0.62[a]
SRP + PRO (gp5)	48.61±2.09[b]	20.10±1.13[c]	17.77±1.19[c]	10.52±1.07[c]	7.49±1.63[c]

The results are expressed as the mean±standard deviation. [a-c]The footnote letters in the same column indicate significant differences among the treatment groups (ANOVA and Tukey's test; *P*<0.05)

Table 3: Mean and standard deviation (M±SD) of the serum MDA concentration (ng/ml) according to treatment group and time point

Treatments groups	Time points MDA (ng/ml)				
	0 days	3 days	7 days	14 days	21 days
C (gp1)	56.8±4.38[a]	56.56±0.72	57.67±1.46	57.86±1.98	55.83±0.91
Vehicle (gp2)	216.63±4.61[b]	150.99±6.11[a]	144.22±1.96[a]	130.40±1.14[a]	120.20±0.83[a]
SRP + vehicle (gp3)	218.34±1.64[b]	119.29±1.36[b]	113.40±1.72[b]	95.00±1.34[b]	86.01±1.98[b]
PRO (gp4)	215.55±1.59[b]	149.40±2.75[a]	144.73±1.85[a]	128.83±2.67[a]	119.03±2.35[a]
SRP + PRO (gp5)	219.16±2.25[b]	109.61±0.91[c]	102.63±1.43[c]	80.72±1.82[c]	63.59±1.11[c]

The results are expressed as the mean±standard deviation. [a-c]The footnote letters in the same column indicate significant differences among the treatment groups (ANOVA and Tukey's test; $P<0.05$)

In the present study, IL-1β and TNF-α were chosen because their expression occurs in periodontitis in conjunction with the loss of connective tissue and bone.[26] Regarding the effect of the different local treatments used in this study, the animals that received local treatment by SRP with irrigation (gp3 and gp5) showed significantly lower serum values of IL-1β, TNF-α, and MDA than those that received local irrigation alone (gp2 and gp4) at all-time points. The data presented here clearly demonstrate periodontal disease remission after SRP treatment; thus corroborating the consensus in the literature that SRP treatment is effective in inducing periodontitis remission.[27] Although SRP exhibited satisfactory results in the present study, this approach is frequently insufficient because it cannot completely eliminate microbial factors and does not have a direct effect on the host response.[28] Considering the important roles of inflammation and oxidative stress in periodontal disease, it seems interesting to use pharmacological agents that exert anti-inflammatory and antioxidant actions. Hence, PRO was used in the current study over other products because it is purely natural, readily available, safe, and nontoxic. Furthermore, it possesses a broad spectrum of biological activities, including anti-inflammatory, immunomodulatory, and antioxidant properties.[9,10] In the present study, significant decreases in serum IL-1β, TNF-α, and MDA levels were detected in rats that received treatment with PRO irrigation as an adjunctive therapy to SRP (gp5) when compared with rats that were treated by SRP with vehicle irrigation (gp3). This observation provides direct evidence that PRO has anti-inflammatory and antioxidant effects that are effective in ameliorating periodontitis induced by ligature placement. The anti-inflammatory and antioxidant effects of PRO can be attributed to its associated constituents: flavonoids, phenolic acids and their esters, and terpenoids.[14,29]

These findings are consistent with those of Machado *et al*.,[30] who showed that PRO administration inhibited proinflammatory cytokine production, enhanced anti-inflammatory cytokine production, and minimized the increase in the MDA level in experimental animal models.[31] The proinflammatory cytokine-inhibiting effect of PRO was also supported by an in-vitro study performed by Huang Wen-Chien *et al*.[32] They showed that PRO suppressed TNF-α-induced inflammatory responses, including the expression of IL-1β and TNF-α in human periodontal ligament fibroblasts. These findings may be a critical step toward successful periodontal therapy, since TNF-α and IL-1β act as potent osteoclastogenic cytokines.[33] However, Aral *et al*.[1] showed that plasma IL-1β and TNF-α levels were not significantly altered upon treatment by systemic PRO administration. In addition, the results of a histometric study in rats performed by de Freitas *et al*.[34] showed that subgingival irrigation with solutions containing 11% PRO extract produced nonsignificant effects when used as an adjunct to basic mechanical therapy. This discrepancy in results may be attributed to differences in methodology, route of administration, and method of extraction or may be because the chemical composition of PRO is complex and depends on the flora at the site of collection.

Clinical studies performed by several authors[35-38] have demonstrated that subgingival irrigation with PRO as an adjunctive therapy to SRP in periodontal treatment is more effective than mechanical treatment with a saline solution in terms of clinical and microbiological parameters.

PRO was used as adjunct to SRP in gp5, and the results showed significantly lower serum values of IL-1β, TNF-α, and MDA in gp5 compared with gp4, in which PRO was used as a monotherapy. This can be explained by increased penetration of PRO into periodontal tissues, through the epithelium and connective tissue, after the predicted removal of the sulcular epithelium following SRP procedures.

On the other hand, the present study showed that local PRO irrigation as a monotherapy, as performed in gp4, was not beneficial for the treatment of LIP; nonsignificant decreases in the serum levels of IL 1β, TNF-α, and MDA were observed in gp4 compared with gp2 at all-time points. These results probably reflect the maintenance of a biofilm on the rat tooth surface, which is considered the primary etiologic factor of periodontitis, and PRO was unable to penetrate this biofilm. However, a study by Doganli[39] showed that PRO was effective against established biofilms,

and this variation in results may be attributed to differences in methodology, as Doganli's study was performed in vitro.

PRO has been used mainly in traditional and folk medicine. Therefore, it is difficult to pinpoint the exact recommended standard dose. Different doses of PRO extract have been used by researchers; for evaluating new bone formation at an expanded suture in rats, 25% PRO extract was used by Altan *et al*.[40] For evaluating anti-inflammatory effects, 10% PRO extract was used systemically in the study of Aral *et al*.[1] To evaluate clinical and microbiological characteristics, 20% PRO was used as an adjunct to SRP by Coutinho,[36] and the selection of 10% PRO extract used in the current study was based on the results of our pilot study, which was conducted to compare three different concentrations of PRO: 5%, 10%, and 20%. The results of the pilot study demonstrated that 5% PRO extract was not effective, and 20% was not superior to 10%. Hence, the lower dose of 10% was selected.

It should be recognized that this study has utilized an animal model. However, for every animal model of human disease, there are inherent limitations; in comparison to human periodontitis, the course of disease in the experimental periodontitis is short and follows an acute course, through which tissue trauma and adjacent microbial accumulation accelerate the destructive process. Such pathways of acute inflammation are likely to be different from chronic periodontitis.

Conclusions

According to the results of this study, it can be concluded that local irrigation with PRO as an adjunctive therapy to SRP can be effective in the treatment of LIP in a rat model by reducing inflammatory cytokine and oxidative stress levels.

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Conflicts of interest

There are no conflicts of interest.

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