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ASSESSMENT OF THE EFFECT OF LOCAL ADMINISTRATION OF PLACENTAL EXTRACT GEL ON THE TREATMENT OF EXPERIMENTAL PERIODONTITIS IN RATS: A HISTOPATHOLOGIC AND IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Background: Periodontitis is an inflammatory disease of the teeth supporting tissues brought on by microbial biofilm. Local medication delivery methods work directly on pathogenic bacteria in periodontal pockets, assisting in disease management. Since the early 1900s, placental treatment has been used to aid in disease recovery and tissue regeneration.

Aim of the study: this study aimed to evaluate the effect of placental extract gel (PEG) in the treatment of experimental periodontitis in rats.

Materials and Methods: Periodontitis was induced on thirty Wistar albino rats. The animals were divided into three groups (n=10); negative control, scaling and root planning only (SRP), and SRP combined with (PEG) groups. Animals were sacrificed after 14 days of baseline and examined for epithelial and inflammatory cell status in connective tissue; Image analyzed computer system Image J was used for counting inflammatory cells, alveolar bone, and TNF-α immune expression.

Results: Negative control group showed severe epithelial deterioration, severe alveolar bone destruction, periodontal ligament fibers (PDL) disorganization, and a significant increase in TNF-α. Both treated groups showed proper improvement where best results were reported with SRP in combination with PEG group in the form of significant differences in epithelial, alveolar bone formation, inflammatory cells population, PDL organization, new vascularization, as well as a significant decrease in TNF-α immune expression.

Conclusion: PEG could be successfully used as an adjunct to conventional periodontal based on the histologic and immunohistochemical findings in this study.

KEY WORDS: Periodontitis, Scaling and root planning (SRP), Placental extract gel (PEG), Tumor necrosis factor-alpha (TNF-α).

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INTRODUCTION

Periodontitis is a complex disease that is one of the leading causes of tooth loss. It is characterized by an intricate interplay of biofilm with the host immune-inflammatory response, as well as an alteration in bone and connective tissue homeostasis. A hyperresponsive inflammatory response, while generally protective, may result in increased tissue damage.[1] The release of periodontal pathogen virulence factors e.g. lipopolysaccharides (LPS) to the surrounding periodontal tissues triggers an inflammatory response that results in the secretion of pro-inflammatory cytokines such as interleukin-1(IL-1), tumor necrosis factor (TNF-α) and IL-6 by inflammatory cells, which are mainly responsible for the deterioration of periodontal tissue. The degeneration of periodontal tissue is largely attributable to host-derived processes rather than bacteria proteolytic enzymes.[2] In the periodontology literature, several experimental research has been performed to identify the process of tissue damage produced by periodontal disease, how to avoid destruction, or how to compensate for destruction's implications.[3-6]

Scaling and root planning (SRP) is the basic treatment for periodontitis. SRP aimed at mechanical elimination of the pathogenic biofilm and slowing the recolonization of periodontitis-causing pathogens.[7] However, because of restricted access to bacteria that colonize deep inside the periodontal pocket and the structural complexity of the tooth, SRP alone will not be able to eliminate bacteria.[8] As a result, combining SRP with antimicrobial, anti-inflammatory, and bone and tissue regeneration medications can improve the therapeutic impact.[8]

Antibacterial drugs have been the major wound therapy for many years, yet they have no function in epithelialization, tissue regeneration, vascular regeneration, or wound closure. The placenta is thought to be a component with the potential to change current medicine. It improves healing, boosts collagen production, and helps to improve the tensile strength of regenerating tissue by stimulating neuronal, hormonal, and immune system control.[9]

In India and other countries, the extract of the human placenta is a wound-healing medication that has been licensed under several brand names.[10] This might be because the extract contains a variety of bioactive peptides that possess tissue regeneration potential.[11,12] Furthermore, the extract contains amino acids, nucleotides, polydeoxyribonucleotides, and carbohydrates, all of which are thought to be involved in wound healing.[13] In experimental osteoarthritis[14] and hypoxic-ischaemic brain damage,[15] the human placental extract has immunotrophic, antioxidative, and anti-inflammatory properties. The human placental extract also stimulates the development of human fibroblasts [16] and chondrocytes [9] and promotes type I collagen synthesis and has anti-inflammatory activities, as well as blocks the production of IL6 and IL8, which can lead to bone formation and angiogenesis.[17]

TNF-α is a well-recognized pro-inflammatory mediator associated with periodontitis. It has been established that it is involved in the pathogenesis of periodontitis, including the stimulation of osteoclast development,[18] control of matrix metalloproteinases, connective tissue degrading enzymes, and boosting the host response to periodontal infections by activating signal transduction pathways.[19]

The present study was designed to evaluate the effects of local administration of Placental extract gel (PEG) combined with SRP in the treatment of experimental periodontitis in rats through both histological and immunohistochemical TNF-α expression.

MATERIAL AND METHODS

Experimental model

This research comprised thirty Wistar albino rats whose weight ranged from 180 to 250 g. The study was performed in compliance with the National

Institute of Health's (NIH) recommendations for animal care and use in experimental procedures, as well as the Experimental Animal Research Ethics Committee of Suez Canal University number 487/2022. Before beginning the trial, the rats were acclimated for two weeks and kept in five-rat / cages. They were given a standard laboratory diet to eat. All efforts were taken to ensure that the test animals were not in any pain or discomfort.

Sample size calculation

A total of 30 rats were sufficient for the three groups where the effect size was 0.5 using an alpha (α) level of 0.05 and a beta (β) level of 0.05, i.e., power = 80%.

Induction of periodontal disease:

After the acclimation period, thirty rats were generally sedated intramuscularly with Xylaject (5 mg/kg body weight), ligature wires, and elastic rings were inserted around the cervix of one of the maxillary incisors, and they were left for 10 days for periodontitis induction. The wires were checked every day, and if they were removed by rats, they were replaced with new wires.[20]

Study groups:

The ligatures were removed (baseline) after the experimental periodontitis (EP) has been induced, the rats were divided into three groups:

Group I: (n=10) negative control; animals were sacrificed immediately without any treatment received at baseline.

Group II: (n=10) Scaling and root planning (SRP).

Group III: (n=10) SRP + Placental extract gel (PEG) (PLACENTREX ®, Manufactured by ALBERT DAVID LIMITED 5/11, D. Gupta lane, Kolkata-700050, INDIA).

Treatment procedures

Both groups (II and III) received the following treatment:

In group II, 10 rats were treated with SRP without any further medication. For SRP, Mini curettes (HuFriedy Co. Inc., Chicago, IL, USA) were placed at the mesial and distal surface of the roots within the periodontal pockets and pulled 10 times to eliminate the subgingival plaque.

Using a fine insulin gauge syringe, the periodontal pockets were irrigated with saline. All rats were sacrificed on day 14 from the baseline.

In group III, 10 rats received SRP with the adjunctive subgingival application of PEG. PEG was put into a tiny gauge insulin syringe after the SRP process and administered into the periodontal pocket on the first, third, and seventh days. Starting with the deepest area of the periodontal pocket, injections were made sub-gingivally into four areas around the teeth (buccal, lingual, mesial, and distal). All rats were sacrificed on day 14 from the baseline.

Histopathological analysis:

The maxilla of all rats was removed after euthanization with a lethal dosage of thiopental (150 mg/kg),[21] and they were preserved in 10% neutral buffered formalin and demineralized with a 10% EDTA solution containing 5% sodium sulfide. The samples were dehydrated in alcohol and then cleaned in xylene before being embedded in paraffin wax blocks. In a mesiodistal plane, they were sectioned longitudinally along with the incisors. 5 micrometer thick sections were mounted on glass slides and stained with hematoxylin and eosin for light microscopy examination and photographed by (E-330 Olympus) digital camera.[22]

The scoring system was derived and adjusted from *Adolfo et al.,* study,[23] examining epithelial and inflammatory cells status in connective tissue; Image analyzed computer system Image J was used

for counting inflammatory cells, and evaluation of alveolar bone as follows:

The score for connective tissue inflammation: Score 1= Few inflammatory cells in the connective tissue (5-15 cells), Score 2 = moderate inflammatory cells in the connective tissue (15-30), Score 3= more than 30 inflammatory cells in the connective tissue.[23]

The score for alveolar bone: Score 1= regular borders, no signs of osteoclastic activity, Score 2 = irregular borders but no signs of osteoclastic activity, Score 3= irregular borders with occasional osteoclastic activity, Score 4 = irregular borders, numerous osteoclasts, and sequestration.[23]

The score for epithelium: Score1 = 2-3 layers neatly arranged, Score 2 = atrophy of epithelium and several layers slightly disorganized, Score 3= epithelial atrophy and ulceration, several layers intensely disorganized.[23]

Immunohistochemical analysis:

Commercial indirect immunoperoxidase streptavidin/biotin kits were applied to detect TNF-α expression (ThermoScientific, CA, USA). After 30 minutes in the oven at 60°C, paraffin sections were deparaffinized in xylol and rehydrated in an alcohol series and distilled water. To eradicate endogenous peroxidase activity, a 0.1% H_2O_2 solution was then dripped over the sections and incubated at room temperature for 10 minutes. Antigen retrieval was accomplished by boiling sections in Citrate Buffer (pH 6.0) solution for 20 minutes, and the Ultra V Block solution was used for 10 minutes to finish the protein blocking phase. The primary antibody (TNF-α) appropriate for the antigen to be evaluated was applied to the sections without washing the protein-blocking serum and incubated for 60 minutes at room temperature in a humidity chamber. After employing biotin-labeled anti-mouse, rat, and rabbit polyvalent secondary antiserum (nNovacastra, catalog no: RE7103, USA) and the streptavidin-peroxidase enzyme for 15 minutes, the sections were stained with AEC chromogen for color reaction. For contrast staining, Mayer's hematoxylin was applied to the sections for 1 minute before being sealed with a water-based mounting solution. Immunohistochemical data were evaluated and microphotographs were taken using an Olympus BX50 light microscope with a DP70 digital camera mounted. Image analyzer computer system (image J / figi 1.46) was used to count the number of immune-positive cells as well as the number of the remaining unstained cells. The fraction of the positive cells was calculated.[24]

Statistical analysis:

A normality test (Kolmogorov-Smirnov) was performed to check the variables' normal distribution. Descriptive statistics were calculated in the form of Mean ± Standard deviation (SD). One-way ANOVAs were used to compare groups in each variable under study. Bonferroni's post hoc test was performed for the evaluation of statistical significance among the groups. P-value ≤ 0.05 is considered to be statistically significant. All Statistical analyses were carried out using SPSS software version 26.0 for Windows (Statistical Package for Social Science, Armonk, NY: IBM Corp).

RESULTS

Histopathological and immunohistochemical results

Epithelial score

Group I showed atrophic, and ulcerative epithelium with hydropic degeneration (Figure 1-A), group II showed less atrophy, and ulceration in the epithelium (Figure 1-B), while group III showed a normal epithelial pattern. (Figure 1-C)

The mean epithelial score in group I was (2.6), while it was (2.0) in group II and (1.7) in group III. (Table1)

TABLE (1) Comparison between groups for histopathologic score:

	Groups	Mean scores	ANOVA test	Pair-wise Comparisons	
				Bonferroni's post-hoc test	
			P-value (F-valu)	*Pair-wise Comparisons*	*P-value*
Epithelial score	**Group I**	2.6[a]	< 0.02* ** (F=4.36)	GI vs GII	< 0.0001**
	Group II	2.0[b]		GI vs GIII	< 0.0001**
	Group III	1.7[c]		GII vs GIII	< 0.0001**
Inflammatory cells score	**Group I**	2.0[b]	< 0.001* ** (F=10.88)	GI vs GII	< 0.0001**
	Group II	2.60[a]		GI vs GII**I**	< 0.0001**
	Group III	1.3[c]		GII vs GII	< 0.0001**
Alveolar bone score	**Group I**	3.10[a]	< 0.001* ** (F=9.14)	GI vs GII	< 0.0001**
	Group II	2.20[b]		GI vs GII**I**	< 0.0001**
	Group III	1.5[c]		GII vs GII	< 0.0001**

a,b and *; means significant different (p< 0.05)

Different letter in the same column indicating statistically significant difference (p< 0.05).

Inflammatory cells and alveolar bone score

Group I showed severe inflammatory cells infiltrate, dilatated blood vessels, sequestration, and irregular borders of alveolar bone with Howship's lacunae and osteoclasts lying inside them. Periodontal ligaments fibers (PDL) showed severe disorganization and deterioration. (Figure 1-D)

Group II showed moderate inflammatory cell infiltrate with dilatated blood vessels, in addition to irregular alveolar bone borders with empty Howship's lacunae however osteoclasts may be seen occasionally. PDL showed moderate organization with areas of detachment. (Figure 1-E)

Group III showed minimal inflammatory infiltrate with less blood vessel dilatation. New bone formation is evident with regular alveolar bone borders without any bone resorption. Newly formed bone has reversal lines denoting past osteoclastic activity, osteoblastic rimming, and prominent new vessels formation with proper periodontal ligaments organization with the near absence of fibers detachment. (Figure 1-F)

Scoring of inflammatory cells showed that at baseline, the mean score was (2.0) in group I while, it was (2.6) in group II, and (1.3) in group III. (Table1)

Scoring of alveolar bone at the baseline, the mean score in group I was (3.1) while it was (2.2) in group II and (1.5) in group III. (Table 1)

TNF-α immunohistochemical score

The immunohistochemical expression showed the following findings. Group I, has intense immunopositive staining in alveolar bone, and periodontal ligament. (Figure 1-G) Group II, has moderate immunopositive staining in alveolar bone,

and periodontal ligament. (Figure 1-H) Group III, has mild immunopositive staining in alveolar bone, and periodontal ligament. (Figure 1-I)

The results in table (2) showed that there is a clear significant difference between the studied groups for the TNF immune expression ($p< 0.0001$). The high value was recorded in group I (204.40±15.62) followed by group II (167.36±10.18) while group III was the lowest one (113±6.54). (Fig.1 & Table 2)

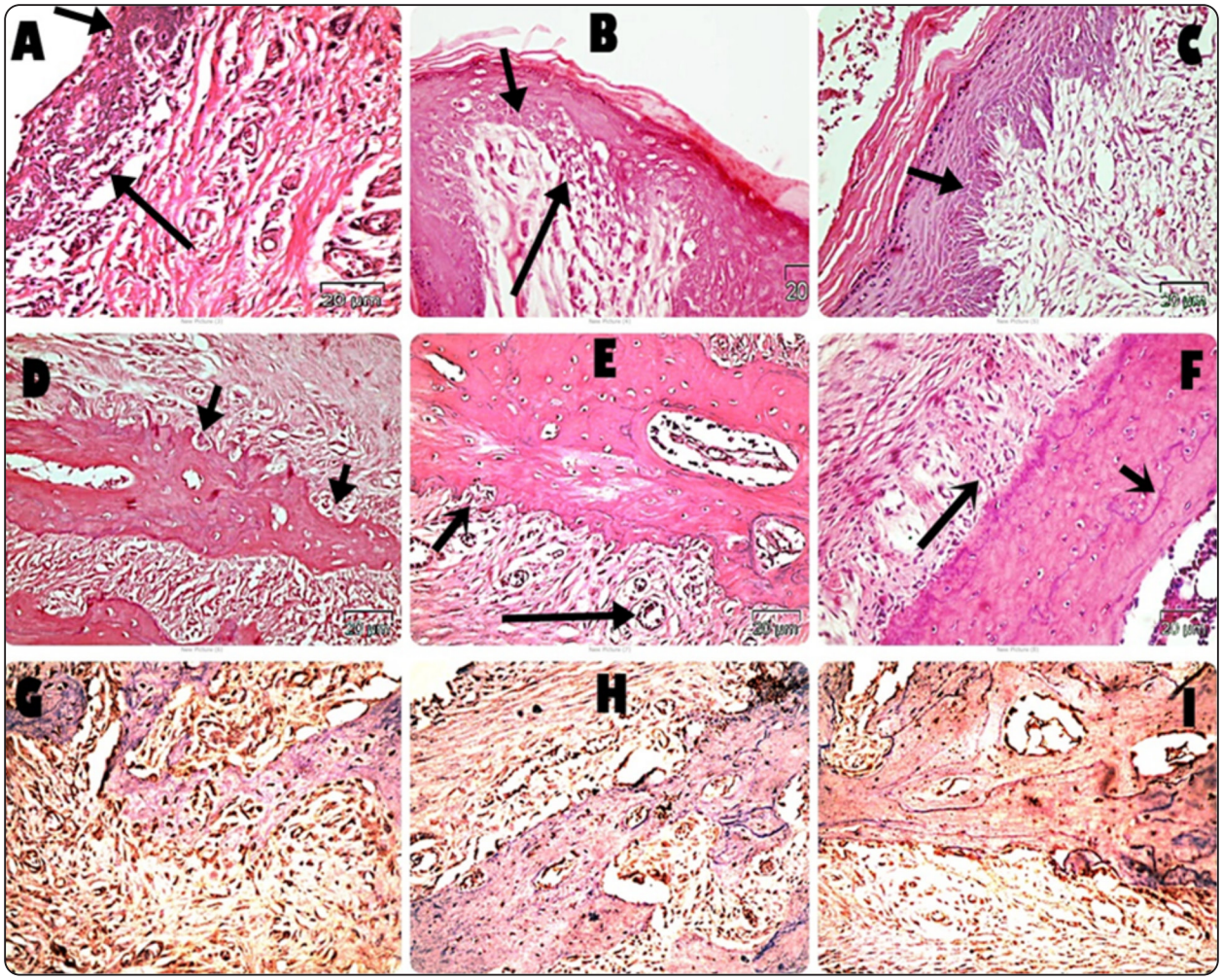

Fig. (1) Histopathological photomicrograph shows; (**A**) Group I: Epithelial atrophy (short arrow), hydropic degeneration, and intense inflammatory cells infiltrated in underlying connective tissue (long arrow) with areas of epithelial ulcerations. (**B**) Group II: Atrophy of epithelium (short arrow), moderate inflammation in underlying connective (long arrow). (**C**) Group III: Normal epithelial lining (arrow), with minimal inflammation of the underlying connective tissue. (**D**) Group I: Excessive bone resorption with prominent osteoclastic activity, irregular bone border (arrows), and disorganization of periodontal ligament fibers. (**E**) Group II: Alveolar bone resorption, moderate osteoclastic activity (short arrow), and well-vascularized periodontal ligament (long arrow). (**F**) Group III: Regular bone border, absence of osteoclastic activity, reversal lines denoting past osteoclastic (short arrow) activity, proper organization of periodontal ligament fibers (long arrow), and newly formed blood vessels. TNF's immunohistochemical photomicrographs show; (**G**) Group I: Alveolar bone (AB) and periodontal ligaments (PDL) show intense immune staining. (x40). (**H**) Group II: Moderate immune staining in AB and PDL (x40). (**I**) Group III: Low immune staining in PDL and AB (x40).

TABLE (2) Comparison between groups for the TNF –α immune expression test

Groups	Measurements Mean ± SD	ANOVA test	Pair-wise Comparisons	
			Bonferroni's post-hoc test	
		P-value (F-valu)	*Pair-wise Comparisons*	*P-value*
Group I	204.40±15.62[a]	< 0.0001** (F=31.89)	GI vs GII	< 0.0001**
Group II	167.36±10.18[b]		GI vs GIII	< 0.0001**
Group III	113.83±6.54[c]		GII vs GIII	< 0.0001**

a,b and *; means significant different (p< 0.05)

Different superscript in the same column reveals significant difference at p< 0.05.

DISCUSSION

The control of etiologic agents has been the focus of conventional periodontal treatments, supporting tissue healing and repair. Therapeutic substances delivered locally into the periodontal pocket operate as drug reservoirs, altering pathogenic flora and promoting wound healing.[25] PEG is now well recognized as a successful treatment for persistent non-healing wounds, burn injuries, post-surgical dressings, and bedsores.[12] PEG improves wound healing directly via active biomolecules such as human fibronectin type III peptide, which has high effectiveness in encouraging cell migration and wound repair. Placental extracts might be a promising treatment in the periodontal environment because of their ability to accelerate wound healing.[26] Because of these benefits, this study was intended to evaluate the effectiveness of the application of PEG combined with SRP in the treatment of experimental periodontitis in rats by examining the histopathologic changes and the expression of TNF-α in the periodontium of the animals included in this study.

According to the literature, ligature-induced experimental periodontitis is a well-understood procedure that can take from around 7 to 42 days to develop. Ligature induces subgingival plaque buildup throughout this time, and as a result, alveolar bone deterioration is detected with periodontal ligament fibril, much as it occurs in human periodontitis.[5, 27, 28, 29] In this experimental investigation, the ligatures were permitted to remain for 10 days based on the information published in the relevant literature. The histopathologic study verified the loss of alveolar bone surrounding all experimental teeth after 10 days.

Histopathologic analysis of the EP group was symmetric with the findings of previous investigations.[3,5,30] The current study showed severe epithelial deterioration, inflammatory cells infiltration, alveolar bone destruction, and disorganization of PDL. The difference in the histologic scores measured in this study was significantly higher (P≥0.001) in the EP group than in groups II and III in terms of the severity of these findings.

It is well known that the gold standard treatment of periodontitis is the SRP.[31] In the current investigation, group II was treated with SRP alone, which showed a minimal effect on the improvement of epithelium, decreasing the population of inflammatory cells, as well as alveolar bone, and PDL recovery. Cobb 2008,[32] reported that SRP improves periodontitis by lowering bacterial numbers, which may not be sufficient for alveolar bone reconstruction or correct PDL arrangement.

The topical administration of different medications enables the periodontium to heal more quickly, reduce microbial burden, and reduce periodontal disease deterioration.[33] PEG has been found to reduce not only the inflammatory condition and the microbiological load on the wound,[34] but also enhance cell migration,[35] matrix production, and tissue regeneration guaranteeing that the wound heals sequentially and continuously. As a topical medication, the placental extract is effective in the treatment of chronic non-healing wounds. The extract has been proved to promote fibrogenesis, neoangiogenesis, and epithelialization.[36]

In the current research SRP combined with local application of PEG was applied for the treatment of EP in group III. The histopathologic findings showed that PEG in combination with SRP proved a high capability in decreasing inflammation in the form of decreasing inflammatory cells population, increasing vascularization, improving alveolar bone formation, and the improvement of improvement of PDL general status. In line with the findings of the present investigation, in vitro study by *Akagi et al,* 2016 [17] on primary human gingival fibroblasts showed that human placental extracts boosted collagen type-1 formation, which is linked to periodontal tissue regenerating capacity. Also, it has been shown that PEG enhances vascular proliferation in regenerated tissues and is beneficial in accelerating healing.[37]

To the best of the author's knowledge, no previous experimental studies examined the effect of PEG on experimental periodontitis. On the other hand, a randomized controlled clinical trial by *Aakriti et al., 2020* [38] compared the clinical parameters in patients with periodontitis treated with SRP with or without local delivery of PEG. They observed a significant improvement in clinical parameters in the group treated with SRP and Placental extract, with a significant decrease in the depth of periodontal pocket with a gain in clinical attachment level.

TNF-α is a prime regulator in periodontitis pathogenesis, playing a significant role in tissue degradation and osteoclastogenesis.[39] The resorption of alveolar bone was associated with the increased activation of multinucleated osteoclast, whereas TNF-α has been proved to be implicated in osteoclastogenesis and disrupting alveolar bone repair as periodontitis progressed.[40] TNF-α has been shown to have deleterious effects on mesenchymal stem cells' osteogenic differentiation and bone repair in several studies. [41,42,43]

The immunohistochemical analysis of TNF-α in this research showed an increase in the expression of TNF-α in both periodontal ligaments and alveolar bone in the EP group which was statistically significant (P≥0.001) compared to SRP treated group and the group treated with SRP + PEG. These findings are consistent with the histopathologic feature of the experimental periodontitis group that showed increased osteoclastic activity and irregular borders of alveolar bone with Howship's lacunae and osteoclasts lying inside them. These changes in the EP group were in agreement with the study of Fujita *et al. 2012* [44] who concluded a significant increase in TNF-α in the gingival crevicular fluid (GCF) of the diseased sites of chronic periodontitis. Moreover, a systematic review by *Madureira et al. 2018* [45] found a link between chronic periodontitis and elevated TNF-α levels in GCF, indicating that the use of TNF-α as a possible biomarker for the diagnosis of periodontitis, which is consistent with the findings of this investigation.

Treatment of EP with SRP combined with PEG in the present research showed mild immunopositive staining of TNF-α in both PDL and alveolar bone which was statistically significant (P≥0.001) compared to the group treated with SRP alone that show moderate immunopositive staining. This finding was confined to the histopathologic finding of both groups and may be attributed to the bioactive components of PEG, it contains a variety of growth factors such as Granulocyte-Colony stimulating factor, Granulocyte-macrophage colony-stimulating

factor, Epidermal growth factor, Fibroblast growth factor, Hepatocyte growth factor, insulin-like growth factor, Platelet-derived growth factor, transforming growth factor, and Vascular endothelial growth factor, all of which have physiological effects ranging from immunomodulation to wound healing, cellular proliferation, and regeneration.[46-48] In addition, PEG also served as natural anti-oxidants because it comprised superoxide dismutase, catalase, and glutathione peroxidase, enzymes that neutralize free radicals and prevent cellular damage and the development of disease.[49]

The outcomes of this study concluded that PEG might be employed as a local drug delivery system in conjunction with SRP for the treatment of periodontitis due to its high capability in decreasing inflammation in the form of decreasing inflammatory cells population with decreasing TNF-α immune expression, increasing vascularization, improving alveolar bone formation as well as improving periodontal ligament fibers' general status. However, further studies are needed to determine the antimicrobial effect of PEG in experimental periodontitis, also, further controlled trials in humans on patients with periodontitis are necessary to investigate the significant clinical benefits of PEG.

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