Evaluation of IL-32 levels in gingival tissue and serum of experimental periodontitis model

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Abstract

Aim: Interleukin (IL)-32, a recently discovered proinflammatory cytokine, is demonstrated in several infectious diseases. The goal of this study is to investigate the levels of IL-6, IL-10 and IL-32 in gingival tissue and serum of rats with experimental periodontitis.
Material and Methods: Experimental periodontitis was induced by placing a silk ligature around the cervix of both sides of mandibular first molars in each male rat except for control group (Group 1). Thirty male Wistar albino rats were randomly divided into three groups of ten animals each as experimental periodontitis groups (Group 2, ligated for seven days; Group 3, ligated for fourteen days) and periodontally healthy control group (Group 1). At the end of experimental period, rats were sacrificed, and histomorphometric analyses were performed on the mandibles. IL-32, IL-10 and IL-6 levels were measured in gingival tissue and serum samples by ELISA.
Results: Alveolar bone and attachment loss were statistically higher in all experimental groups than those in control group (P<0.001). It was found that the levels of IL-32 and IL-6 (P<0.01) and IL-10 (P<0.05) in gingival tissues were higher in Groups 2 and 3 than those in Group 1 except for IL-32 and IL-10 levels in Group 3. There was a positive correlation between levels of IL-32 and IL-6 in the serum and gingival tissues in all groups (P<0.05).
Conclusion: The present results reveal that IL-32 values are locally increased in periodontitis. Proinflammatory cytokines properties, which are linked to periodontal tissue destruction, are also associated with IL-32.

Keywords: Interleukin-32; Experimental Periodontitis; Gingiva; Serum.

INTRODUCTION

Periodontal disease can be regarded as an infectious disease which is mainly caused by periodontal microorganism (1). Severity and progression of the disease is based on the host immune response which can also be observed by the production of mediators including cytokines, prostaglandin, and acute-phase protein (2). There are several studies reporting that periodontal destruction is directly linked with the biological activity of cytokines (3-5). Significant increase in the level of interleukin (IL)-6 in gingival tissue and serum is associated with inflammatory reaction (3, 6, 7). IL-10 can also be regarded as a vital anti-inflammatory cytokine which is responsible for regulating the production of pro-inflammation cytokines including IL-1β, IL-6 and IL-8 (5, 8) even though it can be found in healthy and inflamed periodontal tissues (4).

IL-32 which was currently discovered and was originally labelled natural killer (NK) cell transcript4 (NK4) is a proinflammatory cytokine (9). There are different cells that produce IL-32 including endothelial cells, monocytes, T cells, and epithelial cells (10). Alternative mRNA splicing results in the production of six isoforms of IL-32 (α, β, γ, δ, ε, and ζ) and immune and non-immune cells both can express these (11). IL-32 produces pleiotropic effects on the cellular function (9).

These functions are apoptosis, cell death, cell differentiation induction, and induction of pro- and\ or anti-inflammatory cytokines (9). IL-32 has been implicated in several inflammatory disorders, including rheumatoid arthritis (RA) (12), inflammatory bowel disease (13), and atherosclerosis (14). The expression of IL-32 is considerably up-regulated by Porphyromonas gingivalis (P.gingivalis)-derived lipopolysaccharide (LPS),

in comparison to the unstimulated cells in monocytes and the researchers have suggested that IL-32 may be a prime contributing factor in pathogenesis of periodontal disease as in chronic periodontitis (CP) (15). On the contrary, IL-32 levels in gingival tissue of periodontitis patients were found lower in comparison to healthy controls (16). However, there is no clear elucidation of the process during periodontal inflammation that describes the regulation of expression of IL-32. Moreover, in the experimental periodontitis, the relationship between IL-32, IL-6, and IL-10 has not been identified.

Thus, a hypothesis was created as the serum and gingival tissue levels of IL-32, IL-6 and IL-10 change with experimental periodontitis in rats and in different time periods. Therefore, the present study aimed to evaluate systemic and local effects of IL-32 in the periodontal tissue destruction. This study also compared the serum and gingival tissue levels of IL-6 and IL-10 to understand the role which is played by IL-32 for causing periodontitis in different periods.

MATERIALS and METHODS

Animals
The study evaluated a group of thirty Wistar albino rats, each 8 weeks old and weighing between 200-250 grams. The subjects were individually interned within plastic cages at a room temperature of 22±1°C within 50% humidity conditions. The lighting was maintained at a 12:12-h light−dark cycle, with the required food and water being provided ad libitum. The internment of the subjects was ensured to be in compliance with the protocols and guidelines recommended by the Ethical Committee of Animal Research of Bulent Ecevit University, in accordance with Guide for the Care and Use of Laboratory Animals (Protocol No. 2013-13-05/06). Upon stratifying them on the basis of their weights, they were arranged within 3 experimental groups of 10 subjects each. The groupings ultimately consisted of Group 1, healthy control; Group 2, ligated for 7 days; Group 3, ligated for 14 days.

Induction of periodontal disease
The experimental periodontitis effect was induced after tying 3.0 sterile silk ligatures within the cervical areas on both the left and the right of the mandibular first molars of individual subjects, barring those animals constituting the control group. Each of the subjects was therefore provided with two ligatures. General anesthesia was used during the procedure, which was intraperitoneally delivered along with 100mg∕kg ketamine and 0.75mg∕kg chlorpromazine. The ligatures contributed to periodontal diseases, facilitating the movement and passage of bacteria within the gingival cavities (3).

Sample Collection
At the conclusion of the experimental phase, the subjects were denied food or water in the evening prior to the concluding day of the study. On the proceeding day, 5mL of venous blood was drained out through cardiac punctures under general anesthesia conditions, which were forwarded for serum analyses. On the conclusion of the study, subjects with experimental periodontitis, and the periodontally healthy rats were all decapitated. Their blood samples were placed within centrifuges (Shimadzu UV160A, SNo:28006648, Kyoto, Japan) at 3000g within room temperatures over a period of 10 minutes, enabling the collection of serums, which were then placed at −70°C prior to analysis. The mandibles were thereafter surgically removed, along with the gingiva within the surroundings. The gingival tissue samples were collected from within the buccal region located within the mandibular right first molars, prior to storage at -700C for subsequent biochemical analysis.

Biochemical Analysis
The gingival tissue was blotted, prior to being weighed upon a microbalance. The tissues were cryogenically frozen using liquid nitrogen, before being subsequently grounded manually. This was done by placing them within eppendorf tubes having a required volume of PBS (pH 7.4, 10mM), diluted to 10 mg. tissue/mL PBS. This was sonicated (METU Electromechanical, Serial No.30607, Berlin, Germany) for 10 minutes at +4°C with 220V. On the day of evaluation, homogenates defrosted within the room from the samples were centrifuged (SIGMA 3K30, Serial No.76262, Osterode am Harz, Germany) at +4°C for 5 minutes with 15000g and supernatants were arranged for subsequent IL-32, IL-6 and IL-10 analysis. Gingival tissue and serum IL-32, IL-6 and IL-10 concentrations were evaluated using commercially marketed enzyme-linked immunosorbent assay (ELISA) kits (Hangzhou Eastbiopharm Company, Zhejiang, China (Mainland)). The quantum of protein present within the tissues was concluded by the Lowry method (17), with the results expressed as mg. per protein. The conclusions were derived as picogram (pg) of cytokine∖mg of protein (pg∖mg.protein) within the gingival tissues, and as pg∖mL within the serum.

Histomorphometric analysis
The left of the mandible so detached from within the gingiva was fixated within 10% neutral buffered formalin. The samples collected were decalcified within 8% formic acid (14 days), and subsequently embedded within paraffin. Serialized paraffin sections (5 μm) were concluded from within the mesiodistal aspects within the mandibular first molars. Three of the sections reflective of the central parameters of individual tooth were observed and thereafter stained with hematoxylin and eosin (H&E). Histomorphometric analysis was concluded utilizing a small microscope (BX50 research microscope, Olympus, Tokyo, Japan). The images were digitized using a camera (DP26 Digital Camera, Olympus, Tokyo, Japan) and subsequently analyzed using software (OLYMPUS DP2-BSW, Center Valley, PA) through a calibrated examiner (B.A.), not knowing the experimental design. As stated within the earlier study (18), individual sections were stained with H&E wherein the paramaters

assessed included: 1) the percentage of alveolar bone in furcation area, 2) alveolar bone loss (ABL), and 3) clinical attachment level (CAL). The percentage ratios of alveolar bone area upon individual specimens were concluded as a ratio of the alveolar bone area versus the furcation area. The alveolar bone area was concluded as a mix of the trabecular bone area and the bone marrow area in furcation. The levels of the alveolar bone were concluded through a measure of the distances within the cemento-enamel junction (CEJ) and the alveolar bone crest. CAL was concluded to be the distance within the CEJ, versus the coronal extent of the connective tissue attachment to cementum. ABL and CAL values were concluded within mesial and distal regions of the mandibular first molars. All averages of the measurements concluded were utilized towards analyzing the data.

Intra-examiner reproducibility
Prior to histomorphometric analyses, the examiner (B.A.) evaluated 20 specimens twice, with a week's interval between the measures. Bland-Altman plots along with intraclass correlation coefficients were utilized towards concluding the intra examiner agreement and reliability measures (19). Bland–Altman plots reflected the agreements within the two values concluded within a week's interval in the histomorphometric parameters. The intraclass correlation coefficients (95% confidence interval) were concluded to be 0.981 (0.907-994) relative to the alveolar bone area measures, 0.988 (0.958-0.996) for alveolar bone loss and 0.992 (0.978-0.997) for clinical attachment level.

Statistical Analysis
The Kolmogorov–Smirnov test was used to evaluate the normality of the data. One-way analysis of variance and Tukey's post hoc test were employed to evaluate histomorphometric and biochemical parameters within the groups in the aftermath of the normality within the data distribution concluded. SPSS software (SPSS Inc., Chicago, IL, USA), version 19.0 was used for the tests, with $p<0.05$ being considered to be a statistically significant measure.

RESULTS

Histomorphometric Findings
Values associated the alveolar bone area in the furcation region, ABL, and CAL is stated within Table 1. Significant increases in the ABL and CAL were observed in all experimental periodontitis groups when compared with the healthy control group; however, this increase was greater in ligated group for 14 days ($P<0.001$). The alveolar bone area was higher in all experimental periodontitis groups than in the control group ($P<0.001$). The histologic appearances are reflected within Figure 1.

Biochemical Findings
IL-32, IL-6 and IL-10 levels in gingival tissue and serum are showed in Table 2. The levels of IL-6 in gingival tissue was concluded to be statistically lower in control group when compared to that in all experimental periodontitis groups ($P<0.01$), while there was no statistically difference between Groups 2 and 3 ($P>0.05$). Significant increases in the levels of IL-32 and IL-10 were determined in Group 2 when compared to Group 1 ($P<0.01$). However, no significant difference in the levels of IL-32 and IL-10 was observed in Group 3 when compared to Groups 1and 2 ($P>0.05$). The levels of IL-32 and IL-6 in serum were higher in all experimental periodontitis groups than control group ($P<0.01$), whereas there was no statistically significant difference in the serum levels of IL-10 among groups ($P>0.05$).
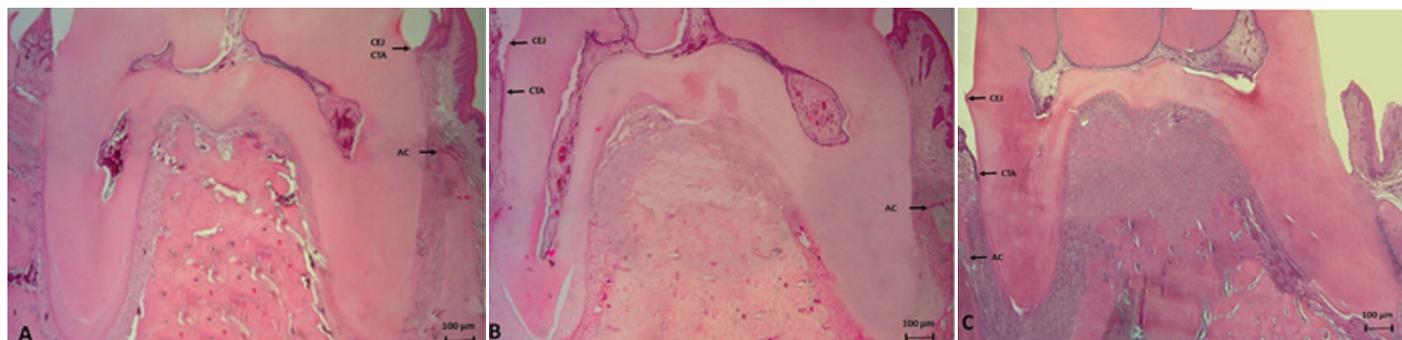
Figure 1. (A) The sections from the mesio-distal aspects throughout the mandibular first molars in control group (Group 1) (H&E, 4x), (B) The sections from the mesio-distal aspects throughout the mandibular first molars in ligated group for 7 days (Group 2) (H&E, 4x), (C) The sections from the mesio-distal aspects throughout the mandibular first molars in ligated group for 14 days (Group 3) (H&E, 4x), CEJ: Cemento-enamel junction, CTA: Connective tissue attachment, AC: Alveolar crest

Correlations
The correlation coefficients are stated within Table 3. Upon examining all the groups simultaneously, a strong degree of positive correlation was observed within levels of IL-32, IL-6 and IL-10 in the gingival tissue ($P<0.001$). A positive and significant degree of correlation is also recorded within levels of IL-32 and IL-6 in the serum and gingival tissue ($P<0.05$). Further, strong negative correlations are concluded within the ABL, alveolar bone area and CAL ($P<0.001$). Also, significant correlations were found in among the histomorphometric values, serum levels of IL-32 and serum and gingival tissue levels of IL-6 ($P<0.001$).

Table 1. The percentage of alveolar bone in furcation area, alveolar bone loss, and clinical attachment level			
Groups	Alveolar bone area (%)	Alveolar bone loss (µm)	Clinical attachment level (µm)
Control group (Group 1; n=10)	67.638±4.80	433.459±32.11	114.214±20.43
Ligated group for 7 days (Group 2; n=10)	54.523±2.79*	751.671±42.67*	231.02±45.16*
Ligated group for 14 days (Group 3; n=10)	46.31±3.46*†	1037.02±59.46*†	495.28±48.02*†
Data are expressed as the mean ± standard deviation. One-way ANOVA and post-hoc Tukey's test. p<0.05 was considered to be statistically significant. * Statistically significant difference from control group (p<0.05) †Statistically significant difference from ligated group for 7 days (p<0.05)			

Table 2. Levels of IL-32, IL-6 and IL-10 in rat gingival tissue and serum						
Groups	IL-32		IL-6		IL-10	
	Gingival tissue (pg\mg.protein	Serum (pg\mL)	Gingival tissue (pg\mg.protein)	Serum (pg\mL)	Gingival tissue (pg\mg.protein)	Serum (pg\mL)
Control group (Group 1; n=10)	14,70±1,97	13.91±2.20	31,93±8,23	43.40±11.05	60,10±34,18	47.01±11.38
Ligated group for 7 days (Group 2; n=10)	31.99±7.13*	22.86±1.40*	135.36±28.04*	101.20±9.84*	105.91±32.97*	41.44±9.99
Ligated group for 14 days (Group 3; n=10)	21.60±7.53	22.38±1.51*	91.77±32.14*	89.66±12.16*	77.43±24.67	48.03±8.89
Data are expressed as the mean ± standard deviation. One-way ANOVA and post-hoc Tukey's test. p<0.05 was considered to be statistically significant. * Statistically significant difference from control group (p<0.05)						

Table 3. The Pearson correlation (r) among groups with respect to IL-10, IL-6, IL-32 levels and alveolar bone loss, alveolar bone area and clinical attachment level in all groups									
		Alveolar bone loss	Clinical attachment level	S IL-32	S IL-6	S IL-10	G IL-32	G IL-6	G IL-10
Alveolar bone area	r	-0.841**	-0.805**	-0.724**	-0.613**	-0.215	-0.169	-0.355*	-0.127
	p	0.000	0.000	0.000	0.000	0.134	0.242	0.012	0.379
Alveolar bone loss	r		0.896**	0.672**	0.583**	0.024	0.202	0.380**	0.145
	p		0.000	0.000	0.000	0.867	0.159	0.007	0.315
Clinical attachment level	r	-0.896**		0.521**	0.435**	0.070	0.050	0.220	0.031
	p	0.000		0.000	0.002	0.631	0.732	0.124	0.833
S IL-32	r	0.672**	0.521**		0.876**	-0.037	0.461**	0.621**	0.321*
	p	0.000	0.000		0.000	0.801	0.001	0.000	0.023
S IL-6	r	0.583**	0.435**	0.876**		-0.140	0.510**	0.682**	0.367**
	p	0.000	0.002	0.000		0.332	0.000	0.000	0.009
S IL-10	r	0.024	0.070	-0.037	-0.140		-0.279*	-0.255	-0.234
	p	0.867	0.631	0.801	0.332		0.050	0.074	0.102
S IL-32	r	0.202	0.050	0.461**	0.510**	-0.279*		0.953**	0.876**
	p	0.159	0.732	0.001	0.000	0.050		0.000	0.000
G IL-6	r	0.380**	0.220	0.621**	0.682**	-0.255	0.953**		0.820**
	p	0.007	0.124	0.000	0.000	0.074	0.000		0.000
** Correlation is significant at the 0.01 level *Correlation is significant at the 0.05 level S, serum; G, gingival tissue; IL, Interleukin									

DISCUSSION

In the current study, the role played by inflammatory cytokines within the incidence of periodontitis was observed, utilizing biochemical analysis processes within two varying experimental periods. To the best of the authors' knowledge, this is the first experimental study that has examined in gingival tissue and serum IL-32 levels in rats with experimental periodontitis and that has also investigated the association among IL-32, IL-6 and IL-10 in periodontal health and disease.

The ligature-induced experimental periodontitis model was utilized within the study. This enabled significant efficiencies in handling, costs and in its similarities to human diseases. Ligature-induced periodontitis within rats is considered amongst the more common experimental models related to periodontitis (3). Sobaniec and Sobaniec-Lotowska (13) stated that alveolar bone loss was markedly observed within 7 days of ligature placement in rats. Within the study, periodontitis was induced through the use of silk thread ligatures over 7 days. Brito et al. (3) determined that systemic inflammation markers at 28 days after ligature placement were as much as at baseline. They suggested that rats have a great ability to adapt front of inflammatory stimuli (3).

The primary objective of the study using the current model was to evaluate the local effects of the lesions and the systemic consequences too. Hence, the subjects were evaluated for 14 days after the ligature. Besides, the changes brought onto the periodontium were initially evaluated for both 7 and 14 days once the ligatures were removed within the rats. The current research revealed that in contrast with unligatured control rats, position of ligature led to considerable amount of alveolar bone loss and apical migration of the junctional epithelium. For about fourteen days in the ligated groups there was extensive amount of alveolar bone and attachment loss, which contrasted with our perception of the control and experimental groups. The outcomes are in accordance with the previously conducted studies (3,5,13).

Higher levels of IL-6 in periodontitis in the gingival tissue (8,20,21) and serum (22) were previously found and this demonstrates that IL-6 is an important factor in the immunopathology of periodontal illness by provoking bone resorption. Serum IL-6 levels are known to raise within 14-28 days after ligature in contrast with the control group (3), on the other hand Hatipoğlu et al. (23) found that there was no statistically significant difference in serum IL-6 levels between control group and ligated diabetic group in 21 days. According to Dutzan et al. (24), in periodontal disease-affected tissues a major over-expression of IL-6 was identified in contrast with healthy gingival tissues, nevertheless, IL-10 was down regulated in periodontal lesions. IL-10 is a solid inhibitor of IL-6 fabrication by P.gingivalis LPS-stimulated human gingival fibroblasts (HGF) and resultantly has a major influence on controlling bone resorption in periodontal disease (20). According to Gümüş et al. (22), serum IL-10 levels and IL-6 has no connection. However, another research showed an increase in expression of IL-10 against untreated control when gingival fibroblasts were handled with P.gingivalis LPS for 48 hours (25). As per Napimoga et al. (26), IL-10 in the gingival biopsies prominently increased at mRNA in CP group against periodontally healthy group. The present study demonstrated that gingival tissue and serum IL-6 levels were greater in all experimental groups compared with control groups whereas gingival IL-10 levels in control group were lower than those of ligated group in 7 days. Moreover, there was a progressive relationship between IL-6 and IL-10 in the gingival tissue, however, not in serum. Concurring with prior reports it is established that IL-6 and IL-10 is an important factor in the periodontal disease.

IL-32 shows proinflammatory characteristics in several ailments (12,27,28). Though, lower levels of IL-32 was identified in the gingival tissue of periodontitis patients than in healthy patients (16). Similarly, by controlling of IL-8 expression in HGF stimulated by P.gingivalis led to the down regulation of mRNA expression of every isoform of IL-32 (IL-32α, β, γ, δ), that leads to the induction of an anti-inflammatory outcome (16). Accordingly, the small sample of patients were appropriate to evaluate the relationship between healthy and periodontitis tissue because it can be affected by the cytokine of genetic background or single nucleotide polymorphism (SNP), however, seemingly IL-32 has an anti-inflammatory impact in strong gingival tissue (16). Whereas, it has been revealed from a study of airway inflammatory diseases that TNF-α suggestively upregulated the IL-32α, β, γ, and δ expression in human lung fibroblasts (29). The disparity between the discoveries in the writing is credited to Ouhara et al. (16) that certain bacterial elements might have reacted with HGF and lower the generation of IL-32 by creating a negative sign to decrease IL-32 mRNA expression and IL-10 may interfere in the bacteria-facilitated concealment of IL-32 generation from HGF. Similarly, there were two distinctive kinds of IL-32 receptors to stimulate cell signalling as a proinflammatory signal and an anti-inflammatory signal (16).

It has been revealed that gingival and serum IL-32 levels were considerably higher in ligated group for 7 days groups than in control group. Furthermore, a strong relationship was found among serum levels of IL-32 and the gingival IL-32 levels and the serum levels of IL-6, as well as the alveolar bone and attachment loss. It was previously demonstrated that IL-32 was considerably upregulated by the periodontal pathogens, P.gingivalis and Fusobacterium nucleatum, in an oral epithelial cell line H400 (30). Moreover, IL-32 was expressed by monocytes via P.gingivalis-derived LPS and supposedly IL-32 is a factor that leads to the formation of periodontal disease (15). Furthermore, significant IL-32 levels in the GCF and saliva were exhibited in the patients with

CP in contrast with healthy panels and this increase is linked with proinflammatory characteristic of the IL-32 (31). Our data has been indicated that IL-32 operates as a proinflammatory cytokine and is a crucial factor in periodontitis. Also, levels of IL-32 in gingival tissue and serum may work as an analytical indicator for periodontitis.

There is a distinct feature of the biological activity of each different isoform of IL-32. IL-32γ is the most biologically operational isoforms among others and helps in increasing proinflammatory cytokines and chemokines in inflamed tissues (32). It has been indicated by Heinhuis et al. (33) that IL-6 generation is amplified through intracellular IL-32γ expression by THP1 cells after lipopolysaccharide stimulation. Accordingly, generation of IL-6 is directly related with IL-32γ level in RA patients (33,34). Additionally, sIL-6R operates as an upstream administrative element for IL-32 amid viral disease and endorses IL-6 generation through IL-32 (35). Our study indicated that there was a strong relationship between IL-32 and IL-6, both in serum and in gingival tissues. This result showed that IL-32 may take part in the development of periodontitis by increasing IL-6 generation. Perhaps, the relationship between IL-32 and sIL-6R applies a vital effect on the periodontal reaction. In the current study sIL-6R levels were not observed. Also, the relationship between IL-6 and IL-32 should be explained for the understanding of periodontitis. Reportedly, IL-32β upregulated the IL-10 generation by interfering with the discharge/action of protein kinase C in myeloid cells and that IL-32δ lessened the generation of this cytokine in a dosage expanding way in U937 cells (36). However, there are 6 subtypes of IL-32, and until now their part in periodontitis is undefined. The IL-32 role in periodontitis might be best explained by researching the particular roles of its distinctive isoforms.

In conclusion, this study provides further evidence of a possible role of IL-32 upregulation in experimental periodontitis. Thus, it may be concluded that the proinflammatory impact of IL-32 might be associated with the development of periodontitis. However, the consequence of these findings may not provide to be a diagnostic and prognostic marker of IL-32 in periodontal disease. Consequently, more research has to be done to explain the correlation of periodontal inflammation and IL-32 expression in gingival tissue in research constituting bigger sample of patients with CP.

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