



Analysis of orosomuroid and c-reactive protein levels in gingival tissue and serum of rats with experimental periodontitis: Comparison at different time points in disease progression

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ARTICLE INFO

ABSTRACT

Article History

Received 05 / 12 / 2016
Accepted 26 / 01 / 2016
Online Published Date 10 / 10 / 2019

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Keywords:

Acute-phase proteins
Gingiva
Orosomuroid
Periodontitis

Acute-phase proteins are thought to trigger periodontal tissue destruction. The core purpose of this study is to analyze the levels of Orosomuroid1 (ORM1) and C-reactive protein (CRP) in gingival tissue and serum of rats with experimental periodontitis. Thirty rats were divided randomly and Group 1 was specified as control group, while the remaining groups were classified as ligature-induced experimental periodontitis groups. Each group was constituted 10 rats and Group 2 was evaluated the impact of ligature for the term of 7 days. Group 3 was classified on the basis of 14 days ligature. With the help of ELISA, gingival tissue and serum samples was utilized for measuring CRP and ORM1 levels. Alveolar bone and attachment loss was statistically higher in all experimental periodontitis groups than those in control group ($P<0.001$). The levels of CRP and ORM1 in gingival tissue were significantly higher in Group 2 than in Groups 1 and 3 ($P<0.05$). Also, a statistically significant positive correlation was found between CRP and ORM1 levels in the gingival tissue ($P<0.001$). The present results reveal that tissue destruction at earlier periods of inflammatory periodontal disease may be associated with ORM.

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1. Introduction

Periodontal disease can be regarded as an infectious disease which is mainly caused by periodontal microorganism (Feng and Weinberg, 2006). 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 (Keles et al., 2012). Acute-phase proteins are effective markers for the identification and evaluation of inflammatory diseases and they are increased in case of microbial infection including periodontitis (Keles et al., 2012).

In case of acute and chronic inflammation, C-reactive protein (CRP) is considered to be the most

accurate marker of acute phase protein and periodontal disease is also linked with significant increase in CRP level (Gornik and Lauc, 2008; Gupta et al., 2015). Significant increase in the level of CRP in gingival tissue and serum is associated with inflammatory reaction which result in excessive production of CPR by hepatocytes (Brito et al., 2013; Lu and Jin, 2010; Shimada et al., 2010).

Orosomucoid (ORM) is an acute-phase plasma protein, which is also termed as alpha-1-acid glycoprotein, and this glycoprotein is inflammation-sensitive (Fournier 2000). In different pathological conditions such as physical trauma, any kind of bacterial infection, the malignant diseases and rheumatoid arthritis (RA), ORM serum concentrations increases many folds in different pathophysiological problems (Fournier et al., 2000; Luo et al., 2015). In the gene expression of ORM, the major regulatory mediators are glucocorticoids, IL-6, and IL-6 related cytokines in liver cells from rat and human (Luo et al., 2015). It is a matter of fact that its main functions are unknown; however, it regulates immunomodulatory and anti-inflammatory functions, inhibits the activation of the polymorphonuclear neutrophil and results in the modulation of monocytes-macrophages dependent secretion of LPS-induced cytokine (Fournier et al., 2000; Sai et al., 2014). Two genes control the expression of ORM and these are ORM1 and ORM2 (Sai et al., 2014). Rangé et al. (2013) demonstrated that ORM is a better inflammatory marker as compared to CRP and the reason behind is that periodontitis is an infectious chronic disease. Though, there is no data regarding comparison of ORM and CRP in the gingival tissue in periodontitis.

Thus, a hypothesis is made that the in the rats with experimental periodontitis, there may be a difference in the gingival tissue and serum level of ORM1 and CRP in different periods. Therefore, the purpose of this study is to analyze the serum and gingival tissue levels of ORM1 and CRP in rats with experimental periodontitis at different periods as well as healthy control group. Also, the relationship between these biomarkers and histomorphometric findings is investigated

2. Material and methods

Animals

The study evaluated a group of fifty 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 $22\pm 1^{\circ}\text{C}$ 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 was ligated for 7 days; Group 3 were 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 (Brito et al., 2013).

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 preceding 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 $+4^{\circ}\text{C}$ 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 $+4^{\circ}\text{C}$ for 5 minutes with 15000g and supernatants were arranged for subsequent CRP

and ORM1 analysis. Gingival tissue and serum CRP and ORM1 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 (Lowry et al., 1951), 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, barring CRP levels. CRP levels was represented as ng/mg.prot within the gingival tissues, and as ng/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 μ m) 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 (Balli et al., 2014), 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 (Donatelli et al., 2013). 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.

3. Results

Histomorphometric findings

Values associated the alveolar bone area in the furcation region, ABL, and CAL is stated within Table 1. Major increases within alveolar bone and attachment loss was observed within the experimental periodontitis groups when compared with the healthy control group ($p < 0.001$). There were major differences within the experimental periodontitis groupings associated with alveolar bone and attachment loss ($p < 0.001$). The alveolar bone area was larger within the experimental group, than in the control group ($p < 0.001$). Within the experimental groups, the alveolar bone area within ligated groups over 14 days was much high when compared with the ligated group over 7 days ($p < 0.05$). The histologic appearances are reflected within Fig. 1.

Table 1. The percentage of alveolar bone in furcation area, alveolar bone loss, and clinical attachment level.

Groups	Alveolar bone area (%)	Alveolar bone loss (μ m)	Clinical attachment level (μ m)
Group 1 (n=10)	67.638 \pm 4.80	433.459 \pm 32.11	114.214 \pm 20.43
Group 2 (n=10)	52.003 \pm 3.47*	879.644 \pm 71.91*	361.153 \pm 58.59*
Group 3 (n=10)	44.770 \pm 4.84**	1263.50 \pm 45.87**	708.92 \pm 59.68**

Data are expressed as the mean \pm standard deviation. One-way ANOVA and post-hoc Tukey's test.

$p < 0.05$ was considered to be statistically significant.

* Statistically significant difference from group 1 (control group) ($p < 0.05$)

‡ Statistically significant difference from group 2 ($p < 0.05$)

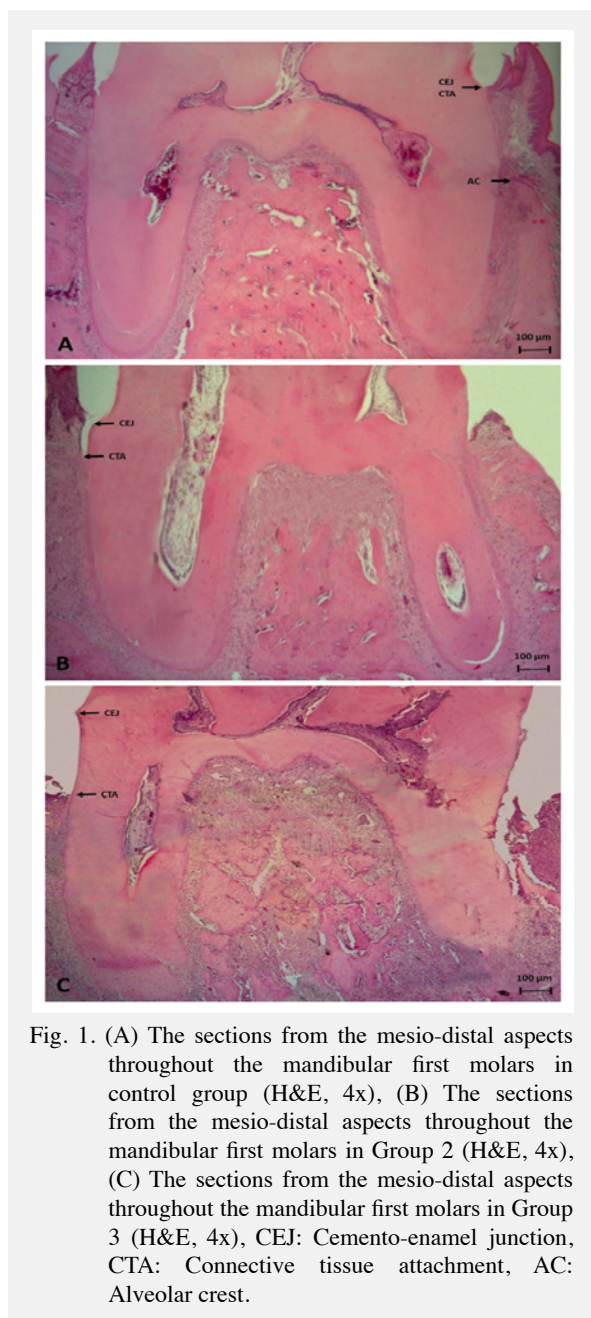


Fig. 1. (A) The sections from the mesio-distal aspects throughout the mandibular first molars in control group (H&E, 4x), (B) The sections from the mesio-distal aspects throughout the mandibular first molars in Group 2 (H&E, 4x), (C) The sections from the mesio-distal aspects throughout the mandibular first molars in Group 3 (H&E, 4x), CEJ: Cemento-enamel junction, CTA: Connective tissue attachment, AC: Alveolar crest.

Biochemical findings

Conclusions against CRP and ORM1 within gingival tissue and serum are reflected in Tables 2. The levels of CRP within gingival tissue and serum were observed to be statistically lower within Group 1 when compared against experimental periodontitis groupings, other than that observed against Group 3 in gingival tissue ($p < 0.05$). Major differences were also observed within Group 3 versus Group 2 with regard to the gingival tissue levels associated with CRP, and relative to Group 3 versus Group 2 with regard to the serum levels of CRP ($p < 0.05$). Gingival tissue ORM1 levels were observed to be lower within the control group in comparison to that for Group 2. It was nevertheless concluded to be higher

within Group 2 when compared to Group 3 ($p < 0.05$). Significant variations were not observed within serum ORM1 levels of the experimental periodontitis and within the control group ($p > 0.05$).

Table 2. Levels of CRP and ORM1 in rat gingival tissue and serum.

Groups	CRP		ORM1	
	Gingival tissue (ng/mg.prot)	Serum (ng/mL)	Gingival tissue (pg/mg.protein)	Serum (pg/mL)
Group 1 (n=10)	27.53±9.31	22.00±4.38	560.98±94.00	463.76±159.09
Group 2 (n=10)	73.38±14.64*	46.62±5.79*	1155.29±277.58*	459.45±109.71
Group 3 (n=10)	44.18±19.10 [‡]	57.35±6.17**	736.57±273.41 [‡]	396.91±160.42

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 group 1 (control group) ($p < 0.05$)

[‡] Statistically significant difference from group 2 ($p < 0.05$)

Correlations

The correlation coefficients are stated within Table 3. Upon examining all the groups simultaneously, a strong degree of positive correlation is observed within levels of CRP and ORM1 in gingival tissue ($p < 0.001$). Further, strong negative correlations are concluded among the alveolar bone level, alveolar bone area and attachment level ($p < 0.001$). There was a positive correlation between serum levels of CRP and histomorphometric values ($p < 0.001$).

Table 3. The Pearson correlation (r) among groups with respect to ORM1, CRP levels and alveolar bone loss, alveolar bone area and clinical attachment level in all groups.

	Alveolar bone loss	Clinical attachment level	S CRP	S ORM1	G CRP	G ORM1
Alveolar bone area	r -0.858**	-0.810**	-0.722**	0.085	-0.199	-0.107
	p 0.000	0.000	0.000	0.555	0.166	0.460
Alveolar bone loss	r -0.925**	0.708**	-0.219	0.193	0.142	
	p 0.000	0.000	0.127	0.180	0.326	
Clinical attachment level	r -0.925**	0.670**	-0.157	0.039	0.011	
	p 0.000	0.000	0.275	0.790	0.942	
S CRP	r 0.708**	0.670**	0.068	0.330*	0.219	
	p 0.000	0.000	0.640	0.019	0.127	
S ORM1	r -0.219	-0.157	0.068	0.093	-0.037	
	p 0.127	0.275	0.640	0.521	0.796	
G CRP	r 0.193	0.039	0.330*	0.093	0.848**	
	p 0.180	0.790	0.019	0.521	0.000	
G ORM1	r 0.142	0.011	0.219	-0.037	0.848**	
	p 0.326	0.942	0.127	0.796	0.000	

** Correlation is significant at the 0.01 level.

* Correlation is significant at the 0.05 level.

S, serum; G, gingival tissue; CRP, C-reactive protein; ORM, Orosomucoid

4. Discussion

In the current study, the impact of acute-phase proteins within the incidence of periodontitis was observed, utilizing biochemical analysis processes within four varying experimental periods. The study concluded efficiencies within periodontal tissue breakdown, in addition to alveolar bone loss in periodontitis related to four varying experimental periods relative to histomorphometric analysis. As per our knowledge, this is a pioneering study evaluating the systemic and local levels of ORM within the periodontium utilizing biochemical analysis processes within the context of experimental periodontitis models.

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 (Brito et al., 2013). Alveolar bone loss is observed to be markedly observed within 7 days of ligature placement (Sobaniec and Sobaniec-Lotowska, 2000). Within the study, periodontitis was induced through the use of silk thread ligatures over 7 days. Brito et al. (2013) concluded how systemic inflammation markers concluded to basal levels within 28 days after the ligature. It was concluded how rats are greatly adaptable to inflammatory stimuli (Brito et al., 2013). 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. 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 (Sobaniec and Sobaniec-Lotowska, 2000; Peruzzo et al., 2008; Brito et al., 2013).

According to Gupta et al. (2015), within a day or two after the acute tissue breaks CRP increases in serum or plasma and declines with the resolution of inflammation or disturbance. Amid periodontitis, rise of CRP in serum or plasma have already been noted (Bain et al., 2009; Lu and Jin, 2010). However, it has been indicated by Lu and Jin (2010) CRP is also developing in human gingiva and it constitutively expresses CRP. They demonstrated that human gingiva consists of a local source of CRP and could possibly affect the CRP levels in gingival crevicular fluid (GCF), saliva and serum (Lu and Jin, 2010). Similarly, Maekawa et al. (2011) revealed that in contrast with gingivitis the expression of CRP mRNA was greater in periodontitis

tissues. It was expected that the CRP's local expression of in gingival tissues may possibly be a critical factor in the advancement and development of periodontitis (Maekawa et al., 2011). In the same way, the levels of the gingival tissue and serum CRP were considerably low in control group as compared to experimental group according to the current study. Additionally, the variation in levels of CRP in the gingival tissue is observed, where the levels of CRP in the gingival tissue were less in Group 3 that that in Group 2 and there were higher levels of CRP in the serum in Group 3 than that in Group 2. Brito et al. (2013) concurred that within fourteen days serum CRP levels rises after ligature in rats and within 28 days restore to basal level. CRP increases faster with inflammation and quickly decreases after its resolution this is because in contrast with other acute phase proteins CRP has a short half-life (Vermeire et al., 2006). As stated, CRP generated by endothelial cells through pro-inflammatory cytokines is considerably low than that observed in blood (Maekawa et al., 2011). According to literature (Maekawa et al., 2011), our data indicates the rise of CRP levels in gingival tissue of the rats at the onset of inflammation and lowers within fourteen days with resistance to inflammation stimuli of the rat.

The study revealed that for 7 days ORM1 levels in the gingival tissue were greater in the ligated groups than for 14 days in the control group, however no considerable variation was observed in the serum ORM1 levels among groups. Moreover, in the gingival tissue there was a great relationship among ORM1 and CRP levels. Until now, no research has been found on the said subject, hence this is the only research that been conducted which indicates ORM1 levels in the gingival tissue increases with inflammation. According to Range et al. (2013), in mild-moderate periodontitis group ORM in the plasma was lower than the severe periodontitis group. After conformity for age, gender and smoking ORM level (in contrast with CRP and IL-6) was linked with periodontal inflammation severity (Range et al., 2013). Also, the acuteness of periodontitis, in morbidly obese patients, is linked with the rise of orosomucooid levels (Range et al., 2013). Mohmoud and Abbas (2002) indicated that ORM was constantly less on the gingival fluid immunograms, but was unidentified in plasma. According to Hochepped et al. (2003) ORM1 has the ability to systemically and locally release soluble IL-6 (sIL-6) through mononuclear leukocytes. Moreover, determining the level of ORM and identifying its glycoforms is more effective in measuring the intensity of an illness rather than assessing the CRP levels (Luo et al., 2015). It is proposed that ORM1 levels in gingival tissue could help in assessing the pathophysiologic procedures related to periodontitis.

Our research suggests that confined relentless infection may impact universal levels of inflammatory intermediaries. Inflammatory state can be provoked through periodontal infection by raising the levels of gingival of ORM1. Though it serious inflammation has been revealed from the model of the ligature-induced

rat periodontitis and is not associated with the chronic illness in humans. Consequently, more research has to be done to explain the correlation of periodontal inflammation and the commitment to the periodontal disease of ORM1 by killing constraints in long haul research constituting bigger sample of patients with CP.

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