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From Cell Biology to Tissue Engineering

Histopathological and clinical expression of periodontal disease related to the systemic inflammatory response

Anca Ionel[1], Ondine Lucaciu[1], Flaviu Tăbăran[2], Cristian Berce[3], Septimiu Toader[3], Lucia Hurubeanu[4], Cosmina Bondor[5] and Radu S. Câmpian[1]

[1]Department of Oral Rehabilitation, Oral Health and Dental Office Management, Faculty of Dental Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, [2]Department of Anatomic Pathology, Necropsy and Forensic Medicine, Faculty of Veterinary Medicine, University of Agricultural Science and Veterinary Medicine, [3]Centre for Experimental Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, [4]Department of Oral and Cranio-Maxillofacial Surgery, "Iuliu Haţieganu" University of Medicine and Pharmacy and [5]Department of Medical Informatics and Biostatistics, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania

Summary. Periodontal disease with its systemic implications is highly prevalent among the population, and this correlation could have an impact on the quality lives of many humans. The purpose of this study was to assess the clinical and histopathological changes of the periodontium correlated with the systemic inflammatory response in periodontal disease. An experimental study was performed on male Wistar rats which were subjected to a procedure of periodontitis induction through placing silk thread ligatures around the lower incisors, under general anesthesia. Clinically, the changes of the periodontal tissue induced by the periodontitis progression were daily assessed. Two blood samples were obtained from each animal, at baseline and on completion of the experiment. The plasma level of the cytokine IL-6 and haematological parameters such as leukocytes, neutrophils, lymphocytes, monocytes, and platelets were determined. After seven days the animals were sacrificed, and samples were prepared for histological evaluation. Clinical manifestations such as changes in the color, contour and consistency of the gingival tissue and the bleeding on probing were registered. Histopathological analysis showed an intense inflammatory cell infiltration, the presence of osteoclasts and an obvious bone resorption activity. A significant increase in IL-6 values during the progression of periodontitis in rats ($p<0.001$) was also observed. The results of this research demonstrated that the clinical and histological changes in the rat's periodontium are correlated with a notable systemic inflammatory response. Therefore, periodontitis control can be inserted as part of the programs of systemic disorders prevention, in clinical practice.

Key words: Periodontitis, Histological aspects, Clinical features, IL-6, Systemic inflammation

Offprint requests to: Anca Ionel, Department of Oral Rehabilitation, Oral Health, and Dental Office Management, "Iuliu Haţieganu" University of Medicine and Pharmacy, 15 Victor Babeş street, 400012, Cluj-Napoca, Romania. e-mail: anca_ionel@yahoo.com or Ionel.Anca@umfcluj.ro

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Introduction

Periodontal disease is a very common chronic disorder involving inflammatory damage of the periodontium, being the leading cause of tooth loss in adults. The changes in the anatomical structure of the gingival tissue, the resorption of the marginal alveolar bone accompanied by the loss of the clinical attachment represent the main features of periodontitis (Lindhe et al., 2003).

The evidence reported in the literature revealed a growing interest in exploring the link between periodontitis and multiple chronic systemic diseases, with considerable repercussions on human health status (Gulati et al., 2013; Gurav, 2014; Rydén et al., 2016). Several assumptions were proposed to support this association, and most of them involved an inflammatory response (Akshata et al., 2012; Cullinan and Seymour, 2013; Carramolino-Cuéllar et al., 2014).

Experimental research conducted in periodontology

has proposed a variety of animal models to study the inflammatory process in periodontal disease, the systemic implications and the treatment of this disorder (Graves et al., 2008; Oz and Puleo, 2011; Do et al., 2013).

Rodents, especially rats are very commonly used as an animal model for experimental studies in periodontology. This is due to the similarities of rats gingival structures with those observed in humans, excepting the keratinization of the sulcular epithelium in rats (Struillou et al., 2010). The bacterial inoculation and ligatures placed around the teeth can produce periodontal pathology in rats. The ligatures increase plaque retention and produce gingival epithelium injuries, enhancing the local inflammation, osteoclast activation, and bone resorption process (Oz and Puleo, 2011).

Considering the continuous growth and migration of teeth in rats, this model is not the best option for studying the evolution of periodontal disease over extended periods, being widely useful for clinical, microbiological and immunological studies (Struillou et al., 2010).

Numerous biochemical and physiological investigations involving experimental studies that have used *in vitro* tests and animal models and clinical trials have demonstrated the significant effect of periodontal pathogens and their endotoxins on some intrinsic pathogenetic mechanisms, such as systemic inflammation and oxidative stress (Chistiakov et al., 2016).

Given these assumptions, the present study aimed to associate the clinical and microanatomical changes in the periodontium with the systemic inflammatory response in an experimental model of periodontitis in the rat.

Materials and methods

Animals and study design

Experiments were performed on forty male Wistar rats (6 weeks of age, weighing 180-200 g) obtained from the center of experimental animal studies of our university. The study was approved by the Institutional Ethics Committee (Protocol no. 324/2015), and the procedures were performed considering animal welfare and ethics in animal experiments. Acclimatization was made a week before the procedures. During the period of the experiment, the animals were housed in wire cages, under standard laboratory conditions (12 h light/dark alternation and controlled temperature, 22±1°C) with laboratory food and water available *ad libitum*.

The periodontal pathology was induced in rats anesthetized by intramuscular injection of a mixed solution of Ketamine 10% and Xylazine 2% (2:1), 0, 12 ml/100 g body weight. A silk thread (4/0) was placed around the inferior incisors as ligatures in "8". Ligatures were maintained for seven days and clinical signs such as gingival bleeding and swelling, associated with periodontitis were observed daily. At the end of the experiment, the animals were sacrificed, and the samples were prepared for histological evaluation.

Two blood samples of 1.5-2 mL were obtained from each animal by retro-orbital sinus puncture, under general anaesthesia - the first, before applying the ligatures on the first day of the experiment (T1) and the second one, on the 7 seventh day (T2), at the end of the experiment. The blood was stored in tubes with ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. Total blood count was completed within 2 hours of the sampling, and the rest of the blood was centrifuged 10 minutes / 3000 rpm. Plasma was separated and kept in Eppendorf tubes at -20°C, until further use in the immunoassays.

Analytical methods

Clinical investigation

Clinically, we daily assessed the structural changes of the periodontal tissue caused by periodontal disease progression. Thus, we evaluated gingival inflammation by recording some parameters: the colour of the tissues, the consistency, the volume and the bleeding on probing. The presence of bacterial biofilm, puss infiltrate in the sulcus and dental mobility were also recorded.

Laboratory assays

Haematological analysis assessed the following parameters: total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets. We also performed IL-6 plasma concentration measurements.

Blood was analysed using a Sysmex XT-1800i automated hematology analyser (Sysmex Corporation, Japan). The iL-6 assay was performed using a commercial Rat IL-6 ELISA kit (Abbexa, Cambridge, UK).

Histological analysis

The specimens were cleaned and fixed in 10% neutral buffered formalin for 72h. The decalcification was performed in a mixture of 8% formic acid and 8% chlorhydric acid (1/1) for 3 weeks. The samples were sectioned longitudinally and dehydrated through successive baths of Isopropyl alcohol. Then, the samples were clarified in xylene and impacted in paraffin wax (Prophet et al., 1992). Sections of 4 μm thickness were obtained with a rotary microtome (Leica RM2135). Subsequently, these sections were stained with haematoxylin-eosin (H&E) and examined under an Olympus BX41 microscope. The bright field microscopic images were taken with an Olympus UC30 camera and processed using Olympus Stream Basic image analysis software (Ionel et al., 2015).

Statistical analysis

The statistical processing was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and the Microsoft Excel application. The data are presented as mean±standard deviation of the mean (SD). Normal distribution was assessed using Shapiro-Wilk test and p-values <0.05 were considered as statistically significant. The comparison between T1 and T2 for the same parameters was performed with paired Student's t- test for paired samples if data were normally distributed or Wilcoxon signed -rank test if data were not normally distributed.

Results

Clinical aspects

The clinical signs related to periodontal disease progression are illustrated in Fig. 1. On the first day of the experiment, no clinical signs of gingival inflammation were observed among the rats. The gingival tissue had a normal and healthy aspect, smooth-textured and light pink color. The marginal gingiva was firm and tightly bound to the underlying supporting structures. The gingival sulcus appeared as a thin space around the surface of the tooth (Fig. 1a,b).

Clinical signs of periodontitis were recorded on the second day after applying the ligature. The gingiva changed its normal aspect, becoming erythematous and edematous. The irregular surface of the marginal gingiva had an intense red colour. Bleeding on probing was also recorded. These local inflammatory alterations were associated with an early stage of gingivitis (Fig. 1c).

At the end of the experimental period, after seven days, the clinical appearance of the tissues was characteristic of periodontitis. The cyanotic aspect of the periodontal tissue and a marked swelling were observed. The presence of bleeding on probing is an expression of gingival fragility. The surface presented thick and extensive necrotic ulcerations, covered by fibrinoid deposits and a large amount of food debris. An increased teeth mobility accompanied the identified clinical changes (Fig. 1d).

Histopathological aspects – descriptive histology

Representative sections from rats' periodontium, obtained by histopathological analysis demonstrates that the presence of a silk ligature around the lower incisor, in a submarginal position, induced a characteristic microscopic aspect for periodontitis. Intense inflammatory cell infiltration, the presence of osteoclasts and an obvious bone resorption activity were detected (Fig. 2).

The systemic inflammatory response induced by periodontitis

The mean values and standard deviations for haematological parameters (leukocytes, neutrophils, eosinophils, basophils, lymphocytes, monocytes, platelets) and immunological marker (IL-6) were compared both in T1 and T2 (Table 1).

A statistically significant difference ($p<0.001$) was detected regarding the mean values of the inflammatory marker (IL-6) between the two moments of evaluation, T1, and T2. Thus, the average value at T1 (4.84±7.02)

Table 1. Comparative analysis of haematological parameters in rats, before and after periodontal disease induction.

Variables	Values		p
	T1	T2	
IL-6 (pg/mL)	4.84±7.02	19.29±31.00	<0.001
Leukocytes (x 10^3 μL)	6.99±1.87	7.17±2.50	0.71
Neutrophils (%)	24.27±8.62	32.41±14.29	0.003
Eosinophils (%)	1.69±2.56	1.11±1.19	0.78
Basophils (%)	0.01±0.04	0.02±0.05	0.43
Lymphocytes (%)	64.03±10.69	56.77±13.24	0.01
Monocytes (%)	10.03±4.25	10.16±5.21	0.35
Platelets (x 10^3 μL)	953.31±227.21	1175.11±210.85	<0.001

Data are given as mean ± SD.

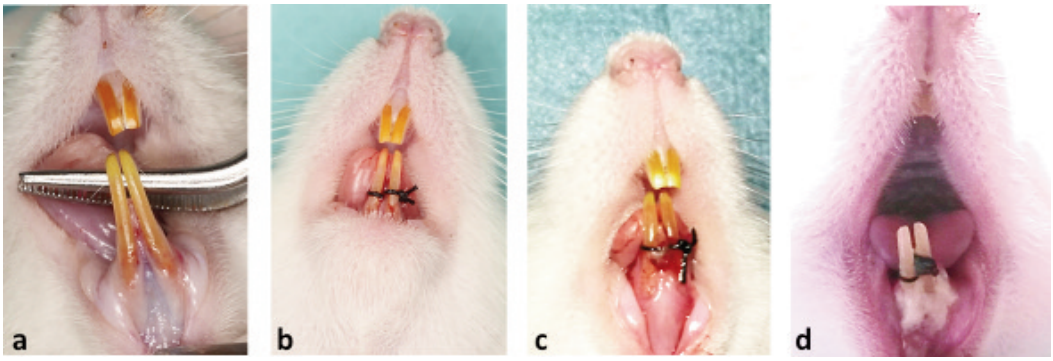

Fig. 1. Clinical aspects of the periodontal tissue of rats; the first day of the experiment - healthy gingival tissue before and after the ligature placement **(a)** and, **(b)**; the clinical changes that suggest periodontal disease progression, on the second day **(c)** and the seventh day **(d)** of the experimental disease induction

was lower compared to that at T2 (19.29±31.00).

No statistically significant difference between T1 and T2 could be observed in the number of total leukocytes (p=0.71). An increase of neutrophils between T1 (24.27±8.62) and T2 (32.41±14.29) was observed, with statistically significant differences (p=0.003). The value of eosinophils decreased from T1 (1.69±2.56) to T2 (1.11±1.19)(p=0.78), while regarding monocytes the values showed a slight increase from T1(10.03±4.25) to T2(10.16±5.21)(p=0.35). There was a statistically significant decrease of lymphocytes in T2 compared to T1(p=0.01). A statistically significant increase in platelet numbers could also be observed from T1 to T2 (p<0.001).

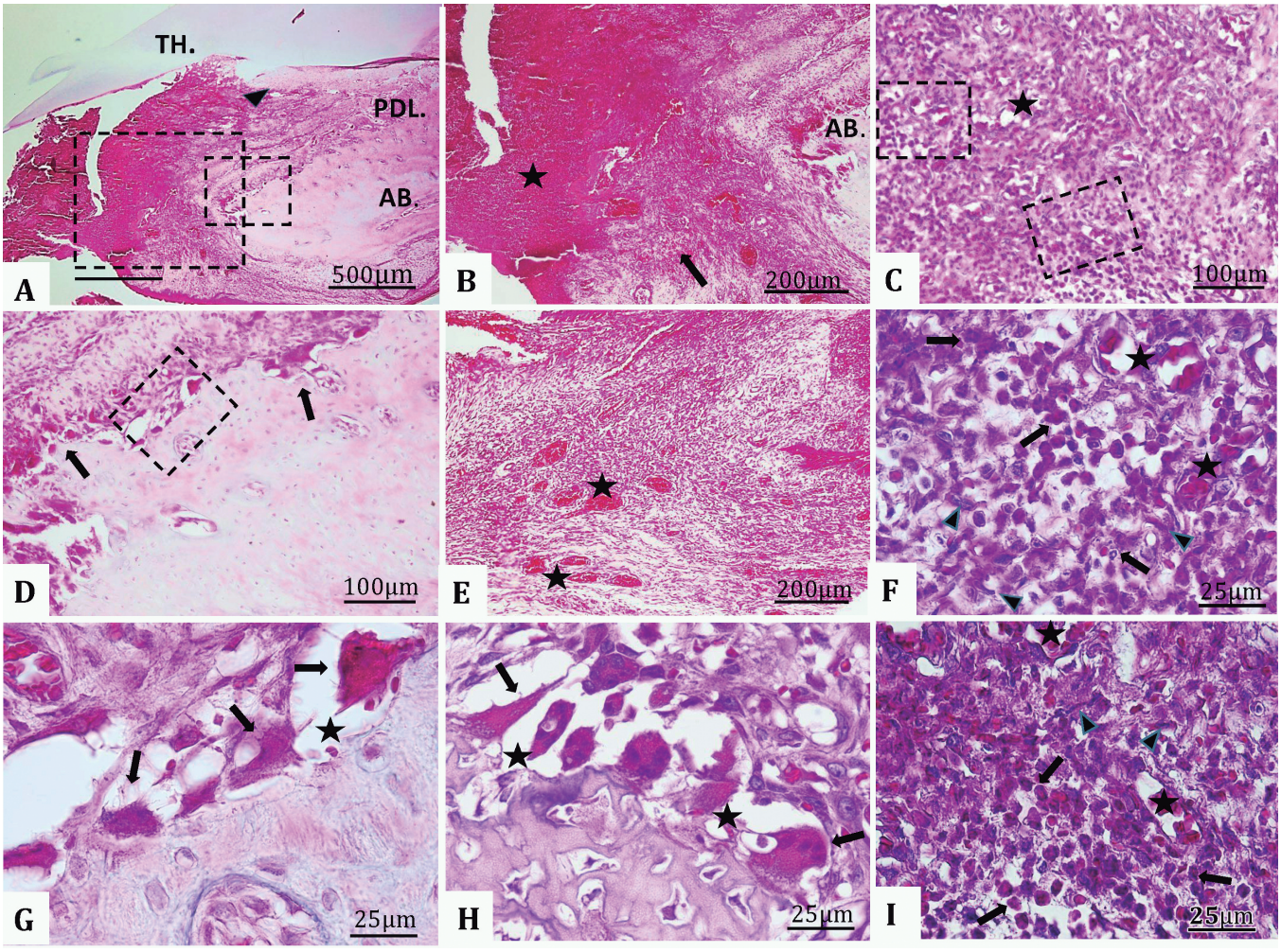

Fig. 2. Histopathological aspects of the periodontal region of rats after periodontal disease induction. **A.** The section of the periodontal space of rat incisor- tooth (TH), periodontal ligament(PDL) and alveolar bone (AB). **B.** This image captures a histopathological detail of the area marked by the large square from figure A, which reveals the presence of a rich mixture of cellular debris and neutrophils (noted by a star). The arrow marks the presence of granulation tissue with an inflammatory character, in different stages of maturity, with numerous leukocytes. **C.** The subjacent region is dominated by the inflammatory granulation tissue; blood vessels with different calibers (star), leukocyte infiltration and a fibrous connective tissue with lax aspect can also be observed. **D.** Detailed aspect of the area delimited by the small rectangle from panel A; a severe alveolar osteolysis was detected characterized by the presence of a border of osteoclasts (black arrow) involved in the bone matrix resorption. **E.** The image presents a histopathological detail of the periodontal ligament; granulation tissue with inflammatory character, pronounced leukocyte infiltration, and numerous congested blood vessels (star) mark the inflammatory process. **F.** This image is a detail of the area demarcated by the upper rectangle from image C; we notice the presence of granulation tissue by the polymorphonuclear inflammatory infiltrate (arrows), young blood vessels (stars), fibroblasts and fibrocytes (arrowheads) and a lax fibrous extracellular matrix. **G.** In this detailed picture of the area delimited by the rectangle from panel D, can be observed a pronounced bone resorption marked by numerous osteoclasts (indicated by arrows) located in the resorption lacunae ("resorption pits")(indicated by stars). **H.** Numerous activated osteoclasts (arrows) are observed in the bone resorption gaps ("resorption pits") (stars). **I.** This image captured a detail of the area demarcated by the lower rectangle from image C; numerous polymorphonuclear cells (PMN) (arrows), fibroblasts and fibrocytes (arrowheads), young blood vessels (stars) in a lax fibrous extracellular matrix characterize the inflammatory granulation tissue. H&E staining.

Discussion

The present study assessed the correlation between clinical signs, histopathological features of periodontitis and the systemic inflammatory response.

The natural occurrence of periodontal disease in rats is not commonly found, so ligature placement around teeth has been suggested to obtain experimental periodontitis (Do et al., 2013). The experimental procedure of inducing periodontitis in rats used in this study is an original and reproducible one and has been confirmed by preliminary research (Ionel et al., 2015). Briefly, the silk ligatures placed around the rats' lower incisors acted as a gingival irritant, favoring bacterial colonization, gingival inflammation, and bone loss process.

Clinically, some characteristic signs of acute inflammation appeared on the second day after the induction of the periodontal pathology. Changes in color, contour and consistency of the gingival tissue and bleeding on probing that we have noticed in rats are similar signs of gingivitis in humans (Carranza et al., 2002).

The main cause of bone resorption in human periodontitis is the extension of inflammation from the marginal gingiva into the supporting periodontal tissues. The inflammation infiltrates from the bone surface, and the initial bone loss that follows marks the evolution from gingivitis to periodontitis (Carranza et al., 2002). The histopathological analysis performed in the present research showed a significant alveolar bone loss in rats after seven days; the data obtained being in accordance with the findings of other previous studies from the literature (de Souza et al., 2011; Graves et al., 2012; Herrera et al., 2015). The determinant factor in causing periodontal disease in rats is the bacterial invasion as in humans, but some assumptions suggested that the mechanical trauma produced by the ligatures could also contribute to the bone loss process (Graves et al., 2008).

An important finding of this study is a significant increase in values of the inflammatory marker (IL-6) during the progression of periodontitis in rats ($p<0.001$). The mean value of Il-6 at baseline (4.84±7.02) was significantly lower compared with that observed at the end of the experiment (19.29±31.00). The association of these values with the clinical and microanatomical aspects which confirm the disease suggests an inflammatory response of the host organism caused by the presence of periodontal pathology.

Our findings are in agreement with other studies, which advanced the hypothesis that periodontitis increases circulating cytokines and inflammatory mediators levels, thereby contributing to the occurrence of systemic diseases, particularly cardiovascular diseases, as an independent risk factor (Libby at al., 2002; Seymour et al., 2009; Chistiakov et al., 2016).

The influence of local inflammation on the host organism homeostasis was also evaluated by counting the number of total leukocytes. In early stages of inflammation, one of the systemic effects is the increased number of total leukocytes, particularly neutrophils. In our study, there was noted a slight rise in the total leukocytes count at the end of the experimental period, but without significant differences from baseline ($p=0.71$). Instead, the neutrophils, as primary defence cells, showed a statistically significant increase associated with the local periodontal lesions ($p=0.003$). These results are consistent with previous evidence according to which the initial inflammatory process, mainly due to the bacterial infection, is characterized by extended neutrophil migration into tissues, to neutralize the pathogens by phagocytosis (Freire and Van Dyke, 2013).

The blood platelets number is also associated with the inflammatory process, being involved in the antibacterial defence in the tissue repair processes. They migrate to the injury site and release substances that regulate the leukocytes' chemotaxis, such as platelet activating factor and macrophage inflammatory protein. Thus, the activated platelets have been proposed as being involved in the pro-inflammatory mediator and receptor activity, followed by the platelets' binding to leukocytes and endothelial cells (Kamath et al., 2001). The results of our research indicate a higher mean platelet count due to the periodontal pathology induction (953.31±227.21) compared with baseline (1175.11± 210.85). The detected differences were found to be statistically significant ($p<0.001$). This is in agreement with previous studies which reported that the platelets level and activation is higher in the periodontal disease evolution (Shi et al., 2008; Nicu et al., 2009).

The increased level of systemic inflammatory markers has been associated with the local periodontal inflammatory process, although they are not distinctive for periodontal disease, making it difficult to determine the distinct contribution of periodontitis in the occurrence of systemic disorders. While most research shows a positive relationship between periodontal disease and heart diseases, there are still some inconsistencies, requiring the development of further studies in this direction of research to support the assumptions outlined above.

The ligature-induced periodontitis model has some limitations and cannot reproduce all aspects of human disease. However, experimental animal models represent an indispensable link between hypotheses and human patients (Graves et al., 2012). Despite the limits of this study, we could highlight that the inflammatory alterations of the periodontium, achieved through this experimental protocol, had a significant impact on the general condition of the rat's organism.

Considering the increased prevalence of chronic periodontitis and cardiovascular diseases in recent years, and based on the systemic involvement of periodontal status, the preventive measures for these disorders should be a priority in public health programs. As a holistic approach in heart disease control, attention must also be focused on the independent risk factors, such as

periodontal disease. Thus, extrapolating the findings of this study, we support that periodontal therapy can be inserted as a part of the preventive cardiovascular programs, in clinical practice.

In summary, the results of this research support the hypothesis of periodontal inflammation resolution on systemic conditions, proving that the clinical and histopathological changes in rat's periodontium are correlated with a notable systemic inflammatory response.

Conflict of interest. The authors declare no conflict of interest.

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