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Increased serum interleukin 6 in experimental periapical periodontitis associated with metabolic bone disease

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Abstract

Background. Inflammatory cytokines such as interleukin 6 (IL-6) have implications in the pathogenesis of metabolic bone disorders and also in periapical disease. Both metabolic bone diseases and periapical periodontitis (PP) share a common feature represented by inflammation-mediated bone loss; however, the specific role of these cytokines is not always consistent.

Aims. The aim of the study was to assess the serum IL-6 levels and investigate the associations with histomorphometric changes in the alveolar bone around the molar roots in a rodent experimental model of PP.

Methods. The research was conducted on 3 groups of animals: group A – animals without any pathology, group B – animals with PP, and group C – animals with PP and simulated metabolic bone disease treated with alendronate (ALD). Peripheral blood was collected for biochemical analysis of IL-6 interleukin levels and the lower jaws were removed for histomorphometric analysis.

Results. Serum IL-6 levels were significantly increased in the experimental groups compared to the control group ($p < 0.05$). In group B, a moderate inflammatory infiltrate and a medium number of osteoclasts were present, while in group C, discrete inflammatory infiltrates accompanied by a low number of osteoclasts were confirmed by histomorphometric analysis.

Conclusions. The induction of PP determines elevated circulating levels of IL-6 with repercussions on the systemic state, maintaining a low degree of inflammatory response. The administration of ALD in female rodents contributes to the diminishing rate of extension in PP, limiting the destruction of the tissues.

Keywords: interleukin 6, periapical periodontitis, bone metabolism, estrogen deficiency.

Introduction

Periapical periodontitis (PP) is characterized by an inflammatory response of the periodontium at the root apex of teeth and concurrent progressive alveolar bone destruction as a consequence of pathogenic bacteria persistence in the endodontic biofilms (Nikolic et al., 2019; Romualdo et al., 2018). The supporting tissues of the teeth including alveolar bone, periodontal ligament and cementum are all affected during the development stages of the periapical disease.

Under physiological conditions, the complex remodeling process that appears in bone is controlled by the balance between bone formation by osteoblasts and degradation by osteoclasts. The main factor responsible for this activity is the receptor activator for NF-κB ligand (RANKL)/ osteoprotegerin (OPG) ratio, expressed by the bone cells (Blaschke et al., 2018; Al-Daghri et al., 2017). Osteoblasts are responsible for the construction of bone material by generating collagen, osteopontin and osteocalcin, and transforming them into an osteoid matrix. Then, the mineralization of the newly formed matrix is enhanced by calcium phosphate, hydroxyapatite and calcium carbonate. Osteoclasts manifest enzymatic activity contributing to the degradation of the bone matrix through resorption pits filled with acidic proteases (McDonald et al., 2017; Harmer et al., 2019).

Under pathological conditions, the bone cells release various inflammatory cytokines, such as interleukin 6 (IL-6) which plays a major role in the development of metabolic bone diseases (Hunter & Jones, 2015;

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Schet et al., 2018; Eftekhari et al., 2018) and in the progression of periapical pathology (Balto et al., 2001; Azuma et al., 2014). IL-6 cytokine acts by reducing bone mineral density, affecting normal bone metabolism, and is assumed to have implications associated with osteoporosis (OP) (Yan et al., 2015; Kany et al., 2019; Saribal et al., 2019; Ding et al., 2008; Li et al., 2016) through different pathways: increasing osteoclast differentiation, activation, survival, enhancing RANKL expression and inhibiting osteoblast survival (Al-Daghri et al., 2017; Harmer et al., 2019). The uncoupling of the mechanism behind bone formation and resorption may induce metabolic bone diseases, including OP. The reduction of estrogen levels due to the loss of ovarian function in postmenopausal women determines bone loss and OP (Harmer et al., 2019; Eftekhari et al., 2018). Experimental studies investigating the relationships between hypoestrogenic conditions induced by ovariectomy and bone changes showed a decrease in mineral bone density of jaws in rodents (Romualdo et al., 2018) and also, structural modifications in trabecular alveolar bone (Tanaka et al., 2003; Duarte et al., 2004).

Pharmacological agents including alendronate (ALD), neridronate, ibandronate and other similar compounds are the primary drugs currently used in the treatment of metabolic diseases (Raj et al., 2016). These drugs exert effects on osteoclast activity, inhibiting bone resorption by lowering osteoclast numbers and influencing the RANKL/OPG ratio, as well as on osteoblasts.

Hypothesis

This study aimed to assess the serological modifications of IL-6 levels and also, to investigate the associations between serum IL-6 values and histomorphometric changes in the alveolar bone around the molar teeth roots in an experimental model of PP.

Material and methods

Research protocol

a) Period and place of the research

The present study received the approval of the Ethics Committee of the "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca. The experimental protocol was in agreement with the ethical research principles regarding the handling of laboratory animals, and took place between January and June 2016 in the Experimental Laboratory of the Physiology Department of the "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca.

b) Subjects and groups

A total of thirty female rodents (Rattus norvegicus) with a mean age of 3 months and a weight ranging between 250 g and 280 g were obtained from the Centre of Experimental Medicine of our University.

The study groups (10 animals / group) comprised:

- group A - rodents without any pathology;
- group B and group C, in which occlusal cavities in the lower molar teeth with dental pulp chamber opening were created, using a round steel bur, carefully avoiding furcal perforations. Group C was also subjected to the surgical procedure of ovariectomy, according to the technique described by Khajuria et al., 2012, following which bone metabolism deficiencies were induced as a result of the reduction of estrogen levels. After 10 days postoperatively, alendronate sodium treatment was administered in group C by subcutaneous injection, in two weekly doses of 1 mg/kg body weight until the end of the experiment.

c) Tests applied

For evaluation at different moments of the experiment, peripheral venous blood was collected at 2, 4 and 8 weeks in test tubes, centrifuged at 4°C for 15 min and preserved at -20°C until biochemical analysis. The quantitative detection of IL-6 in serum samples was performed using Rat IL-6 ELISA kit (Boster Biological Technology Co., Ltd.), according to the manufacturer's instructions.

At the moments mentioned above, the animals were sacrificed and their lower jaws were excised in order to perform histomorphometric investigations. The specimens were fixed by immersion in 10% buffered formalin for 24 h and preserved in 4% EDTA solution until complete decalcification. After paraffin embedding, the blocks containing the molar teeth were longitudinally sliced along their axis, resulting in 5 μm thick serial sections. They were stained with hematoxylin and eosin (HE) and subjected to light microscopy for evidencing the apical and periapical regions of the molar teeth. The inflammatory cellular infiltrate and the degree of bone loss were investigated histomorphometrically by quantification of the inflammatory cells and osteoclasts present in the perimeter of the analyzed regions.

d) Statistical processing

The data of this study were statistically analyzed using Starts Direct v 2.7.2 software and Excel (Microsoft Office 2010).

Results

The analysis of serum IL-6 levels considering the three groups of rodents at different experimental moments and their statistical significance were presented in Table I.

Table I

Comparative analysis of the studied groups and their statistical significance.

Moments	Group	Mean ± SD	Statistical significance (p)			
2w	A	2.82 ± 0.18	A-B	0.0020	2w-4w	0.0038
	B	2.50 ± 0.23	A-C	$8.14x10^{-5}$	2w-8w	$2.17x10^{-6}$
	C	5.70 ± 1.33	B-C	$1.98x10^{-5}$	4w-8w	$8.97x10^{-5}$
4w	A	2.82 ± 0.18	A-B	0.5639	2w-4w	0.002
	B	2.95 ± 0.40	A-C	5.52×10^{-12}	2w-8w	0.1641
	C	6.84 ± 0.44	B-C	$6.29x10^{-14}$	4w-8w	0.3482
8w	A	2.82 ± 0.17	A-B	$3.21x10^{-4}$	2w-4w	0.0309
	B	4.02 ± 0.43	A-C	0.0020	2w-8w	0.8526
	C	5.85 ± 2.22	B-C	0.0284	4w-8w	0.2364

The statistical analysis of IL-6 values evidenced the following for unpaired samples:

- at 2 weeks - highly statistically significant differences between groups A and C ($p< 0.001$) and also between groups B and C ($p<0.001$);
- at 4 weeks - similar results to those obtained at 2 weeks;
- at 8 weeks - very statistically significant differences between groups A and C ($p<0.01$) and also between groups B and C ($p<0.05$).

The statistical analysis of IL-6 values showed the following for paired samples:

- in group B - highly statistically significant differences between weeks 2-8, weeks 4-8 ($p< 0.001$) and very statistically significant differences between weeks 2-4 ($p<0.01$);
- in group C - statistically significant differences between weeks 2-4 ($p<0.05$).

Regarding the descriptive histological analysis of tooth sections, in group A, tooth sections showed no inflammation or bone resorption (Figs. 1A, C). Group B, at 2 weeks, showed PP in acute stages with a higher intensity, necrosis of periodontal ligament, resorption of mineralized tissues (bone and radicular cement) (Fig. 1B). At 4 weeks, there was a predominantly moderate inflammatory infiltration of mononuclear cells (macrophages, lymphocytes) and greater bone loss, and at 8 weeks, the intensity of PP decreased, chronic inflammatory cell infiltrate was present and bone destruction was stabilized. In group C, PP appeared at 2 weeks, with inflammatory infiltrate mainly consisting of neutrophils and macrophages. Also, reduced alveolar bone resorption was observed. Frequently encountered, dental pulp lesions were represented by septic pulp necrosis. In parallel, a conjunctive reaction was observed with the multiplication of fibrous tissue (Fig. 1D). At 4 weeks, the same lesions were present, specifically bone loss, pulp necrosis, desmodontal space widening and fibrous tissue multiplication, and at 8 weeks, PP changes were identical to those previously mentioned, but their intensity was more reduced.

The statistical analysis of inflammatory infiltrate evidenced the following for unpaired samples:

- at 2 weeks – very statistically significant differences between groups B and C ($p<0.01$);
- at 4 weeks – no statistically significant differences between the groups of rodents;
- at 8 weeks – highly statistically significant differences between groups B and C ($p<0.001$).

The statistical analysis of inflammatory infiltrate evidenced the following for paired samples:

- in group B – no statistically significant differences between the time moments ($p>0.05$);
- in group C – statistically significant differences between weeks 2-4 and weeks 4-8 ($p<0.01$).

The statistical analysis of alveolar bone resorption evidenced the following for unpaired samples:

- at 2 weeks – very statistically significant differences between groups B and C ($p<0.01$);
- at 4 weeks – highly statistically significant differences between groups B and C ($p<0.001$);
- at 8 weeks – statistically significant differences between groups B and C ($p<0.01$).

The statistical analysis of alveolar bone resorption evidenced the following for paired samples:

- in group B – no statistically significant differences between the time points ($p>0.05$); in group C - statistically significant differences between weeks 4-8 ($p<0.01$) and weeks 2-4 ($p<0.05$).

In group B, IL-6 levels were positively correlated with the number of osteoclasts, having the correlation coeficient $r= 0.5522$, and with the inflammatory infiltrate, $r= 0.5618$, at 2 weeks, and negatively correlated with the inflammatory infiltrate at 8 weeks, $r= - 0.8528$. In group C, IL-6 was negatively correlated with the number of osteoclasts, $r= - 0.8381$.

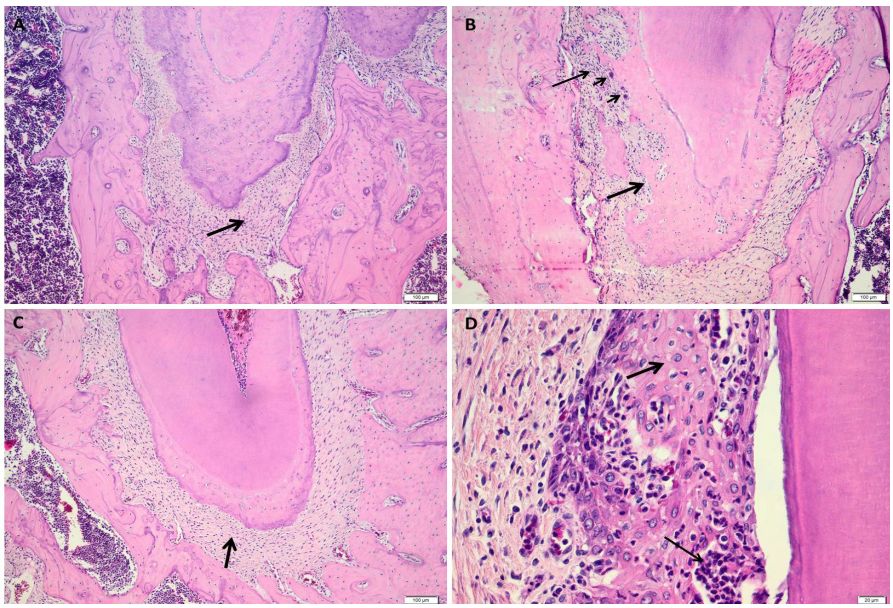

Fig. 1 – **A, C**. Periodontal space and apical regions with normal aspect (arrows), H&E x 100; **B.** Cementum and bone resorption (thick arrow), osteoclasts (short arrows), H&E x100; **D.** Periodontal inflammatory infiltrate (thin arrows), epithelialization (thick arrows), H&E x 400.

Discussions

Both PP and OP are pathological conditions affecting bone, with an imbalance in the turnover process. PP is a localized process reflected in alveolar bone (Azuma et al., 2013), whilst OP manifests as a metabolic dysregulation in the entire skeleton with the excessive activity of osteoclasts. In the case of PP, the locally accumulated inflammatory cells in the affected area generate the cytokine and inflammatory mediators, acting as causative factors in alveolar bone destruction, concomitantly with the hindering of the repair or formation of new bone. In the case of OP, a longer time period is needed for the emergence of structural modifications in cancellous bone and the involved mechanisms have a greater complexity (Mundy et al., 1993).

The available literature emphasizes the role of bone cells in maintaining bone homeostasis (Jeong & Kim, 2016; Schett, 2018; Metzger & Narayanan, 2019). The activity of these cells influences the equilibrium between bone formation and resorption. The major implication of osteoclasts in bone loss is well known: their activation following various pathological conditions determines an increased production of inflammatory cytokines, such as IL-6 (Kany et al., 2019; Eftekhari et al., 2018). A recent study observed a high risk of bone resorption in postmenopausal women with OP as a consequence of estrogen deficiency and release of IL-6 cytokine activating osteoclasts through RANKL (Al-Daghri et al., 2017).

The current study revealed that serum IL-6 levels of ovariectomized rodents treated with ALD were increased in all stages when compared with control and PP groups. These results are in agreement with other studies reporting elevated concentrations of inflammatory cytokines in peripheral blood associated with PP (Zhang et al., 2015; Georgiou et al., 2019).

PP and OP are characterized by bone loss coinciding with increased IL-6 secretion. In an endodontic context, IL-6 acts as an important mediator in modulating the response to bacterial infection (Nibali et al., 2012), being synthesized locally by bone cells (Balto et al., 2001), and reflected later in systemic peripherical blood (Georgiou et al., 2019). It is also known as a regulatory factor in the differentiation activity of osteoblasts and osteoclasts (Xie et al., 2018; Yan et al., 2015; Harmer et al., 2019). IL-6 overexpression may be linked to the extent of chronic inflammatory lesions, resulting in alveolar bone resorption and damage to the periodontal ligament (Nibali et al., 2012).

The pathological role of IL-6 as a mediator in bone resorption has been subjected to controversy over the last decades. Clinical and experimental studies investigating IL-6 expression report the release of this cytokine from osteoblasts (Xie et al., 2018; Yan et al., 2015), with a pivotal role in the formation of osteoclast precursors (Balto et al., 2001; Romaldo et al., 2018), leading to an increased number of osteoclasts and activating the bone resorption pathways (Ji et al., 2019; Harmer et al., 2019). Other studies addressing the impact of IL-6 in the pathogenesis of PP found high IL-6 levels correlated with the extent of tissue damage (Abdolsamadi et al., 2008). Cintra et al., 2016 found elevated serum levels of IL-6 in a rat model of apical periodontitis. Bresil et al., 2017 suggested that prolonged estrogen deficiency influences the progression of apical periodontitis, causing greater lesions in ovariectomized rats, due to the persistence of the inflammatory process in the bone. Inflammation-induced osteolysis proved to be present in both postmenopausal OP and periapical diseases, as a possible systemic damaging factor (Lopez et al., 2015; Romualdo et al., 2018). A recent study conducted on ovariectomized rodents with induced PP showed more elevated levels of proinflammatory cytokines, hypothesizing the role of estrogen activity in post-menarche women with apical disease (Romualdo et al., 2018). The property of bisphosphonates to bind the crystals of hydroxyapatite makes them useful in the treatment of certain pathologies associated with increased bone resorption, such as OP (Xiong et al., 2010).

According to the literature, little evidence concerning the impact of PP is available. Xiong et al., 2010 focused on the effects of bisphosphonate administration on alveolar bone resorption, using one of the most efficient agents, alendronate, in experimentally induced PP. Their study suggests the possible implication of alendronate in the inhibition of osteoclast activity. The reduction of bone damage in PP was observed in ovariectomized rats as a consequence of this therapy, suggesting the possible protective role of these agents (Wayama et al., 2015) in the inhibition of osteoclastogenesis. Our study subscribes to these previous findings regarding the effects of bisphosphonates on the development of PP.

Concerning the histological analysis of the periapical area in the present study, moderate inflammatory changes in animals with induced PP were detected when compared to reduced inflammatory changes in ovariectomized animals treated with alendronate. A moderate number of osteoclasts in direct contact with the alveolar bone surrounding the affected area in PP animals was observed when compared to the low number of osteoclasts in treated ovariectomized animals. Future studies are needed in order to elucidate the complex mechanisms involved in the pathogenesis of PP, with focus on the implications of cytokines in inflammatory alveolar bone alterations.

Conclusions

1. Elevated circulating IL-6 levels were identified in association with alveolar bone resorption in the experimental model of PP.

2. A negative correlation was observed between bone loss and IL-6 values in PP and simulated metabolic bone disease, probably due to the action of ALD in diminishing the rate of alveolar destruction.

Conflicts of interest

Nothing to declare.

Aknowledgements

The article exploits the data from the first author's doctoral thesis, defended in 2016, at the Iuliu Hatieganu University of Cluj-Napoca.

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