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Evaluation of osteoclasts' distribution on the periodontal ligament of rabbits

Avaliação da distribuição de osteoclastos no ligamento periodontal de coelhos

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Abstract
Objective: *assess the distribution and quantity of osteoclasts on periodontal ligament of rabbit's molars in physiological conditions.* ***Methodology:*** *one hundred and seventy-six histological sections were analyzed, obtained through bone blocks removed from the first molar regions on both sides of the mandible of 44 rabbits. Sections were stained with TRAP technique.* ***Results:*** *osteoclasts were found in 53,98% of the slides and were absent in 46,02%. These cells were usually located on the cervical and median third. Non-parametric Friedman Exact test evidenced a statistically significant difference between the thirds, and Dunn's a Posteriori test indicated such difference between apical and cervical thirds and apical and median thirds (p<0,05).* ***Conclusion:*** *physiological re-modeling of the alveolar bone occurs when stress generated is sufficient to induce monocytes differentiation in osteoclasts. These cells are more concentrated on the cervical and median thirds due to the direction of the dental movement.*
Keywords: *Osteoclasts. Periodontal ligament. Bone Remodeling .*

Resumo
Objetivo: avaliar a distribuição e a quantidade dos osteoclastos no ligamento periodontal de molares de coelhos em condições fisiológicas. **Metodologia:** foram analisadas 176 secções histológicas obtidas através de blocos de osso removidos da região de primeiro molar de ambos os lados da mandíbula de 44 coelhos. As secções foram coradas pela técnica de TRAP (Fosfatase ácida tartarato-resistente). **Resultados:** na análise dos resultados, em 53,98% das lâminas não havia osteoclastos, enquanto que em 46,02% a presença desta célula foi detectada. Quando presentes localizaram-se mais nos terços cervical e médio. O teste não-paramétrico Exato de Friedman evidenciou diferença estatisticamente significante entre os terços e o teste a Posteriori de Dunn indicou essa diferença entre os terços apical e cervical, e apical e médio (p<0,05). **Conclusão:** conclui-se que o remodelamento fisiológico do osso alveolar acontece quando o stress gerado sobre ele é suficiente para induzir a diferenciação de monócitos em osteoclastos. Estas células concentram-se mais nos terços cervical e médio devido à direção do movimento dentário.
Palavras-Chave: Osteoclastos. Ligamento periodontal. Remodelação Óssea.

INTRODUCTION

The periodontal ligament is a well vascularized and innerved tissue, which hosts osteoblasts and osteoclasts among various cells[1,2]. Osteoclasts are stable multinucleated cells whose origin is related to hematopoietic progenitor cells of the endothelial reticulum system. They are found on the absorption sites or at the Howship's lacunae[3]. These cells are responsible for bone absorption, and therefore, release enzymes capable of acidifying the micro-environment, making the tissue soluble and digesting matrix proteins. As a result, growth factors previously deposited by osteoblasts which are dissociated at the matrix are released from the tissue[1,2].

The cytokines which are crucial to the differentiation and maturation of monocyte derived osteoclasts are the interleukins IL-1, IL-3, IL-6, IL-11, tumor necrosis factor (TNF), granulocytes macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and Kappa B nuclear factor (RANK-L). In physiological conditions, osteoblasts and macrophages are the main cells in osteoclastogenesis. In inflammatory conditions, T-helpers cells are responsible for secretion of cytokines which stimulate osteoclasts and other immunological cells differentiation[4,5,6].

Considering the importance of osteoclasts in bone metabolism and the need to better understand its physiological mechanisms, the purpose of this work was to assess

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the distribution and quantity of these cells in the periodontal ligament of rabbit's first molars.

MATERIALS AND METHODS

The protocol of this work was in accordance to the Ethics Committee for Use of Animal of Bahiana School of Medicine and Public Health in Salvador, Bahia, Brazil, under protocol number 02/2006. Forty-four adult albino male rabbits, weighing between 2Kg and 3 kg were used. A bone fragment was obtained from each side of the animals' mandibles, containing the first inferior molar.

Procedures were conducted under general anesthesia. For cardiovascular protection, atropine (2mg/kg) was administered. Sedation, analgesia and muscular relaxing were achieved with acepromazine (1mg/kg) and ketamine (10mg/kg). General anesthesia was confirmed by the abscense of palpebral reflex or animal reaction on non invasive procedures during the preparation for surgery. After shave and asepsis, bupivacaine 0,5% with 1:200.000 adrenaline, was infiltrated in the surgical beds. Mandible section, containing the first molar, was removed with a 701 bur. Euthanasia was conducted under general anesthesia with excessive administration of the anesthetic medicine.

Tissues were decalcified in EDTA and embedded in paraffin, on such way, that the vestibular and lingual apex of the inferior molar root, as well as both corticals of its alveolar process could be observed. Two sections of 5 µm of each block were stained by the TRAP (Tartar Resistant Acid Phosphatase) technique. Through this technique osteoclasts stain in red. Slides were counterstained with hematoxylin.

One hundred and seventy-six histological sections were obtained. Each one of them was carefully examined by two calibrated examiners. Under 40X magnification on light microscopy osteoclasts were quantified in each one of the radicular thirds in which they were present.

A descriptive analysis was performed (absolute/relative frequency, median, maximum and minimum, first and third quartiles) aiming at identifying the general and specific characteristics of the studied sample. In order to verify the existence of significant differences between the thirds, cervical, median and apical, non-parametric Friedman Exact test was used, followed by Dunn's a Posteriori test. The level of significance adopted was 95%. Correlation between the thirds was measured by Spearman´s test, at a 95% significance level.

Results were presented in a descriptive manner and through comparative tables.

RESULTS

After detailed reading of all analyzed sections in every animal, it was possible to evidence that 08 (18,2%) rabbits did not present osteoclasts in their periodontal ligaments. In the animals where osteoclasts were observed (Figures 1 and 2), these cells could be found on one first molar in 19 (43,2%) rabbits and on both first molars in 17 of them (38,6%) (Figure 3). Table 1 shows the distribution of frequency and percentage values of osteoclasts on periodontal ligament on the total histological sections studied. From the 176 sections analyzed, 81 (46,02%) sections presented osteoclasts on the periodontal ligament, whereas 95 (53,98%) there was none.

Through the analysis of the quantity of osteoclasts in each radicular third, cervical, median and apical, the median third showed the highest quantity of these cells, whereas the apical third had the lowest quantity (Figure 4). There was a statistically significant difference between the apical and cervical thirds, and the median and apical thirds when compared to each other ($p<0,05$) (Table 2). When analysis was performed considering the combinations of radicular thirds, it was verified that they were more frequently situated on the cervical and median thirds simultaneously. No osteoclasts appeared when cervical and apical thirds were studied together (Figure 4).

The best correlation was observed between apical and medium thirds, $r=0,7432$ $p<0,0001$, (Figure 5) followed by the association between cervical and medium thirds, $r=0,0557$ $p<0,0001$, (Figure 6). Considering that no osteoclast was found at the apical and cervical when they were analyzed together, the correlation between these thirds has no biological plausibility.

***Figure 1**. Osteoclast presentation (arrows) at periodontal ligament of radicular third. TRAP X100.*

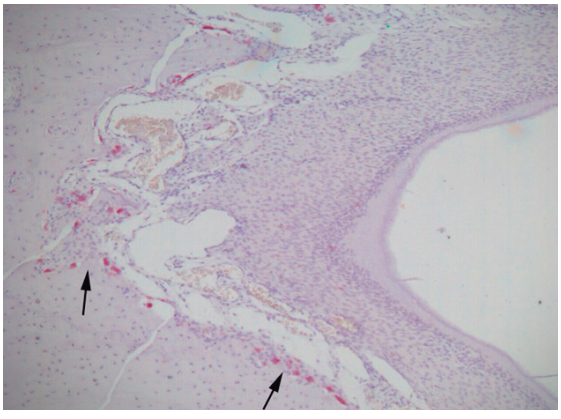


***Figure 2**. Osteoclasts occupying Howship's lacune at the alveolar bone. TRAP X400.*

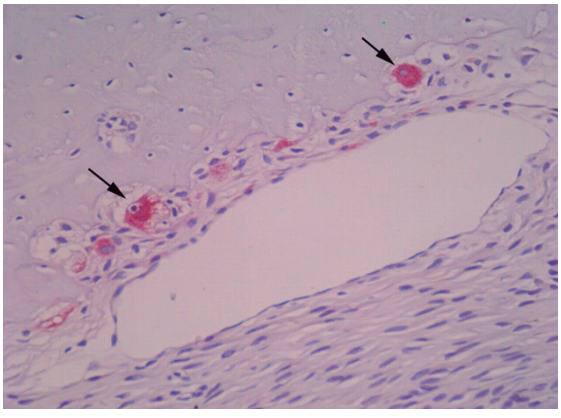


***Figure 3**. Osteoclasts distribution at the molar of the rabbit.*

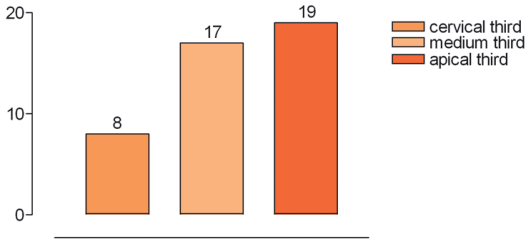


***Table 1** Frequency of osteoclasts at the total histological sections analyzed.*

Osteoclasts	*Frequency*	*(%)*
No	95	53,98
Yes	81	46,02
Total	***176***	***100,0***

***Figure 4**. Distribution of osteoclasts at radicular thirds and at the combination of them.*

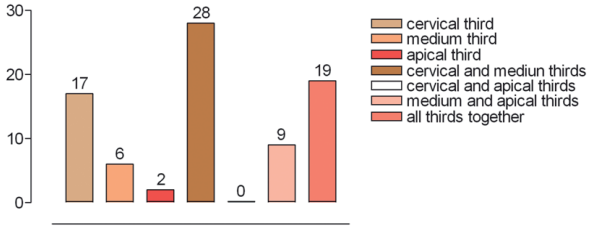


***Table 2.** Analysis of osteoclasts distribution at radicular thirds.*

Group	*Median*	*(Minimum -Maximum)*	*(Q1 -Q3)*	*p*
Cervical [1,3]	5,0	(0 - 80,0)	(3,0 – 16,0)	
Median [2,3]	7,0	(0 - 115,0)	(1,0 – 12,0)	< 0,001*
Apical [1,2]	0	(0 - 62,0)	(0,0 – 6,00)	

** Friedman Exact Test*

[1,2]Divergent Pairs; [3]Congruent Pairs - p<0,05 – Dunn's Posteriori Test

***Figure 5**. Correlation between presence of osteoclast at the apical and medium third. Spearman´s test, 95% significance level.*

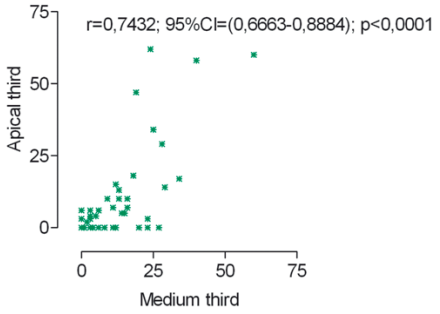
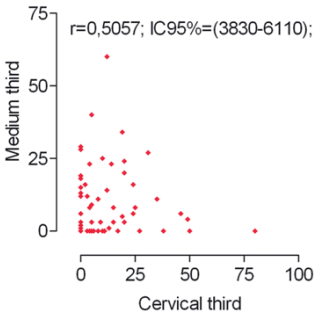


***Figure 6**. Correlation between presence of osteoclast at the medium and cervical third. Spearman´s test, 95% significance level.*



DISCUSSION

The knowledge about the osteoclastogenesis mechanism is important for the understanding of physiological bone absorption. Literature lacks studies that demonstrate the behavior of these cells, as well as of cytokines and growth factors that induce the differentiation of monocytes in osteoclasts. The present work, therefore, offers a significant knowledge of these cells biology and confirms that its presence on tissues will be evident on areas where metabolism is active, due to all the different processes that impel bone remodeling.

The majority of rabbits from this research presented osteoclasts in at least one tooth, confirming the relevance of these cells on the physiology of the periodontal ligament and surrounding bone. However, in relation to the frequency of osteoblasts on the periodontal ligament, the sections that did not present osteoclasts were more numerous.

This suggests that physiological stress, on regions where osteoclasts were absent on the periodontal ligament, was not sufficient to induce differentiation of monocytes onto these cells. This result disagrees from the opinion of other authors who affirmed that the alveolar process is in constant remodeling, regardless on the amount of stress generated[1,2]. However, a stronger tension on the periodontal ligament is necessary in order to induce osteoclastogenesis in the entire radicular perimeter[7,8,9].

Regions where osteoclasts were more concentrated, after being impelled to physiological remodeling, were cervical and median thirds, together. This suggests micro-movement direction that the first inferior molars were impelled to make during the chewing stress of these rodents. According to the results, the fulcrum point of these teeth during chewing was probably at the radicular apex, whereas the amplitude of displacement was more intense on the cervical and median thirds, which intensifies the metabolism at the region. Based on the inferences made by Andrade et al[3], the presence of osteoclasts on bone tissue is directly dependent on the intensity of this metabolism.

As soon as stress occurs on the periodontal ligament, RANK-L is secreted by osteoblasts and macrophages, and acts on RANK receptor found on the cellular surface of osteoclasts progenitor cells[10,11,12,13,14,15,16,17,18,19]. The RANK-L/RANK pathway seems to be the main trajectory responsible for the regulation of osteoclastic differentiation, however it is known that the activation of the osteoclast does not solely depend on RANK's activation[20,13,14]. Cytokines also act on the activation of cells responsible for the absorption of the alveolar bone, such as macrophage and granulocyte-macrophage colony-stimulating factor (M-CSF and GM-CSF), interleukins (IL-1 and IL-6), tumor necrosis factor alfa (TNF-alfa) and prostaglandin (PGE)[10,21,22,15,23].

Based on the findings reported here, it is possible to infer that the synthesis and concentration of these factors were more intense on regions where precursors of osteoclasts were more widely recruited, that is, on the median and cervical thirds. It is possible to affirm that this process is not uniform in the whole periodontal ligament, where areas of higher concentration of these molecules co-exist with areas of lower periodontal metabolism. On bone metabolism, the IL-1 dependent release of RANK-L, is balanced by the synthesis of osteoprotegerin, which outlines tissue's homeostasis[15,17]. Under the same conditions, osteoblasts and macrophages are the main inducers of osteoclastogenesis[14,16,24,25]. If the stress generated on the bone is not enough to induce the action of these cells on osteoclasts precursors, the alveolar bone will not undergo physiologic remodeling. Under these circumstances, osteoclastogenesis would be unnecessary, since tissue's metabolism this would be balanced and remodeling would represent an irrelevant energetic cost for the organism, as it was observed in some rabbits and on most part of the analyzed sections.

Among the local inhibitory mediators of the absorptive process that obstructs the spontaneous osteoclastic differentiation, there are osteoprotegerin, IL-10 and IL-18, interferon-gama (IFN-gama) and transforming growth factor beta (TGF-b) [3,15,17,21,25]. It is possible to suppose that, on periodontal ligament, the activity of these cells is more important to bone remodeling homeostasis. Their functions contradict the activity of those that induced osteoclastogenesis and could promote bone loss with no apparent clinical reason.

Medium and apical thirds, at rabbit's molars, operate together in the process of dispersion of the forces, originated during mastication, for the alveolar bone ridge. Since the force transmitted by the mastication for that tooth is equally absorbed by those two thirds, possibly the fulcrum, of the micro-movements that occur at this unit during the chewing effort locates between both. Considering, that the cervical third is placed more distant from the fulcrum than others, the dispersion of forces on the alveolar bone ridge in that area is more intense during these moments[26,27,28]. Therefore, it will present a larger amount of osteoclasts and in less congruous way with the other thirds.

CONCLUSION

Based on the results obtained, it is possible to conclude that the osteoclasts will be present on the periodontal ligament, in physiological conditions, when the stress generated is sufficient to stimulate monocyte differentiation. Under these conditions, its distribution on the periodontal ligament is not uniform and will be present in a higher concentration on the cervical and median thirds, due to the direction of dental movement during chewing activity.

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