Journal of Oral Biology and Craniofacial Research 8 (2018) 54–57

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Original Article

Presence of mast cells and the expression of metalloproteinase 9 in the gingiva of ovariectomized rats with periodontal disease

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ARTICLE INFO

Article history:
Received 9 June 2017
Accepted 12 October 2017
Available online 14 October 2017

Keywords:
Periodontitis
Estrogen deficiency
Mast cells
MMPs

ABSTRACT

Background: The host's answer has an important role in periodontal disease, and the mast cells have a prime role. Such cells seem to be influenced by estrogen deficiency. The objective was to evaluate the mast cells and the expression of metalloproteinase(MMP)-9 in periodontal disease induced in ovariectomized rats.

Methods: For that purpose, 36 rats were used; 18 ovariectomized (OVX) and another 18 Sham-operated (SHAM). After 60 days the periodontal disease was induced by a ligature around the first lower right molars (group P). The opposite side was the control group (group C). The euthanasia occurred 3, 7 and 14 days after the placement of the ligature. The gingiva was removed and analyzed histochemically and immunohistochemically to quantify the mast cells and to analyze MMP 9 expression.

Results: By comparing the groups SHAM-P and C and groups OVX-C and P, it was noted that mast cells from group C were higher than P in all experimental periods. When comparing groups SHAM-C and OVX-C, significant factors were not found. When comparing groups SHAM-P and OVX-P, there was an inclination for mast cells reduction with time. The MMP-9 expression was related to the presence of periodontitis.

Conclusions: It was concluded that periodontitis led to mast cells reduction and MMP-9 increase. The ovariectomy itself did not alter the MMP-9 expression and did not influence the presence of mast cells in rat papilla, however, when associated to inflammation led to a reduction of mast cells.

1. Introduction

The mast cells are widely spread over the tissues, next to blood vessels and nerves, and also in subepithelial area. The mast cells cytoplasm contains membrane-linked granules that have several biological functions. The mast cells play a part in many events during the inflammation process[1] and have been widely studied due to their contribution in tumor angiogenesis[2] and participation on periodontal disease pathogenesis.[1,3]

The mast cells are important inflammatory cells because of their cytoplasmic granules that contain histamine, slow-reacting substance of anaphylaxis (SRS-A), heparin, eosinophil chemotactic factor of anaphylaxis and bradykinin, that are released to the gingival tissue in the periodontal disease. Besides that, the mast cells interleukins increase the collagenase activity and may increase the bone resorption.[4]

Jeffcoat et al.[5] demonstrated that the treatment with degranulation inhibitor from mast cells delayed the periodontal disease progression in dogs. Thus, the anti-histaminic abuse can modulate the inflammatory process and host's response to periodontal infection.

After being activated, mast cells may produce fibroblastic growing factor (FGF), nitric oxide, interleukins, acid phosphatases and some cytokines like interferon, alpha tumoral necrosis factor (TNF-α) and metalloproteinases (MMPs).[3]

Considering its participation in inflammation and tissue repair, presence near the surfaces in bone remodeling process and its capacity to supply chemical mediators[6]; it is reasonable to suggest that those cells disclose a relationship with the osteoporosis pathogenesis.[7] Further, mast cells may exacerbate bone resorption.[8]

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https://doi.org/10.1016/j.jobcr.2017.10.002
0976-5662/

Studies demonstrated a possible relationship between the presence of mast cells and the post ovariectomy estrogenic deficiency.[9] Mast cells accumulate in the tibia bone marrow one month post ovariectomy.[9] Chemical induction of mast cell degranulation produces marked changes in the activity of MMPs, among other changes in ovariectomized rats.[10]

The MMPs belong to a family of enzymes that depends on zinc to act. They have several classes, among them: collagenases, gelatinases, stromelysins, matrilisins and membrane-type MMPs. Colagen IV e V are the main subtracts for the gelatinases MMP-2 and -9[11] that have been widely studied due to their participation in tumor progression.[12]

The MMPs have been found in cases of inflammatory periodontal disease and are related to the progress of the periodontal disease.[3] Some authors have investigated the role of the MMPs 2[13,14] and 9[13–15] in the progress of periodontitis,[16] but the participation of those enzymes in the process has not been totally established.

Since mast cells play an important role in the periodontal disease in humans and are related to estrogens and MMPs, the objective of this study was to evaluate the effect of estrogenic deficiency on mast cells and in MMP 9 expression on gingivae of ovariectomized rats with experimental induction of periodontal disease.

2. Methods

The present study was performed according to the Ethical Principles for Animal Experiments and approved by the Ethics Committee in Animal Experiment of the Faculty of Pindamonhangaba under protocol 005/2007. Thirty six adult female rats (*Rattus norvegicus,* variation *albinus, Wistar*), 90 days old, weighing about 300 g supplied by the Faculdade de Pindamonhangaba – FAPI were used.

The animals were divided in two groups: ovariectomized group (OVX) and false operated group (Sham), n = 18. Ovariectomy was performed as previous described.[8] After 60 days, to induce periodontal disease, rats were first anesthetized with an intramuscular injection of ketamine (90 mg/kg) and xilazine (10 mg/kg). A cotton ligature was placed in a subgingival position around the cervix of right first mandibular molars in each animal. The opposite side was used as control.

After the ligature placement, animals were randomly assigned to three experimental groups: 3, 7 or 14 days according to the experimental period (n = 6 animals per group).

Rats were euthanized under general anesthesia and perfusion of formaldehyde 10%. The gingiva around the first molar was removed, storage in formaldehyde 10% for at least 24 h included in paraffin and submitted to standard histological technique to HE stain, histochemistry using Toluidine Blue stain and immunohistochemical technique.

Light microscopy was used to qualitative analysis of HE slides. Ten semi-serial cuts with an average thickness 5 μm and an interval of 100 μm from each animal were obtained and toluidine blue-stained for visualization of the metachromatic mast cells. The mast cells quantification was performed in a light microscope with 400× of magnitude in the lingual papilla. The data obtained were submitted to statistical analysis.

The immunohistochemical reaction was performed by the streptavidin-biotin-peroxidase method (LSAB Systems kit DAKO Carpinteria CA, USA). The primary antibody used was the anti-MMP-9 (Santa Cruz Biotechnology) 1:200, incubated overnight at 4 °C.

The positive control was a case of epidermoid carcinoma. Negative controls, without the primary antibody were performed. Blinded qualitative analysis was performed in light microscopy.

Descriptive and inferential analyses were performed. The variance analysis test (ANOVA, two factors) and the Tukey multiple comparisons tests were applied (5%).

3. Results

In control groups, differential characteristics between groups OVX and SHAM were not seen in the experimental periods. The lining epithelium of the mucosa and gingival epithelium were orthokeratinized stratified squamous epithelium. The gingival epithelium was covered by a thin layer of keratin and the sulcus by the sulcular epithelium. The lamina propria of lingual and vestibular papillae formed by a fibrous connective tissue showed some blood vessels mixed with collagenous and fibroblasts fiber bundles. The dentogingival junction had organized bundles and collagenous fibers fan arranged. Mast cells were found around the mucosa blood vessels, deep in the specimen. Such cells were also seen permeating the lingual or vestibular papilla, but in smaller amount.

In animals with periodontal disease, the more relevant inflammatory alterations were seen in the papilla region (Fig. 1). The sulcular and junctional epithelium showed an intense exocytosis of the neutrophils and mononuclear cells besides the thickness increase. The junctional epithelium showed elongated sometimes extending deeply. In many fragments an analysis of the junctional epithelium was not possible since the tissue seemed to be torn or absent. In the lamina propria, numerous blood vessels were found, many of them exhibiting dilatation and surrounded by inflammatory cells. Edematous areas with bundles of collagenous fibers dissociated were seen in some cuts. Hyalinization areas characterized by eosinophilic and amorphous material were seen mainly in ovariectomized animals.

The inflammatory infiltrate did not show clear modifications during experimental periods. Rare mast cells were found in the papilla region, near the inflamed area. However, they were present along the mucosa subjacent to the lining epithelium and mostly around the major deep blood vessels. Sometimes microbial colonies were found on the surface.

Cytoplasmatic granules on mast cells of the tissue exhibited metachromasia to Toluidine Blue stain. Several granules dispersed in the cellular cytoplasm were seen. The mast cells present in the papilla generally showed pink colored granules easily seen, while the mast cells found in the connective tissue subjacent to the epithelium or even in the submucosa showed many cytoplasmic gathered granules, resulting in a dark purplish color. Sometimes

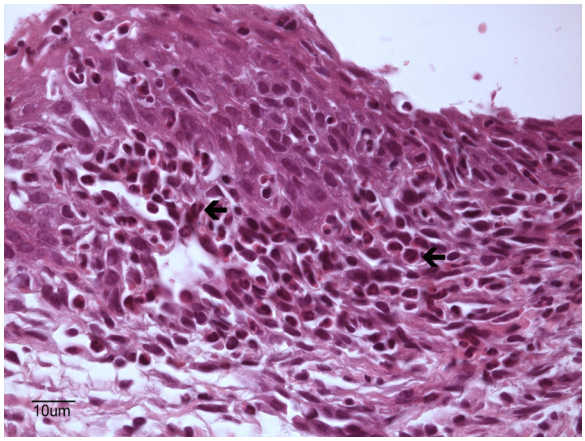

Fig. 1. Intense inflammatory infiltrate composed by neutrophils (arrows) and mononuclear cells in the junctional epithelium and in the subjacent connective tissue in SHAM-P 14 days.

granules were also observed disperse in the tissue. The cellular morphology showed variations; cells with abundant cytoplasm and central oval nuclei or small cells with minor central nucleus.

The histochemical data of the number of mast cells in the papilla lingual region were submitted to descriptive and inferential statistics and are represented in the following graph (Fig. 2). By observing Fig. 2 it was noted that mean values of the control group were higher than the periodontitis, independently of ovariectomy, in all observation periods.

ANOVA from data of SHAM groups showed the periodontitis, time and interaction as significant factors ($p = 0$; $p = 0$; $p = 0{,}07$, respectively). After the Tukey test, it was noted that SHAM-C showed means at 3 (1.77), 7 (1.89) and 14 days (1.26), considerably higher than the SHAM-P, in the same periods (0.64; 0.11; 0.09).

Upon the analysis of OVX groups, only the periodontitis effect ($p = 0$) was recognized as significant effect. The Tukey test revealed that the periodontitis group, independently of time, presented lower average (0.24) comparing with the control group (1.55). The OVX-P animals presented lower means at days 3, 7 and 14 (0.45, 0.12 and 0.14) than OVX-C (1.45, 1.95 and 1.26).

After comparing SHAM-C and OVX-C in 3 periods it was clear that no factors such as time, ovariectomy and the interaction were significant ($p = 0.05$; $p = 0.68$; $p = 0.74$, respectively). Thus resident mast cells did not alter after ovariectomy.

Comparison of SHAM and OVX groups with periodontitis showed the experimental period as significant effect ($p = 0$). Upon Tukey test it was noted that the 3-day group showed the highest average (0.54). Sham and OVX did not differ. Mast cell appears to reduce with time.

On control group, the lining epithelium of gingiva showed a keratin layer with positive immunohistochemical expression for MMP-9. In lamina propria, artery walls and bundle of skeletal muscles fibers expressed MMP-9 and were used as an intern positive control. The acinus of minor salivary glands of the submucosa were negative in all samples, except ductal cells that sometimes showed positivity.

The gingival, junctional and sulcular epithelium sometimes showed MMP-9expresssionon the basal layer. In the papilla connective tissue, some positive fibroblasts were noted. The intensity, in most cases, was higher in the dentogingival junction.

In animals with periodontitis it was observed an intense expression of the junctional epithelium and the collagen fibers of the dentogingival connection beyond the previous remarks. Most of lymphocyte and neutrophil present in the inflammatory infiltrate have positive staining. The same was seen in some fibroblasts of the papilla. In the connective tissue, subjacent to the mucosa lining epithelium, a few inflammatory cells could be seen positively marked. Deeply, some huge and star-shaped marked fibroblasts were noted, besides the nervous fibers that also express the metalloproteinase 9. Many mast cells expressed MMP9, mostly deep inside the tissue. Clear differences were not found between groups SHAM and OVX. A correlation was noted between the intensity of the inflammation and the major expression of the protein in the tissue.

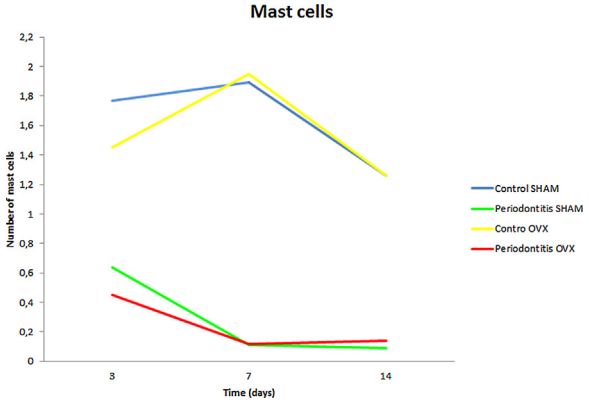

Fig. 2. Graphic. Average of mast cells considering the effect of periodontitis, ovariectomy and the experimental period.

4. Discussion

In this study, the lingual papilla region of animals with periodontitis demonstrated a decrease in the number of mast cells with the passing time. That reduction was noted in both ovariectomized and SHAM rats. Similar results were found by Rodini[14] using similar methodology. Mast cells may have their role reduced in the experimental periodontitis in rats (Table 1).

In agreement with our results, Gemmell et al.[17] confirmed a reduction in the count of mast cells according to the severity of the human periodontal disease. On the other hand the authors noted a Th2 lymphocyte prevalence, and suggested that mast cells may not be the source of cytokines that induce these cells.

However, studies revealed that mast cells participate in the human periodontitis or in the experimental periodontites induced in animals.[14,18] According to Kornman et al.[19] lipopolysaccharide of gram-negative bacteria causes release of mediators from mast cells, like histamine and induces endothelial cells inflammatory response.

The evaluation of MMP9 expression in this study showed a relationship between the presence of periodontal disease and higher intensity of enzyme. The MMP 9 degrades the denatured collagen (gelatins) and the collagen type IV[11] and has been correlated to the pathogenesis of the periodontal disease. An increase of MMP-9 in the crevicular fluid of patients with chronic periodontitis was also seen by Rai et al.[20]. Guan et al.[21] showed that production of MMP-9 by the human periodontal ligament cells can be induced by *P. Intermedia* contributing for a periodontal destruction.

Another finding of this study was the presence of hyalinization areas with diverse extensions, mostly associated to ovariectomized animals with periodontitis. These regions were not evident in SHAM animals with periodontal disease. The influence of estrogens in collagen synthesis have been investigated.[22]

Pirilä et al.[23] evaluated the influence of estrogen on tissue repair of ovariectomized rats. The authors investigated its effects on MMP-2, MMP-1 membrane type and laminin-5 gamma2-chain. They found that the laminin-5 gamma2-chain expression had been reduced in ovariectomized animals, as well as MMP-2 and 9. Estrogen promoted tissue repair in ovariectomized rats normalizing the content and structure of collagen. Estrogen also recovered the expression of laminin-5 gamma2-chain, a key-molecule in the repair process.

Remarkable differences between the MMP 9 in SHAM and OVX rats were not noted in this study. Hu et al.[24] studied the treatment with estrogens associated or not to progestin in several biomarkers of cardiovascular events in women in post-menopause, among

Table 1
Descriptive statistics for the number of mast cells in the lingual papilla region.

Time	SHAM-C	SHAM-P	OVX-C	OVX-P
3	1.77 ± 0.52^{A}	0.64 ± 0.4^{1Ba}	1.45 ± 0.70^{A}	0.45 ± 0.36^{Ba}
7	1.89 ± 0.41^{A}	0.11 ± 0.17^{Cb}	1.95 ± 1.03^{A}	0.12 ± 0.20^{Cb}
14	1.26 ± 0.40^{B}	0.09 ± 0.11^{Cb}	1.26 ± 0.56^{B}	0.14 ± 0.13^{Cb}

*Groups sharing same letters did not presented significant differences.
Upper case letters indicate Tukey test results by testing time and periodontitis as factors.
Lowercase letters indicate Tukey test results by testing estrogen deficiency and time as factors.

them, the MMP-9. The authors also did not see an increase in the MMP activity. Controversial results were found by Zecchin et al.[25] The authors showed that the estrogenic deficiency reduced the expression and activity levels of MMP-2 and 9 and types I and III collagen in the granulation tissue of dental alveolus post-extraction rats. They suggested that the estrogenic deficiency may impair the tissue repair by the interference in the tissue remodeling of the extracellular matrix.

Mamalis et al.[22] investigated *in vitro* the estrogen regulation of the proliferation and osteoblastic differentiation, collagen synthesis and expression of periostin gene, in human periodontal ligament cells. They concluded that the estrogen receptor-beta has an important role in inducing estrogenic effects in the cells of human periodontal ligament, such as proliferation and osteoblastic differentiation and the expression of key-molecules for functional and structural integrity of the periodontium, like the *periostin*.

It is known that mast cells have receptors for estrogen and that estrogenic hormones have also been related to alteration in tissue remodeling.[25,22] Therefore, a regulation of mast cells by estrogens in the tissue remodeling may be possible. But to prove and clarify this relationship, new studies will be necessary.

Lesclous et al. demonstrated that after estrogen depletion, mast cell accumulates in rat bone marrow. The number increase quickly until a peak in 28 days and remains higher in next month. Concomitantly, osteoclasts activate trabecular bone resorption. It is possible that estrogen deficiency targeted a precursor common to both cells.[26] After ovariectomy, due to estrogen absence, histamine, IL6 and TNF-α are release by mast cells and commits precursors in osteoclastic pathway.[27]

According to the experimental conditions of the present work, estrogenic deficiency did not influence the presence of mast cells and the expression of the metalloproteinase 9 on gingivae of rats with periodontal disease.

5. Conclusion

It is concluded that periodontitis lead to a reduction in the number of mast cells e an increase in the expression of metalloproteinase 9 in these animals.

Acknowledgement

This work was supported by the State of São Paulo Research Foundation (FAPESP) [grant no. 08/54771-0].

References

1. Huang S, Lu F, Chen Y, Huang B, Liu M. Mast cell degranulation in human periodontitis. *J Periodontol*. 2013;84:248–255.
2. Ribatti D, Guidolin D, Marzullo A, et al. Mast cells and angiogenesis in gastric carcinoma. *Int J Exp Pathol*. 2010;91:350–356.
3. Steinsvoll S, Helgeland K, Schenck K. Mast cells—a role in periodontal diseases? *J Clin Periodontol*. 2004;31:413–419.
4. Lagdive SS, Lagdive SB, Mani A, et al. Correlation of mast cells in periodontal diseases. *J Indian Soc Periodontol*. 2013;17:63–67.
5. Jeffcoat MK, Williams RC, Johnson HG, Wechter WJ, Goldhaber P. Treatment of periodontal disease in beagles with lodoxamide ethyl, an inhibitor of mast cell release. *J Periodontal Res*. 1985;20:532–541.
6. Dobigny C, Saffar JL. H1 and H2 histamine receptors modulate osteoclastic resorption by different pathways: evidence obtained by using receptor antagonists in a rat synchronized resorption model. *J Cell Physiol*. 1997;173:10–18.
7. Brumsen C, Papapoulos SE, Lentjes EG, Kluin PM, Hamdy NA. A potential role for the mast cell in the pathogenesis of idiopathic osteoporosis in men. *Bone*. 2002;31:556–561.
8. Prado RFD, Silveira VAS, Rocha RFD, Vasconcellos L, Carvalho YR. Effects of experimental osteoporosis and low calcium intake on postextraction sockets of rats. *Int J Exp Pathol*. 2012;93:139–147.
9. Lesclous P, Saffar JL. Mast cells accumulate in rat bone marrow after ovariectomy. *Cells Tissues Organs*. 1999;164:23–29.
10. Chancey AL, Gardner JD, Murray DB, Brower GL, Janicki JS. Modulation of cardiac mast cell-mediated extracellular matrix degradation by estrogen. *Am J Physiol Heart Circ Physiol*. 2005;289:H316–H321.
11. Woessner Jr. JFJr.. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *Faseb J*. 1991;5:2145–2154.
12. Gutschalk CM, Yanamandra AK, Linde N, Meides A, Depner S, Mueller MM. GM-CSF enhances tumor invasion by elevated MMP-2, -9, and -26 expression. *Cancer Med*. 2013;2:117–129.
13. Chen D, Wang Q, Ma ZW, et al. MMP-2, MMP-9 and TIMP-2 gene polymorphisms in Chinese patients with generalized aggressive periodontitis. *J Clin Periodontol*. 2007;34:384–389.
14. Rodini CO, Batista AC, Dionisio TJ, Santos CF, Cunha FQ, Lara VS. Morphologic evaluation and expression of matrix metalloproteinases-2 and 9 and nitric oxide during experimental periodontal disease in rat. *J Mol Histol*. 2008;39:275–282.
15. Smith PC, Munoz VC, Collados L, Oyarzun AD. In situ detection of matrix metalloproteinase-9 (MMP-9) in gingival epithelium in human periodontal disease. *J Periodontal Res*. 2004;39:87–92.
16. Korostoff JM, Wang JF, Sarment DP, Stewart JC, Feldman RS, Billings PC. Analysis of in situ protease activity in chronic adult periodontitis patients: expression of activated MMP-2 and a 40 kDa serine protease. *J Periodontol*. 2000;71:353–360.
17. Gemmell E, Carter CL, Seymour GJ. Mast cells in human periodontal disease. *J Dent Res*. 2004;83:384–387.
18. Batista AC, Rodini CO, Lara VS. Quantification of mast cells in different stages of human periodontal disease. *Oral Dis*. 2005;11:249–254.
19. Kornman KS, Page RC, Tonetti MS. The host response to the microbial challenge in periodontitis: assembling the players. *Periodontology*. 2000;14:33–53.
20. Rai B, Kharb S, Jain R, Anand SC. Biomarkers of periodontitis in oral fluids. *J Oral Sci*. 2008;50:53–56.
21. Guan SM, Shu L, Fu SM, Liu B, Xu XL, Wu JZ. Prevotella intermedia induces matrix metalloproteinase-9 expression in human periodontal ligament cells. *FEMS Microbiol Lett*. 2008;283:47–53.
22. Mamalis A, Markopoulou C, Lagou A, Vrotsos I. Oestrogen regulates proliferation, osteoblastic differentiation, collagen synthesis and periostin gene expression in human periodontal ligament cells through oestrogen receptor beta. *Arch Oral Biol*. 2011;56:446–455.
23. Pirila E, Parikka M, Ramamurthy NS, et al. Chemically modified tetracycline (CMT-8) and estrogen promote wound healing in ovariectomized rats: effects on matrix metalloproteinase-2, membrane type 1 matrix metalloproteinase, and laminin-5 gamma2-chain. *Wound Repair Regen*. 2002;10:38–51.
24. Hu P, Greendale GA, Palla SL, et al. The effects of hormone therapy on the markers of inflammation and endothelial function and plasma matrix metalloproteinase-9 level in postmenopausal women: the postmenopausal estrogen progestin intervention (PEPI) trial. *Atherosclerosis*. 2006;185:347–352.
25. Zecchin KG, Pereira MC, Coletta RD, Graner E, Jorge J. Ovariectomy reduces the gelatinolytic activity and expression of matrix metalloproteinases and collagen in rat molar extraction wounds. *Calcif Tissue Int*. 2005;76:136–145.
26. Lesclous P, Guez D, Llorens A, Saffar JL. Time-course of mast cell accumulation in rat bone marrow after ovariectomy. *Calcif Tissue Int*. 2001;68:297–303.
27. Lesclous P, Guez D, Baroukh B, Vignery A, Saffar JL. Histamine participates in the early phase of trabecular bone loss in ovariectomized rats. *Bone*. 2004;34:91–99.