

Periodontal inflammation induced by chronic ethanol consumption in ovariectomized rats

Inflamação periodontal induzida pelo consumo crônico do etanol em ratas ovariectomizadas

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ABSTRACT

The immune system plays an important role in the pathogenesis of periodontal diseases. The host may modulate periodontal inflammatory reactions and it determines variances in the individual susceptibility and in the periodontal disease progression speed. Osteoporosis and alcoholism are described as risk indicators of periodontal disease among the systemic acquired factors. **Objective:** The current study aims to analyze chronic alcohol consumption influence on induced periodontitis in rats presenting estrogen deficiency. **Material and Methods:** Sixty rats approximately 90 days old were used in the experiment; they were divided into two groups: correlated surgery (OVZ) or surgical ovariectomy simulation (SHAM). Each group was divided into three subgroups: (C) control diet, (A) ethanol containing 20% liquid diet and (I) par-fed control diet. Thirty days after castration the diet and the experimental periodontitis induction were kept for 56 days. Interproximal regions between the first and the second lower left molar and the respective contralateral site without periodontal disease induction were assessed for inflammatory features. **Results:** Hormone deficiency resulted in important inflammatory changes concerning the meaning of SHAM-C and OVZ-C. The ethanol diet has resulted in inflammatory changes to both groups SHAM-A and OVZ-A in the absence of periodontitis, with also greater severity when combined with ovariectomy. **Conclusion:** It was concluded that the association between estrogen deficiency and 20% ethanol was just relevant for sites without periodontitis disease induction, since it induces stronger severity in the inflammatory process in the presence of the

RESUMO

O sistema imunológico tem um importante papel na patogênese da doença periodontal, sendo capaz de modular a resposta inflamatória, determinando variações na susceptibilidade individual e velocidade da progressão da doença periodontal. Entre os fatores sistêmicos adquiridos, a osteoporose e o alcoolismo são descritos como indicadores de risco para a perda óssea associada a doença periodontal. **Objetivo:** O objetivo deste estudo foi analisar a influência do consumo crônico de etanol na periodontite induzida em ratas com deficiência estrogênica. **Material e Métodos:** Foram utilizadas sessenta ratas, com aproximadamente noventa dias, divididas em dois grupos, ovariectomizado (OVZ) ou simulação cirúrgica de ovariectomia (SHAM). Cada grupo foi dividido em três subgrupos: grupo controle (C); grupo etanol a 20% como dieta líquida (A) e grupo dieta controle pareada (I). Após trinta dias da castração, as dietas e a periodontite induzida foram mantidas por 56 dias. A região interproximal entre o primeiro e o segundo molar inferior esquerdo e a região contralateral, sem indução da doença periodontal, foram avaliadas quanto as características inflamatórias. **Resultados:** Os resultados mostraram que, considerando SHAM-C and OVZ-C, a deficiência hormonal resultou em alterações inflamatórias importantes. Considerando os grupos SHAM e OVZ na ausência de periodontite, a dieta com etanol resultou em alterações inflamatórias em ambos os grupos e também maior severidade quando combinado com a ovariectomia. **Conclusão:** Concluiu-se que a associação da deficiência estrogênica e etanol 20% somente foi relevante para sítios sem indução da doença periodontal, induzindo maior severidade

inflammatory cells scattered in the conjunctive tissue and of the disorientation of periodontal ligament fibers.

KEYWORDS

Alcoholism; Osteoporosis; Ovariectomy; Periodontal disease.

do processo inflamatório, com presença de células inflamatórias dispersas no tecido conjuntivo e desorientação das fibras do ligamento periodontal.

PALAVRAS-CHAVE

Alcoolismo; Doença periodontal; Osteoporoze; Ovariectomia.

INTRODUCTION

The impact of abusive ethanol consumption is often stronger on young women, since it causes endocrine and reproductive dysfunctions, among other diseases [1]. However, it is expected an increased impact on older women due to the increased life expectancy and the higher probability of ethanol synergism with factors associated with post-menopause and with medications used to control anxiety and depression [2]. This association between chronic ethanol consumption by women and estrogen deficiency may directly influence the immune potential and change important metabolic functions for bone homeostasis with resorption process prevalence. This prevalence, once associated with periodontal pathogenic bacteria, becomes able to speed up the bone resorption process found in periodontal diseases [3,4].

Studies have showed direct or indirect association between ethanol and periodontal disease [5-7] as well as evidences of direct ethanol effects on osteoclast, osteoclastogenesis and osteopenia [8], and on the activation of genes and pathways correlated with osteogenesis and osteoclastogenesis [9,10].

It is observed in the literature that the animal model has been used to identify periodontal inflammation processes by means of ligature-induced periodontitis in rats [4,11-13].

According to studies conducted in rats associating alcohol diet and/or estrogen deficiency, these variables boost greater periodontal inflammation process severity

at sites with or without periodontal disease induction. Dantas et al. [13] found increased expression of inflammatory marker seven in the absence of periodontal disease and the potentiation of some inflammatory mediators in the presence of periodontal disease, especially in the periodontal ligament. Irie et al. [6], found higher values of alveolar bone loss, insertion loss, oxidative damages, tissue necrosis factor (TNF), and polymorpho nuclear density in the alcohol group than in the isocaloric diet group. Although the polymorpho nuclear density and the TNF production were higher in the alcohol group with induced periodontitis, the damages concerning bone loss were higher in groups without induced periodontal disease.

Souza et al. [12] conducted a study on female rats receiving alcoholic diet. They found ethanol effects on periodontitis progression and increased bone loss in the furcation area of rats subjected to 20% ethanol intake for 8 weeks.

Regarding estrogen deficiency-ethanol association, Marchini et al. [4], conducted a study on ovariectomized rats subjected to 20% ethanol consumption for 8 weeks and they found Ca/P concentration in the alveolar bone of the OVZ alcohol group lower than that found in the sham control group, both groups without induced periodontitis.

The current study aims to evaluate the combined and possibly potentiating effect of estrogen deficiency and chronic alcohol use on induced periodontitis in rats. In addition, it presents the histological features of the interproximal periodontal tissue.

MATERIALS AND METHODS

The present study was approved by the ethics committee of São José dos Campos School of Dentistry, State University of São Paulo – UNESP (Protocol N°001/2007 CA/CEP).

Treatment of animals

Sixty adult female Wistar rats (3 months-old) weighing 300g on average were randomly assigned to 2 groups of thirty rats each: one group was ovariectomized (OVZ: test group, the rats were subjected to estrogen deficiency by removing the ovaries) and the other group was subjected to sham operation (SHAM: control group, simulated ovariectomy, the ovaries were exposed but not removed).

After 30 days, the OVZ and SHAM rats were randomly distributed into three subgroups (10 rats/group), according to their diet: C (control; water), A (rats fed on 20% ethanol v/v, per volume) and I (rats pair-fed with ethanol replaced by isocaloric amounts of carbohydrate).

Group A rats received standard rat chow (Guabi Nutrilador, Mogiana Alimentos, Campinas, SP, Brazil) and ethanol solution (Ecibra, CETUS, Santo Amaro, SP, Brazil) presenting 20% ethanol v/v concentration, both ad libitum. The isocaloric solution concentration contained, in millilitres, the same amount of calories found in the 20% alcohol solution. It was prepared by dissolving 266 g sucrose in 1.000 mL water. The calculations took into account the alcohol concentrations (20%) and the sucrose (4.1 kcal/g) and alcohol (7.1 kcal/g or 5.6 kcal/ml) caloric values. One day after ethanol administration, the control animals were fed with the same amount of rat chow and volume of liquid diet as that consumed by the alcohol groups. Ethanol was replaced by isocaloric amounts of carbohydrate in this liquid diet. Ethanol represented 37% of the total dietary energy intake in group A.

After the adaptation period (5% and 10% ethanol for 7 days each), the rats received diet containing experimental concentrations.

Thirty (30) days after the SHAM or the OVZ surgery, periodontitis was induced in all groups. It was done by placing cotton ligatures around the cervix of the mandibular first molar in a way to leave the contralateral teeth unligated in order to work as control. The ligature was knotted on the buccal side of the tooth. Eight weeks after the beginning of the dietary treatment, the rats were sacrificed and their mandibles were removed.

Histological Analysis

The mandible from each rat was resected and immediately demineralized in 10% tetrasodium-EDTA aqueous solution (EDTA – Titriplex P.A – Merck-KgaA, Darmstadt, Germany). The specimens were then dehydrated, embedded in paraffin, sectioned (5 μ m) along the molars in mesiodistal direction and stained with hematoxylin and eosin (HE). The image was examined under Zeiss Axiophot 2 stereomicroscopy (Carl Zeiss, Oberkochen, Alemanha), in five semi serial sections in two cuts interval per specimen (50X magnification).

A single examiner, who was blind to the assigned treatment, analyzed the interproximal region after making adaptations in the studies by Galvão et al. [14], and Liu et al. [15]. The examiner took under consideration specific aspects of the periodontal ligament, the alveolar bone crest, the gingival connective tissue and of the epithelial tissue. Normality and pathological changes were taken into account in each histologically evaluated aspect.

The junctional epithelium presented standard size when its apical portion met the cementum-enamel junction (CEJ); and it was migrated when it was apically positioned in relation to the CEJ. It was non-proliferated when two to three layers were neatly arranged, showed mild proliferation when several layers

were neatly arranged or slightly organized, and it was intensely proliferated when several layers were randomly arranged, of ten entangled. Hydropic degeneration was also observed. Figure 1 exemplifies some of the here in described epithelial changes.

The gingival connective tissue was evaluated for fibroblast cell quantification, which could be normally or abundantly present. As for inflammation, it could show: 1) No inflammation-absence of inflammatory cells, 2) Mild inflammation-few inflammatory cells near the junctional epithelium, 3) Moderate inflammation-presence of scattered inflammatory cells in the connective tissue, 4) Severe inflammation-more than 1/3 of the interproximal tissue cells were inflammatory cells.

The collagen fibers of the supracristal gingival tissue were assessed for the absence or presence of hyalinization, for parallel or disoriented fibers, and for fibers inserted or not in the cement. Figures 2 and 3 depict the connective tissue changes.

The most coronal portion of the bone crest was assessed for the contour – regular or irregular – with no, few or many bone

resorption lacunae (Howship's lacunae) and with or without osteoclasts. Figure 4 depicts the assessed bone changes.

The periodontal ligament was evaluated for fibroblastic cell qualification – which could be normally or abundantly found – and for the disorientation and the loss of periodontal ligament fibers' insertion in the alveolar bone. Figure 5 depicts the observed changes.

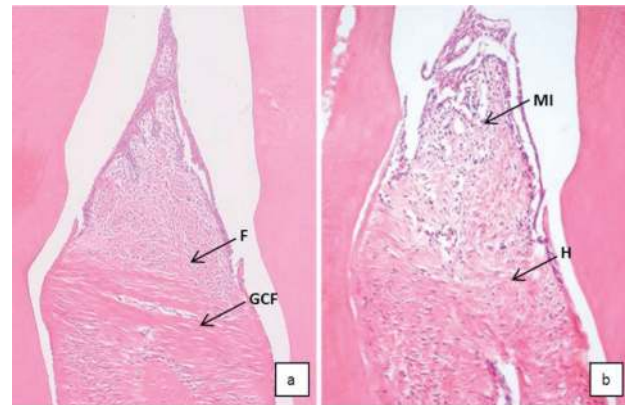


Figure 2 - Photomicrograph: a) Connective tissue with moderate inflammation (MI), with quantification of abundant fibroblast cells (F), gingival collagen fibers (CF) arranged in parallel and no hyalinization. b) Connective tissue with moderate inflammation, with quantification of standard fibroblast cells, disorganized collagen fibers gingival and presence of hyalinization (H) (x200 - H&E).

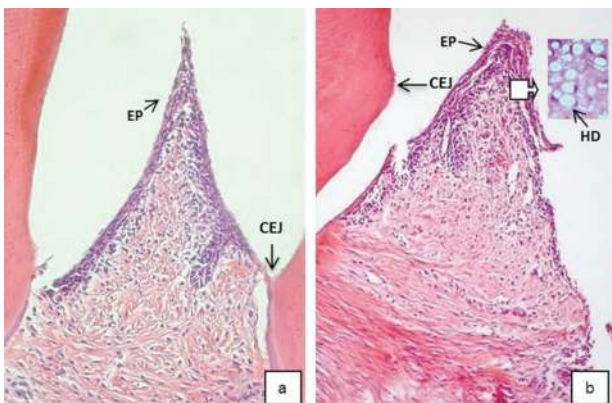


Figure 1 - Photomicrograph: a) Non-proliferated junctional epithelium (JE); positioned in the CEJ; without hydropic degeneration (x200 - H&E). b) Slightly proliferated junctional epithelium (JE); positioned apical to the CEJ; with hydropic degeneration (HD) highlighted at higher magnification (x400 - H&E).

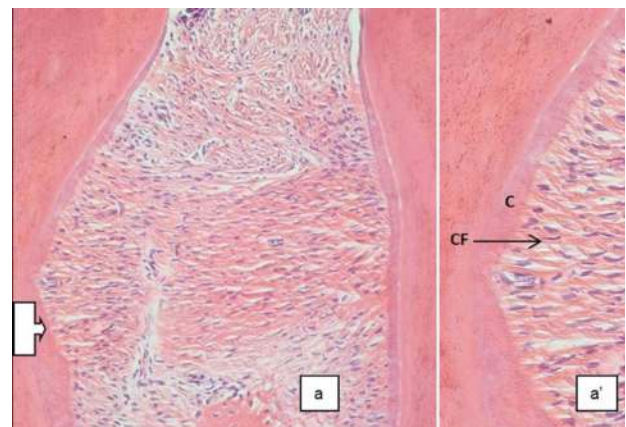


Figure 3 - Photomicrograph: a) Connective tissue with quantification of standard fibroblast cells, organized gingival collagen fibers (CF) and no hyalinization (x200 - H&E); a' represents fibers inserted in the cementum (C) (x400 - H&E).

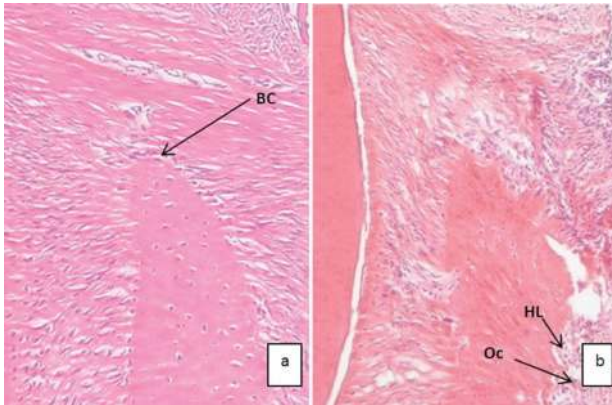


Figure 4 - Photomicrograph: a) Most coronary portion of the interproximal bone crest (BC) showing regular contour and absence of Howship's lacunae (HL) and osteoclasts (Oc) (x200 - H&E); b) irregular contour, presence of Howship's lacunae (HL) aplenty and osteoclasts (Oc) (x200 - H&E).

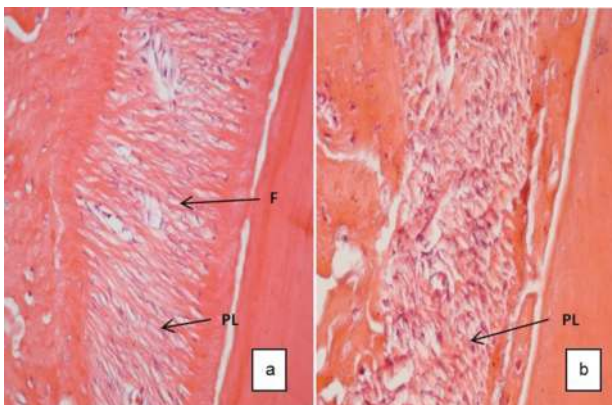


Figure 5 - a) Organized periodontal ligament fibers (PL) embedded in the cementum and alveolar bone with standard amount of fibroblast cells (F) (x200 - H&E); b) disorganized periodontal ligament fibers (PL) not embedded in the cementum and alveolar bone with large quantities of fibroblast cells (F) (x200 - H&E).

Data were estimated as percentage per animal and, subsequently, as percentage per experimental subgroup.

The histological features considered to be representative so the group's profile were divided into two subgroups in each analysis, namely: absent or mild periodontal inflammation and moderate or severe periodontal inflammation. Data were analyzed using the asymmetric correspondence analysis test for sites with and without ligature-induced periodontal disease. The herein used computer software were

Minitab for Windows, version 14.1 (Minitab Inc., State College, PA, USA) and Statistix for Windows version 8.0 (Analytical Software, Tallahassee, FL, USA).

RESULTS

Groups without periodontal disease induction

Histological sections performed in specimens without periodontitis induction showed junctional epithelium positioned in the CEJ or slight migrated in the apical direction. Most specimens showed no or slight epithelial proliferation. However, it was possible to see more pronounced proliferation and loss of organization in the epithelial layers, which were entangled and showed cell adhesion loss. Hydropic degeneration was constantly found. Overall, there was no inflammation or it was possible to see mononuclear inflammatory cells arranged in the connective tissue just below the junction epithelium. The connective tissue of ten exhibited normality pattern regarding fibroblast quantification, which show elongated and evident nuclei. The collagen fiber bundle exhibited parallel pattern forming large bands in the supracristal region. Orientation loss was rarely observed. Blood vessels, sometimes congested, were found.

The alveolar bone crest showed rounded or slightly irregular with Howship's lacunae in the most coronal portion. The periodontal ligament showed vascular channels, elongated fibroblast and well-directed collagen fiber bundles, and the Sharpey's fibers were well inserted in the alveolar bone and in the cementum. Few specimens showed loss of orientation as well as loss of periodontal ligament insertion. Bone tissue exhibited regular and dense aspect, with many osteocytes homogeneously distributed and few medullary spaces.

The Figures 6 and 7 represents the feature distribution profile of SHAM and OVZ subgroups, without periodontal disease. It is possible to see

the representation of the three subgroups(C, A and I) and their histological features, which have been indicated by do tsin a two-dimensional space. Twenty-four histological features ranging among the sub-groups, based on the axes resulting from the correspondence analysis, were taken under consideration. The magnitude regarding distances were, respectively, distance 1:36.64% and distance 2:24.67%. The distances represented the histological features projection percentage for axes 1 and 2. It was possible to observe that the subgroups were well distributed along the main axis, thus reflecting their differences on specific histological features. Subgroups I (SHAM and OVZ) and C (SHAM and OVZ) were relatively homogeneous where as subgroup A(SHAM and OVZ) was not represented by the same histological features. Hormonal deficiency and alcohol diet influenced histological patterns represented by the presence of inflammatory cells scattered in the connective tissue and by periodontal ligament fiber disorientation. The other features showed low inertia, and therefore low variability among the analyzed subgroups.

The histological features were individually evaluated in the SHAM group

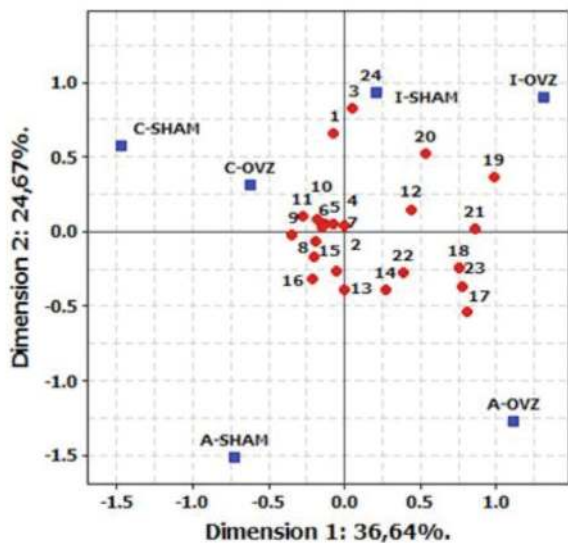


Figure 6 - Representation of distribution profile of the histological features based on subgroups SHAM and OVZ without periodontal disease induction.

without periodontal disease induction. Those histological features those distinguishing subgroups A from the other subgroups were: frequency of intense junctional epithelium proliferation, disorganized gingival collagen fibers and abundant fibroblast cells (these features were consistent with moderate to severe inflammation). As for subgroup C, there was prevalence of specimens without apical migration of the junctional epithelium. Subgroup Is showed: frequency of inflammatory cells scattered in the connective tissue, irregular bone crest contour, presence of Howship’s lacunae,

Epithelial positioning	1	Normality pattern	13	Apical to CJE
Epithelial proliferation (EP)	2	Absent or mild	14	Intense
Hydropic degeneration (HD)	3	Absent	15	Present
Quantification of gingival fibroblast cells (F)	4	Standard quantity	16	Abundant
Presence of interproximal inflammatory cells (IC)	5	Absent or near the junctional epithelium	17	Scattered or occupying more than 1/3 of the connective tissue
Disorientation of supracrestal gingival fibers (GFC)	6	Absent	18	Present
Alveolar bone crest contour (BC)	7	Regular	19	Irregular
Howship’s lacunae (HL)	8	Absent or few	20	many
Osteoclastd (OC)	9	Absent	21	Present
Quantification of periodontal ligament fibroblast cells	10	Standard amount	22	Abundant
Disorientation of periodontal ligament fibers (PL)	11	Absent	23	Present
Loss of periodontal ligament fiber insertion	12	Absent	24	Present

Figure 7 - Captions to the histological features based on subgroups SHAM and OVZ without periodontal disease induction, represented in Figure 6.

osteoclasts, and loss of periodontal ligament fibers insertion (compatible with moderate to severe inflammation). As for the OVZ group without periodontal disease induction, the histological features distinguishing subgroup A from the other subgroups were: frequency of inflammatory cells scattered in the connective tissue, abundant amount of fibroblasts in the gingival connective tissue, apical migration of the junctional epithelium, periodontal ligament fibers' disorientation (compatible with moderate to severe inflammation). Subgroup C did not distinguish from the others by any particular feature. As for subgroup I, the following features were observed: absence of hydropic degeneration, abundant amount of fibroblasts in the periodontal ligament, irregular bone crest contour and disorganized supracristal collagen fibers (mostly compatible with moderate to severe inflammation).

Groups with periodontal disease induction

The sites in which periodontitis was induced by cotton ligature showed flattened junctional epithelium clearly migrated apical to the CEJ. CEJ was sometimes ulcerated or ruptured due to ligature use. The preserved areas were proliferated, presenting degenerating epithelial nests, loss of cell adhesion and hydropic degeneration. Almost all animals showed collagen fiber bundles, loss of orientation and hyalinization; the "band" of collagen fibers was rarely seen in parallel within the supracristal region. The periodontal ligament was modified, constituted by connective tissue and collagen fibers arranged in different directions, of ten disorganized and presenting insertion loss. The bone crest showed very irregular aspect, presence of craters and resorption lacunae, sometimes accompanied by multinucleated giant cells.

The Figures 8 and 9 represent the distribution profile of SHAM and OVZ subgroups with periodontal disease, by taking under consideration the 18 histological features

ranging among the subgroups based on the axes resulting from the correlation analysis. There was heterogeneity in the subgroups distribution along the main axis, thus reflecting significant differences in the histological features. Only subgroups I-OVZ and A-OVZ were relatively homogeneous, and they both showed the presence of much Howship lacunae, whereas the other subgroups were not represented by the same histological features.

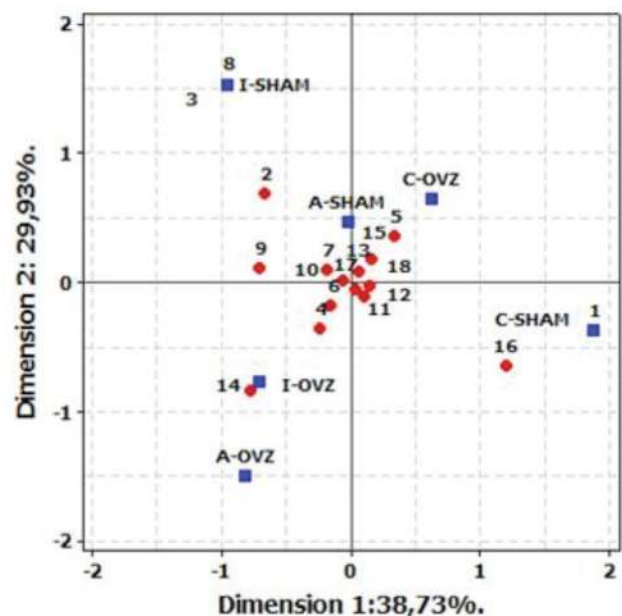


Figure 8 - Representation of histological features based on subgroups SHAM and OVZ with periodontal disease induction.

By separately evaluating the SHAM group with periodontal disease induction, it was found that subgroups C and I showed distinct features and that subgroup A was characterized as an intermediate group between C and I. The histological features that distinguished subgroup C from the others were: abundant periodontal ligament fibroblast cells and slight junctional epithelium proliferation. As for subgroup I: supracristal gingival tissue fibers oriented in parallel, well-oriented periodontal ligament fibers, presence of many Howship's lacunae, no hyalinization of supra cristal collagen fibers, and well-inserted periodontal ligament fibers (mostly consistent with no or mild inflammation). Subgroup A showed no specific histological

Epithelial proliferation (EP)	1	Absent or mild	10	Intense
Hyalinization of gingival collagen fibers (GCF)	2	Absent	11	Present
Disorientation of supracrestal gingival fibers	3	Absent	12	Present
Insertion of supracrestal gingival fibers	4	Absent	13	Present
Howship's lacunae (HL)	5	Absent or few	14	Many
Osteoclasts (Oc)	6	Absent	15	Present
Quantification of periodontal ligament fibroblast cells (PL)	7	Standard quality	16	Abundant
Disorientation of periodontal ligament fibers	8	Absent	17	Present
Loss of periodontal ligament fiber insertion	9	Absent	18	Present

Figure 9 - Captions to the histological features based on subgroups SHAM and OVZ with periodontal disease induction, represented in Figure 8.

features and higher correlation with the absence of osteoclasts. The other features showed low variability among the analyzed subgroups.

As for the OVZ group with periodontal disease induction, subgroups C and A showed very different profiles. The histological features distinguishing subgroup A from the others were: gingival collagen fibers embedded in the cementum, absence of osteoclasts, big amount of fibroblasts in the periodontal ligament (mostly consistent with no or mild inflammation). Regarding subgroup C: few Howship's lacunae. As for subgroup I: no hyalinization of supracrestal collagen fibers. The other features showed low variability among the analyzed subgroups.

DISCUSSION

Post-menopausal hormone deficiency – particularly estrogen – and chronic alcoholism are related to the development of many diseases,

among them: osteoporosis and coronary heart disease [16], cancer, liver and neurocognitive diseases [17]. Similarly to alcoholism, some studies referred to osteoporosis as being a social issue due to its social and physical factors as well as to its economic impact [18-20].

Some studies have demonstrated positive relationship between osteoporosis and periodontal disease [21,22], although other studies have shown conflicting results [18,23,24].

Regarding excessive alcohol consumption, according to a critical review performed by Amaral et al. [25], only 16 out of the 1530 selected indexed studies relating periodontal disease and alcoholism were considered to be relevant to a grounded risk study.

Nowadays, there is no consensus regarding osteoporosis and alcoholism as risk factors for periodontal disease. Thus, ethanol alone or in combination with estrogen deficiency should be classified, for now, as risk indicators, thus requiring further longitudinal studies that preferably demonstrated adjusted to age, gender, smoking, diabetes, amount of dental biofilm and tartar, frequency and type consumed alcoholic beverage.

The current study induced periodontal disease over 56 days. Literature shows different ligature-based periodontal disease induction times [11,14,21,26,28]. According to Rodini et al. [27] and Semenoff-Segundo et al. [28], 15 days would be sufficient to assess gingival inflammation and alveolar bone loss; alveolar bone loss gradually decreases from 42 days until it stagnates. The option for keeping the ligature took under consideration previous studies on chronic ethanol intake for eight weeks [4,11,12,29]. Even using a periodontal disease induction period considered long by some authors, it was possible to identify inflammatory process in the experimental groups as it could be seen by the presence of epithelial cell proliferation, hydropic degeneration, abundant

periodontal ligament fibers and gingival tissue, inflammatory cells, and Howship's lacunae.

The correspondence analysis did not establish the statistical significance of the associations and it did not assess the independent effect of each feature. However, it combines advantages of non-linear and multidimensional methods, a fact that allowed describing the low-dispersion or variability features in the subgroup and identifying differentiated features.

The histological behavior, which was observed by correspondence analysis, showed that I (SHAM and OVZ) and C (SHAM and OVZ) animals without induced periodontal disease were relatively homogeneous, whereas A (SHAM and OVZ) animals had distinct histological features. The hormonal deficiency associated with alcoholic diet induced the greater accumulation of inflammatory cells – which occupied more than 1/3 of interproximal connective tissue – as well as the disorientation of the periodontal ligament fibers.

Similar to the current study, Marchini et al. [4] also found damages at the sites without periodontal disease induction in OVZ alcohol groups in comparison to the sham control group.

As for estrogen deficiency, the histological behavior, which was observed by correspondence analysis, showed that SHAM-C and OVZ-C animals without periodontal disease induction had similar histological profiles, whereas SHAM-C and OVZ-C animals with periodontal disease had different profiles especially marked by the abundant presence of fibroblasts in the periodontal ligament of OVZ animals.

Regarding the used methodology, Amadei et al. [26] also found non-significant results in SHAM and OVZ animals without induced periodontitis. Only groups subjected to ligature for 90 days showed significant ovariectomy-associated bone loss. Differently from Amadei et al. [26], the current study established a waiting time of thirty days between ovariectomy and the beginning of the experiment, since the experiment

was considered to be long; there is the possibility that a factor inherent to SHAM rats' senility could affect the results. In addition, according to Duarte et al. [21], bone loss may be observed right after ovariectomy, and it gradually decreases, thus showing quiescence and exacerbation phases.

It was not possible to identify in the herein presented study which mechanism has changed the pattern when variables such as estrogen deficiency and ethanol consumption were combined. However, it is possible that the inflammatory mediators stimulated by ethanol, as suggested by Irie et al. [6], show synergy or potentiation of the cytokine stimulated by hormone deficiency. These effects are possible to occur in the presence and in the absence of periodontal disease and they show deleterious effects according to the cell types. These findings were also observed in the current study; the periodontal ligament region presented different behavior from that of the other regions, which meets the results found by Dantas et al. [13]. Research using immunohistochemical markers could assist in this interpretation.

Further studies are needed in order to elucidate the mechanism that differentiates the combined effect of ethanol and estrogen deficiency when it comes to periodontal disease severity.

CONCLUSION

The association between estrogen deficiency and 20% ethanol consumption was relevant just for sites without periodontal disease induction, since it induced greater inflammatory process severity, thus showing inflammatory cells scattered in the connective tissue and disorientation of the periodontal ligament fibers.

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