



Influence of chronic alcoholism and estrogen deficiency on the immunohistochemical expression of regulatory proteins of the bone resorption process in the periodontium of Wistar rats

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ABSTRACT

Objective: The aim of this study was to investigate possible changes in immunohistochemical expression of proteins regulating the bone resorption process in the periodontium of rats subjected to alcoholism and/or estrogen deficiency. The investigated proteins were receptor activator of nuclear factor-kappa β ligand (RANKL), a protein that stimulates bone resorption, and osteoprotegerin (OPG), a protein that inhibits bone resorption. At the molecular level, decreased OPG expression and/or increased RANKL expression are consistent with a greater predisposition to bone resorption.

Design: Wistar female rats were divided into ovariectomized (ovx) and non-ovariectomized (sham) groups, and subdivided into ad libitum diet (free diet), alcoholic diet (20% solution), and isocaloric diet (diet with a similar amount of calories as compared with groups ingesting an alcoholic diet). The alveolar bone crest and adjacent tissues were evaluated by immunohistochemical analyses for detection of OPG and RANKL.

Results: A significant decrease in OPG expression and a significant increase in RANKL expression were observed in ovariectomized animals which received alcohol as compared with non-ovariectomized animals which received isocaloric diet (experimental control). When estrogen deficiency was evaluated independently of the diet type, a significant decrease in OPG expression and a significant increase in RANKL expression were observed in ovariectomized animals as compared with non-ovariectomized animals.

Conclusions: Estrogen deficiency associated with alcoholic diet, as well as estrogen deficiency (analyzed independently of diet type), decreased the immunostaining for OPG and increased the immunostaining for RANKL in the periodontium of rats.

1. Introduction

Estrogen deficiency due to menopause is an important contributing factor for osteoporosis in women. Osteoporosis is a skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, which leads to increased risk of fractures (Lupsa & Insogna, 2015).

Despite the importance of the estrogen deficiency as an etiology for osteoporosis, osteoporosis remains a multifactorial disease, and several

other secondary risk factors may also be associated with an increased risk of loss of bone quality (Lupsa & Insogna, 2015). Excessive alcohol consumption is one of these factors (Gaddini, Turner, Grant, & Iwaniec, 2016; Mikosch, 2014).

Osteoporosis is a systemic disease that can affect a wide range of bones, including the alveolar bone. If the alveolar bone is affected by osteoporosis, there may be a higher risk of developing periodontal disease and tooth loss (Jonasson & Rythén, 2016; Penoni et al., 2017; Wang & McCauley, 2016).

Abbreviations: RANKL, receptor activator of nuclear factor-kappa β ligand; OPG, osteoprotegerin; ovx, ovariectomized; sham, non-ovariectomized; RANK, receptor activator of nuclear factor-kappa β

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There is suggestive evidence to support an association between osteoporosis and periodontal disease, especially when it is analyzed in large and well-controlled studies (Genco & Borgnakke, 2013). However, there is still a lack of consensus regarding this relationship, which needs to be further studied (Genco & Borgnakke, 2013; Guiglia et al., 2013). Periodontal disease is a chronic inflammatory alteration that gradually destroys the alveolar bone and soft tissue that support the teeth. Bacterial infection is considered the primary etiologic factor in periodontal disease. However, periodontal disease is also multifactorial in nature, and many other risk factors can modify the individual response to the presence of bacterial infection (Di Benedetto, Gigante, Colucci, & Grano, 2013; Genco & Borgnakke, 2013). Estrogen deficiency (Genco & Borgnakke, 2013; Lerner, 2006b) and alcohol consumption (Amaral Cda, Vettore, & Leao, 2009; Genco & Borgnakke, 2013) have been studied as potential risk factors associated with an increased susceptibility to a faster progression of periodontal disease. Estrogen deficiency (Luo et al., 2014; Macari et al., 2015) and alcohol consumption (Bannach et al., 2015) have been positively correlated with increased alveolar bone loss in animal studies. Accordingly, osteoporosis and periodontal disease are bone resorptive diseases (Guiglia et al., 2013) which share some risk factors, including estrogen deficiency (Genco & Borgnakke, 2013; Lerner, 2006b; Lupsa & Insogna, 2015) and alcohol consumption (Amaral Cda et al., 2009; Genco & Borgnakke, 2013; Mikosch, 2014).

An important mechanism involved in the regulation of bone resorption processes is the RANK/RANKL/OPG (receptor activator of nuclear factor-kappa β / receptor activator of nuclear factor-kappa β ligand / osteoprotegerin) system. The discovery and characterization of this system have resulted in important advances in the understanding of bone biology. RANK is a receptor that can be found on the surface of osteoclast progenitor cells, and RANKL is an osteoclastogenic cytokine. When RANKL binds to RANK there is a stimulus for osteoclast formation, thus increasing bone resorption. OPG is a protein that inhibits osteoclastogenesis and has a high affinity for RANKL. When OPG binds to RANKL, there is a decrease in the number of RANKL molecules available for binding to RANK, which reduces osteoclast formation and decreases bone resorption (Lerner, 2006a; Walsh & Choi, 2014).

It has been suggested that bone loss resulting from estrogen deficiency (Lerner, 2006a), alcohol consumption (Callaci, Himes, Lauing, Wezeman, & Brownson, 2009), or periodontal disease (Lerner, 2006b) could be associated with an increase in RANKL expression and/or decrease in OPG expression.

The aim of this study is to investigate RANKL and OPG immunorexpression in the periodontium of rats subjected to chronic alcoholism and/or estrogen deficiency. Our hypothesis is that estrogen deficiency and/or alcohol consumption increase RANKL expression and/or decrease OPG expression, thus predisposing to greater alveolar bone resorption at the molecular level.

2. Materials and methods

2.1. Animals treatment

This study was conducted in accordance with the ethical principles of animal experimentation and was independently reviewed and approved by the Ethics Committee of the Institute of Science and Technology, UNESP - Univ Estadual Paulista, São José dos Campos, São Paulo, Brazil (protocol # 05/2013-PA/CEP).

Ninety female Wistar rats (*Rattus norvegicus albinus*), three months old, were initially divided into two groups (randomly): ovariectomized (ovx) and non-ovariectomized (sham). In the ovariectomized animals, estrogen deficiency was induced by bilateral removal of ovaries. The non-ovariectomized animals were sham operated (ovaries were exposed but not removed). For all surgical procedures, animals received general anesthesia by intramuscular route. Drugs used in anesthetic procedures were xylazine chloride 2.3 g/100 ml (Anasedan - Vetbrands, Jacaré, SP, Brasil) and ketamine chloride 1.16 g/10 ml (Dopalen - Vetbrands, Jacaré, SP, Brasil). Initially, a master solution was prepared (0.8 ml of xylazine chloride was mixed with 0.5 ml of ketamine chloride). Then, animals received 0.1 ml of the master solution for each 100 g of body weight. After surgery, the animals received two medications: a poly-antibiotic with streptomycins and penicillins (0.1 ml/animal, Pentabático Pequeno Porte - Fort Dodge, Campinas, SP, Brazil) and a nonsteroidal anti-inflammatory, diclofenac sodium (0.3 ml/animal, Voltaren 75 mg/3 ml, Novartis, São Paulo, SP, Brazil). The poly-antibiotic and the anti-inflammatory were administered in a single dose via intramuscular route.

One month after surgery, these groups were subdivided in order to receive the following dietary treatments: ad libitum diet, alcoholic diet, and isocaloric diet. Ad libitum animals received commercial food for rats (freely) and water (freely). Alcohol animals received commercial food for rats (freely) and 20% alcohol solution (freely, as the only source of liquid). Isocaloric animals received commercial food for rats (in a restricted amount), 26.6% sucrose solution (in a restricted amount), and water (freely). Isocaloric animals received a diet containing the average amount of calories ingested the day before by alcohol animals.

At the beginning of dietary treatment, animals receiving alcohol were subjected to an adaptation period of nine days, in which concentrations of the alcoholic solution were increased gradually (5%, 10%, and 15%). From the tenth day, the dietary treatment with the alcoholic solution at 20% was initiated and then maintained for 61 days.

After the dietary treatment, animals were anesthetized and then euthanized by cardiac perfusion with 4% paraformaldehyde. The purpose of this procedure was to obtain better fixation of the tissues in order to improve the quality of Immunohistochemical analysis.

During dietetic treatment, amounts of liquid and solid food consumed by different experimental groups were evaluated. The following liquids were measured (ml/day/animal): a) water (for ad libitum animals), b) 20% alcoholic solution (for alcohol animals), and c) 26.6% sucrose solution (for isocaloric animals). Solid diet was a commercial food for rats (Labina - Purina, Paulínia, SP, Brazil), and it was measured in g/day/animal. Assessments of consumption of liquid and solid foods were performed considering the 61 days of dietary treatment. All animals were weighed at the time of surgical procedures (ovariectomy or sham surgery), and on the day they were euthanized. Weight changes were calculated in percentage.

For the alcohol groups, the percentage of daily calories from alcohol was calculated. Calculations were performed as previously published (Marchini et al., 2012, 2014).

In summary, the animals were divided into six main experimental groups: non-ovariectomized with ad libitum diet (sham/ad libitum), ovariectomized with ad libitum diet (ovx/ad libitum), non-ovariectomized with alcoholic diet (sham/alcohol), ovariectomized with alcoholic diet (ovx/alcohol), non-ovariectomized with isocaloric diet (sham/isocaloric), and ovariectomized with isocaloric diet (ovx/isocaloric).

The dietary aspects of this study, as well the surgical procedures, were based on previously published studies which also analyzed the influence of alcohol consumption (20%) and estrogen deficiency in the periodontium of rats (Alonso, Souza, Balducci, & Rocha, 2016; Marchini et al., 2012, 2014) or other rat tissues (de Deco et al., 2011; Lodi, Marchini, Santo, Rode, & Rocha, 2016; Salgado, Marchini, Tera, Rocha, & Marchini, 2015). However, it is important to highlight that the present study is not part of the abovementioned studies, as we used different animals and different analyses.

2.2. Immunohistochemical procedures

After euthanization, hemimandibles (left side) were placed in 10% formalin for 48 h. Samples were then decalcified in 10%

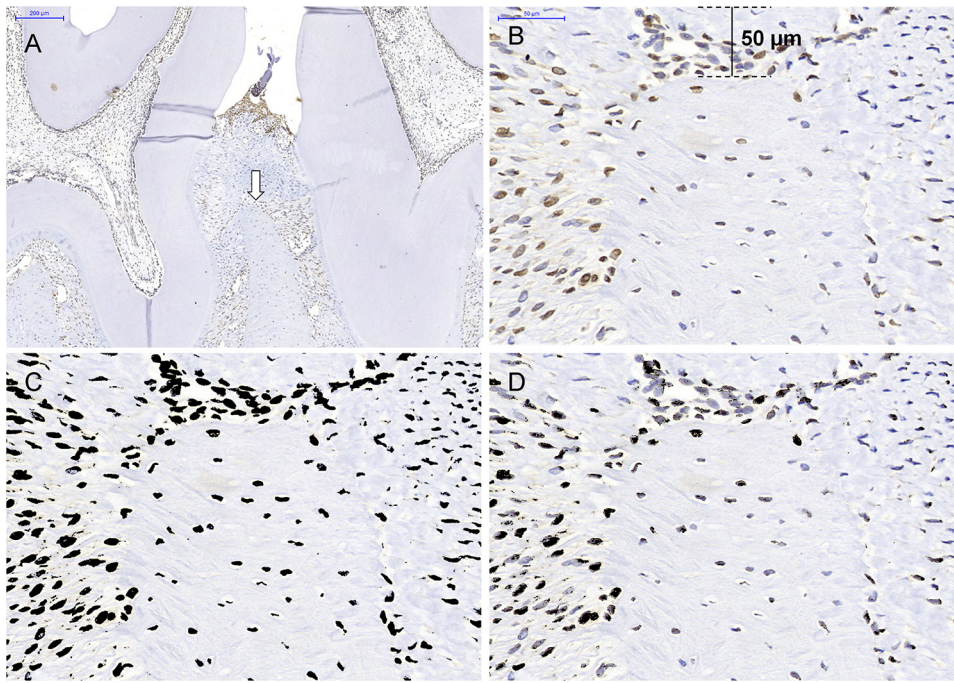


Fig. 1. A. Histological sections standardization (visualization of the alveolar bone crest (arrow) between the roots of the first and second molars (scale bar: 200 µm). B. Apex of the alveolar bone crest. In this region, the quantitative analyses of the percentage of immunostaining and the counting of the immunostained bone cells were performed. The annotation and the scale bar are illustrative and were removed at the time of analysis (sham/alcohol, RANKL; scale bar: 50 µm). C. First analysis - Cells were selected (both immunostained or not). The scale bar has been removed to avoid interference during analysis (sham/alcohol, RANKL). D. Second analysis - Immunostained area (present in the first analysis) was selected. The scale bar has been removed to avoid interference during analysis (sham/alcohol, RANKL). Abbreviations: sham = non-ovariectomized; RANKL = receptor activator of nuclear factor-kappa β ligand. Color online only.

ethylenediaminetetraacetic acid (EDTA) solution for later inclusion in paraffin. Histological sections (3 µm) were sliced at the region of the alveolar bone crest between the first and second mandibular molars and placed on silanized slides. The cuts were made so that it was possible to visualize the distal root of the first molar, the mesial root of the second molar and alveolar bone crest in the center (Fig. 1A).

The primary antibodies used in this study were RANKL (Ms mAb to RANKL, ab45039, Abcam, Cambridge, Cambridgeshire, United Kingdom) and OPG (Rb pAb to osteoprotegerin, ab73400, Abcam, Cambridge, Cambridgeshire, United Kingdom). The RANKL antibody is able to detect the membrane-bound form of RANKL.

Antigen retrievals were performed with pepsin pH 1.8 (initially at 60 °C for 10 min, then at 37 °C for 50 min). Endogenous peroxidase was blocked using hydrogen peroxide (20 vol) and methyl alcohol at a ratio of 1:1 (10 min). The elimination of non-specific antibodies was performed with 1% bovine serum albumin (30 min in humid chamber).

For the primary antibodies, the dilutions used were 1:50 for RANKL and 1:100 for OPG. Both RANKL and OPG were incubated at 4 °C for 18 h. Detection was performed by the EnVision system (DAKO, Carpinteria, California, United States) for 30 min. Staining was visualized by incubation with diaminobenzidine solution (DAB liquid, DAKO, Carpinteria, California, United States) for 5 min. The counterstaining was performed with Mayer's hematoxylin.

The following positive controls were used: for RANKL, lymph node of Wistar rats, which marks the germinal center and lymphocytes; for OPG, liver of Wistar rats, which marks hepatocytes. Negative controls were obtained by omission of primary antibodies.

Slides were processed using an incubator box with a capacity for 18 slides; one for the positive control, one for the negative control, and the remaining 16 spaces were divided among the six experimental groups at a 3:3:3:3:2:2 ratio. This method reduced the likelihood that technical problems had an influence on the final results.

After immunohistochemical procedures, slides were scanned (Pannoramic DESK, 3DHitech, Budapest, Hungary). Histological sections were viewed using Pannoramic Viewer 1.15.4 program. The slides were renamed for a blind analysis.

When the immunostaining in the area of interest was very low, an evaluation of distant areas of the same slide was taken (usually in the mandibular canal region). This procedure was performed in order to

confirm that the lack of immunostaining was not due to technical problems.

2.3. Immunohistochemical analysis in the apex of the alveolar crest

The analyses were performed in a region previously standardized by a picture. First, a picture of each histological section was taken (magnified 40X from the scanned image using Pannoramic Viewer 1.15.4 program). This picture was taken with the apex of the alveolar bone crest located in the center portion, in order to allow the evaluation of cells near the alveolar bone crest, about 50 µm of space was left between the apex of the alveolar crest and the top of the picture (Fig. 1B).

2.3.1. Percentage of immunostaining

The pictures were analyzed in the computer program NIH ImageJ using the tool Image/Adjust/Threshold (public domain, <http://rsb.info.nih.gov/ij/>). Two analyses were performed for each picture. In the first analysis (A1), an estimate of the total area occupied by cells was performed. To do so, a selection of the cells (immunostained or not) was performed (Fig. 1C). In order to standardize the analysis, the selection of the cells stopped when the program began selecting the bone matrix. In the second analysis (A2), only the immunostained area (which was included in the first analysis) was selected (the area marked in brown was kept and the area in blue was excluded) (Fig. 1D). From these data, an estimation of the immunostained area was performed using the following formula $A2/A1 \times 100\%$.

Considering the above-mentioned adaptations, a step-by-step approach to this technique was previously outlined at https://openwetware.org/wiki/?title=Sean_Lauber:ImageJ_-_Threshold_Analysis&oldid=595746 (Lauber, 2012).

2.3.2. Number of immunostained bone cells

Immunostained bone cells were counted at the apex of the alveolar bone crest. This assessment was made using the same standardized pictures which had previously been used for quantitative analysis of the percentage of immunostaining (Fig. 1B). Bone cells were evaluated according to their morphology and location, as previously suggested (Marchini et al., 2014). Uninuclear cells in close contact with the surface of the bone crest were considered to be osteoblasts, cells entrapped

within the bone matrix were considered to be osteocytes, and multinuclear cells in close contact with the surface of the bone crest were considered to be osteoclasts. For this evaluation, the intensity of immunostaining was not considered (the cells were only considered immunostained or not).

The results regarding total number of immunostained bone cells in each picture (sum of the number of immunostained osteoblasts, osteocytes, and osteoclasts) were submitted for statistical analysis.

2.4. Immunostaining analysis of inflammatory cells and fibroblasts

Inflammatory cells were evaluated in the gingival connective tissue located between first and second lower molars. Mononuclear inflammatory cells and polymorphonuclear inflammatory cells were considered in the analyses. In this study, only cells with morphological features similar to lymphocytes or plasma cells were considered as mononuclear inflammatory cells. On the other hand, only cells with morphological features similar to polymorphonuclear leukocytes (segmented nucleus) were considered as polymorphonuclear inflammatory cells.

Fibroblasts were evaluated in a wide area that included gingival connective tissue and the area of the periodontal ligament (region between the first and second lower molars).

These evaluations were performed semi-quantitatively using a numerical scale (scores) from 0 to 3 as follows: 0 (no immunostained fibroblasts or inflammatory cells were observed, or immunostaining was less than 10%); 1 (few immunostained fibroblasts or inflammatory cells, about 10% to 40%); 2 (moderate amount of immunostained fibroblasts or inflammatory cells, about 40% to 70%); and 3 (large amount of immunostained fibroblasts or inflammatory cells, more than 70%).

As the semi-quantitative analyses were considered more susceptible to the interpretation of the examiner (as compared with others analyses performed in this study), these evaluations were performed twice for each animal.

In order to verify the consistency of the measurements, the data obtained in the first and second analyses were used to calculate the intraclass correlation coefficient.

The values obtained in the first and second analyses were also used to calculate the average value for each animal, and the average value was used to perform the other statistical analyses.

2.5. Statistical analysis of diet and weight values

Five animals died before the end of the experiment (one in ovx/alcohol, one in ovx/isocaloric and three in ovx/ad libitum), altering the final sample size. More details regarding the final number of animals at the end of dietetic treatment can be seen as online supplementary materials (as described at the legend of Fig. S1).

The statistics for the data regarding diet and weight were performed using analysis of variance, ANOVA GLM (General Linear Models), and the Tukey test. P-values less than 0.05 were considered statistically significant.

The programs SPSS Statistics for Windows (version 17, IBM, Chicago, Illinois, United States), Minitab (Version 16, Minitab, State College, Pennsylvania, United States), and Microsoft Excel 2007 (Microsoft Corporation, Redmond, Washington, United States) were used to perform the statistical analysis and graphics.

2.6. Statistical analysis of immunostaining

In this study, adequate standardization of histological sections, as described previously in materials and methods and Fig. 1A, was a factor that limited the final number of animals analyzed in each experimental group. Technical difficulties were found due to the reduced size of the area to be analyzed and problems in obtaining adequate angulations at

the moment of inclusion in paraffin as well as during the cuts. After selecting the best histological sections, the final number of animals analyzed per group was seven (for the majority of the analyses) and six (for some analyses). More details regarding the final number of animals analyzed can be seen in the legends of the figures S2, S3, S4, S5 and S6 (supplementary material available online).

The program SPSS Statistics for Windows (version 20, IBM, Chicago, Illinois, United States) was used to perform the calculation of the intraclass correlation coefficients (95% confidence interval).

Data concerning the percentage of immunostaining, total number of immunostained bone cells, analysis of inflammatory cells and analysis of fibroblasts were submitted to normality testing (Kolmogorov-Smirnov test) and were deemed parametric. Statistical analysis was performed using analysis of variance, one-way ANOVA, and the Tukey test. When only two groups were compared (ovariectomized versus non-ovariectomized) the unpaired *t*-test was used. The programs GraphPad Prism 5.1 software (San Diego, California, United States) and Microsoft Excel 2007 (Microsoft Corporation, Redmond, Washington, United States) were used to perform the statistical analysis and graphics. For all the analyses *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Diet and weight

The results regarding liquid diet (ml/day/animal) showed that the group sham/ad libitum ingested statistically more liquids than all other groups ($p < 0.001$; sham/ad libitum vs. ovx/alcohol); ($p < 0.001$; sham/ad libitum vs. ovx/isocaloric); ($p < 0.001$; sham/ad libitum vs. ovx/ad libitum); ($p < 0.01$ (sham/ad libitum vs. sham/alcohol) ($p < 0.001$; sham/ad libitum vs. sham/isocaloric). On the other hand, the group that ingested fewer liquids, ovx/alcohol, was statistically different only from the sham/ad libitum ($p < 0.001$) and sham/alcohol ($p < 0.001$).

The results regarding solid diet (g/day/animal) showed that the groups with ad libitum diet (ovx/ad libitum and sham/ad libitum) ingested the largest amount of solid diet, significantly differing from all groups with controlled diet (sham/alcohol, sham/isocaloric, ovx/isocaloric and ovx/alcohol) ($p < 0.001$; ovx/ad libitum vs. sham/alcohol); ($p < 0.001$; ovx/ad libitum vs. sham/isocaloric); ($p < 0.001$; ovx/ad libitum vs. ovx/isocaloric); ($p < 0.001$; ovx/ad libitum vs. ovx/alcohol); ($p < 0.001$; sham/ad libitum vs. sham/alcohol); ($p < 0.001$; sham/ad libitum vs. sham/isocaloric); ($p < 0.001$; sham/ad libitum vs. ovx/isocaloric); ($p < 0.001$; sham/ad libitum vs. ovx/alcohol). The group that ingested the largest amount of solid diet was ovx/ad libitum, significantly differing from all others groups ($p < 0.001$; ovx/ad libitum vs. ovx/alcohol); ($p < 0.001$; ovx/ad libitum vs. ovx/isocaloric); ($p < 0.001$; ovx/ad libitum vs. sham/alcohol); ($p < 0.001$; ovx/ad libitum vs. sham/isocaloric); ($p < 0.01$; ovx/ad libitum vs. sham/ad libitum). On the other hand, the group that ingested the least amount of solid diet was ovx/alcohol, significantly differing from groups with ad libitum diet (ovx/ad libitum and sham/ad libitum) ($p < 0.001$; ovx/alcohol vs. ovx/ad libitum); ($p < 0.001$; ovx/alcohol vs. sham/ad libitum).

All animals gained weight during the experiment. The results regarding changes in weight (%) showed that the group with the most weight gain was ovx/ad libitum, significantly differing from all others groups ($p < 0.001$; ovx/ad libitum vs. ovx/alcohol); ($p < 0.001$; ovx/ad libitum vs. ovx/isocaloric); ($p < 0.001$; ovx/ad libitum vs. sham/alcohol); ($p < 0.001$; ovx/ad libitum vs. sham/isocaloric); ($p < 0.001$; ovx/ad libitum vs. sham/ad libitum). On the other hand, the group with the least weight gain was sham/alcohol, which was different from all others groups except sham/isocaloric ($p < 0.01$; sham/alcohol vs. ovx/alcohol); ($p < 0.001$; sham/alcohol vs. ovx/isocaloric); ($p < 0.001$; sham/alcohol vs. ovx/ad libitum); ($p < 0.01$;

sham/alcohol vs. sham/ad libitum).

In this study, the ovx/alcohol group ingested an average of 27.06 ml/day/animal of 20% alcoholic solution and 11.66 g/day/animal of solid food. On the other hand, the sham/alcohol group ingested an average of 33.93 ml/day/animal of 20% alcoholic solution and 13.06 g/day/animal of solid food. The proportion of daily calories from alcohol for the ovx/alcohol and sham/alcohol groups was 52.09% and 54.89%, respectively (average = 53.49%).

Graphics with more details regarding the analyses of the liquid diet, solid diet, and weight changes are available as online supplementary materials (Figure S1).

3.2. Immunohistochemical analyses

In the slides immunostained for RANKL and OPG, two quantitative analyses (percentage of immunostaining, and total number of immunostained bone cells) and two semi-quantitative analyses (analysis of fibroblasts, and analysis of inflammatory cells) were performed.

Statistical analyses were performed considering all six main experimental groups; the hormonal condition, analyzed independently of diet type (ovariectomy or sham surgery); and the different diet types, analyzed independently of the hormonal condition (alcohol, isocaloric, ad libitum). For the analysis of hormonal condition and diet type, the same animals from the six main groups were evaluated, then subdivided according to the condition to be analyzed.

When comparing all six main experimental groups, for all analyses (percentage of immunostaining, the total number of immunostained bone cells, analysis of fibroblasts, and analysis of inflammatory cells), the lowest immunostaining for OPG and the highest immunostaining for RANKL were observed in the group with estrogen deficiency that received alcohol (ovx/alcohol). Conversely, the highest immunostaining for OPG and the lowest immunostaining for RANKL were observed in groups without estrogen deficiency that did not receive alcoholic diet (sham/isocaloric and sham/ad libitum). In all the analyses, for both antibodies, the group ovx/alcohol was statistically different from the group sham/isocaloric ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; percentage of immunostaining for OPG); ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; percentage of immunostaining for RANKL); ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; total number of immunostained bone cells for OPG); ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; total number of immunostained bone cells for RANKL); ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; analysis of fibroblasts for OPG); ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; analysis of fibroblasts for RANKL); ($p < 0.05$; ovx/alcohol vs. sham/isocaloric; analysis of inflammatory cells for OPG); ($p < 0.001$; ovx/alcohol vs. sham/isocaloric; analysis of inflammatory cells for RANKL). Considering all the analyses (percentage of immunostaining, the total number of immunostained bone cells, analysis of fibroblasts, and analysis of inflammatory cells) and all antibodies (OPG and RANKL), no difference between the groups sham/isocaloric and sham/ad libitum was observed ($p > 0.05$).

When the hormonal condition was analyzed (independently of diet type) ovariectomized animals had lower immunostaining for OPG and higher immunostaining for RANKL as compared with non-ovariectomized animals, and these differences were statistically significant for both antibodies and for all analyses ($p < 0.001$; ovx vs. sham; percentage of immunostaining for OPG); ($p < 0.01$; ovx vs. sham; percentage of immunostaining for RANKL); ($p < 0.001$; ovx vs. sham; total number of immunostained bone cells for OPG); ($p < 0.001$; ovx vs. sham; total number of immunostained bone cells for RANKL); ($p < 0.01$; ovx vs. sham; analysis of fibroblasts for OPG); ($p < 0.001$; ovx vs. sham; analysis of fibroblasts for RANKL); ($p < 0.05$; ovx vs. sham; analysis of inflammatory cells for OPG); ($p < 0.001$; ovx vs. sham; analysis of inflammatory cells for RANKL).

Considering only the average values, when a comparison was performed between the three types of diets (independently of the hormonal

condition), lower immunostaining for OPG and higher immunostaining for RANKL was observed in alcohol animals as compared with isocaloric and ad libitum animals, for all the analyses. However, the difference between alcohol animals and animals that did not receive alcohol was not statistically different for all the analyses. In the analysis of the total number of immunostained bone cells for OPG, no statistical difference ($p > 0.05$) between the animals which received alcohol and animals which received ad libitum diet was observed. In the analysis of fibroblasts for RANKL, no statistical difference ($p > 0.05$) between the animals which received alcohol and the animals which received ad libitum diet was observed; In the analysis of inflammatory cells for both antibodies (OPG and RANKL) no statistical difference ($p > 0.05$) between the animals which received alcohol and the animals which did not receive alcohol (ad libitum and isocaloric) was observed. The groups isocaloric and ad libitum were not statistically different from each other ($p > 0.05$), for all analyses (percentage of immunostaining, the total number of immunostained bone cells, analysis of fibroblasts, and analysis of inflammatory cells) and for both antibodies (OPG and RANKL).

Graphics with more details regarding the analysis of the percentage of immunostaining, analysis of the total number of immunostained bone cells, analysis of fibroblasts, and analysis of inflammatory cells are available as online supplementary materials (Figs. S2, S3, S4, S5).

3.3. Types of cells immunostained

In this study, several cells considered to be osteoblasts (mononuclear cells in close contact with the bone crest) were immunostained. The immunostaining for osteocytes (cells entrapped in the bone matrix) was more rarely observed. When the immunostaining for osteocytes was observed, it was more frequently for RANKL than for OPG. Osteoclasts (multinuclear cells in close contact with alveolar bone) were rarely observed, some of which showed immunostaining for OPG or RANKL.

Examples of cells considered as osteoblasts, osteocytes, and osteoclasts can be seen in Fig. 2, in slides immunostained for RANKL (Figs. 2A and 2C), and slides immunostained for OPG (Figs. 2B and 2D).

Periodontal ligament fibroblasts and gingival connective tissue fibroblasts were found immunostained for RANKL and OPG. A qualitative analysis showed that for the majority of the slides (regardless the experimental group and type of antibody) the immunostaining was more frequent for periodontal ligament fibroblasts than for gingival connective tissue fibroblasts (Fig. 2E).

Inflammatory cells were found immunostained for RANKL and OPG. However, the immunostaining of inflammatory cells was much more frequent for RANKL than for OPG. Some examples of inflammatory cells found in this study can be seen in the Fig. 2F.

Graphics with more details regarding the types of bone cells found immunostained for each experimental group, analysis of fibroblasts, and analysis of inflammatory cells are available as online supplementary materials (Figs. S4, S5 and S6).

3.4. Intraclass correlation coefficient

The intraclass correlation coefficients were calculated for the semi-quantitative analyses (fibroblasts and inflammatory cells). Considering all the analyses and all the antibodies the intraclass correlation coefficient was 0.90 (p -value < 0.001). Values between 0.75 and 0.90 are indicative of good reliability and greater than 0.90 are indicative of an excellent reliability (Koo & Li, 2016). More details regarding the intraclass correlation coefficients (and respective p -values) for each group, type of analysis and antibody can be seen in Table S1 (available as online supplementary material).

4. Discussion

The results of the immunohistochemical analyses were considered

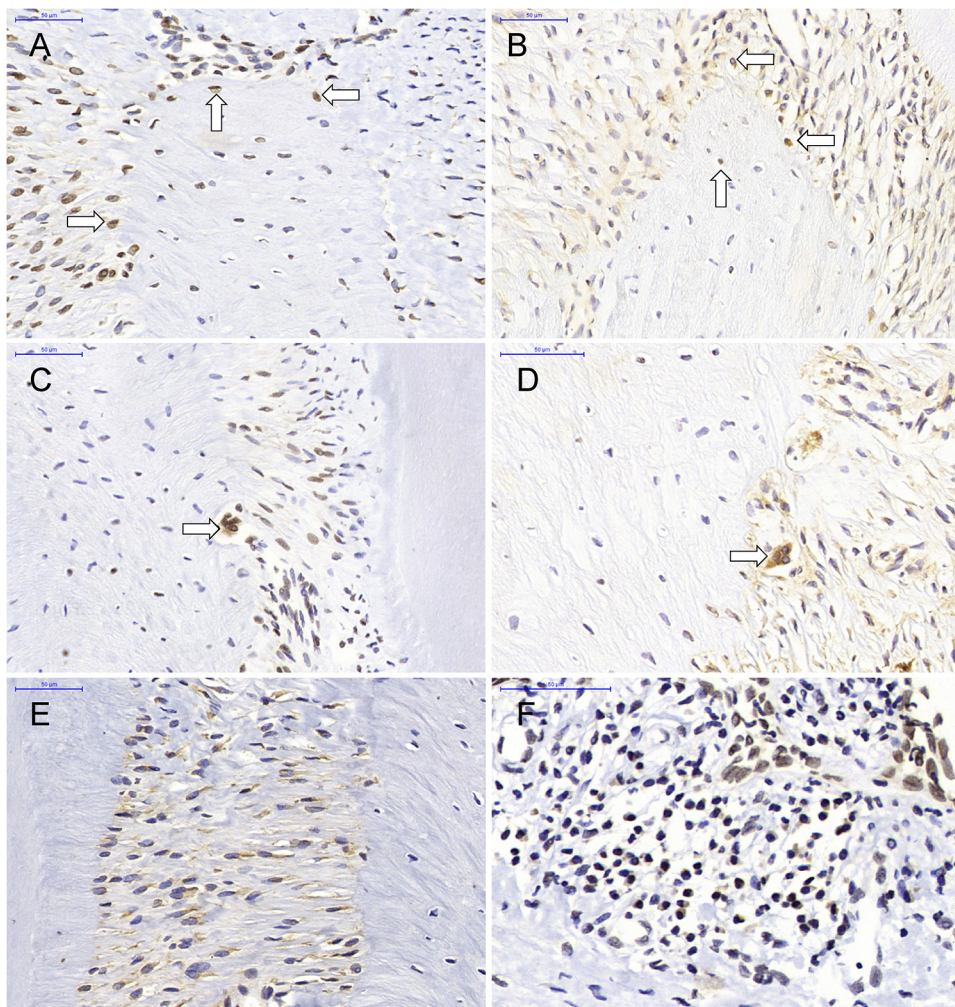


Fig. 2. A. The horizontal arrows show examples of cells considered to be osteoblasts. The vertical arrow shows an example of a cell considered to be an osteocyte (sham/alcohol, RANKL; scale bar: 50 µm). B. The horizontal arrows show examples of cells considered to be osteoblasts. The vertical arrow shows an example of a cell considered to be an osteocyte (sham/isocaloric, OPG; scale bar: 50 µm). C. The arrow shows an example of a cell considered to be an osteoclast (ovx/isocaloric, RANKL; scale bar: 50 µm). D. The arrow shows an example of a cell considered to be an osteoclast (sham/isocaloric, OPG; scale bar: 50 µm). E. Periodontal ligament with several fibroblasts immunostained for OPG (ovx/isocaloric, OPG; scale bar: 50 µm). F. Gingival connective tissue with several inflammatory cells immunostained for RANKL (ovx/alcohol, RANKL; scale bar: 50 µm). Abbreviations: ovx = ovariectomized; sham = non-ovariectomized; OPG = osteoprotegerin; RANKL = receptor activator of nuclear factor-kappa β ligand. Color online only.

important to the conclusions of this study only when all the analyses (percentage of immunostaining, the total number of immunostained bone cells, analysis of fibroblasts, and analysis of inflammatory cells) showed similar results.

For all the analyses, a significant decrease in OPG expression and a significant increase in RANKL expression in ovariectomized animals which received alcohol (ovx/alcohol) as compared with non-ovariectomized animals which received isocaloric diet (sham/isocaloric) were observed. It should be considered that the group sham/isocaloric was the most important experimental control of this study because it allowed controlling not only the hormonal condition but also for a possible influence of the dietary treatment on the results. For all the analyses, when estrogen deficiency was evaluated independently of the diet type, a significant decrease in OPG expression and a significant increase in RANKL expression were observed in ovariectomized animals as compared with non-ovariectomized animals. These results confirmed the initial hypothesis in which estrogen deficiency and/or alcohol consumption could be related to an increase RANKL expression and/or decrease OPG expression.

RANKL is a protein that stimulates the process of bone resorption, and OPG is a protein with high affinity to RANKL which has inhibitory effects on bone resorption (Lerner, 2006a; Walsh & Choi, 2014). Considering this, decreased OPG expression in conjunction with increased RANKL expression could be related to a greater predisposition for alveolar bone resorption (Lerner, 2006b).

It should be considered that both estrogen deficiency (Lerner, 2006a; Lupsa & Insogna, 2015) and alcohol consumption (Mikosch, 2014) are considered possible risk factors for osteoporosis. Other

studies suggest that estrogen deficiency (Eghbali-Fatourehchi et al., 2003; Lerner, 2006a) and alcohol intake (Callaci et al., 2009; Chen, Shankar, Nagarajan, Badger, & Ronis, 2008) may be associated with increased RANKL and/or decreased OPG, which would be compatible with an increased risk of osteoporosis.

In animal studies, treatment with intraperitoneal injections of OPG impaired osteoclast activity and reduced trabecular bone loss in ovariectomized mice (Shimizu-Ishijima, Kawana, & Sasaki, 2002). In another study, intraperitoneal injections of OPG inhibited osteoclastogenesis induced by alcohol consumption and promoted proliferation of osteoblasts (Zhang et al., 2002).

In the present study, OPG and RANKL expression were observed in periodontal tissues. A study in humans found a decrease in OPG expression in gingival crevicular fluid, saliva, and gingival tissues in individuals with periodontal disease (Hassan, El-Refai, Ghallab, Kasem, & Shaker, 2015). In another study, high levels of RANKL and low levels of OPG were observed in patients with periodontal disease (Crotti et al., 2003).

In this study, animals on an alcoholic diet in conjunction with estrogen deficiency (ovx/alcohol) showed the lowest values of immunostaining for OPG and the greatest values of immunostaining for RANKL in all analyses. Other studies have also observed negative effects of alcoholic diet in conjunction with estrogen deficiency in vertebrae (Callaci, Juknelis, Patwardhan, & Wezeman, 2006), femur (Lodi et al., 2016), and periodontal tissues (Alonso et al., 2016; Marchini et al., 2012). MARCHINI et al. (Marchini et al., 2012) found a decrease in the calcium/phosphorus ratio in the apex of the alveolar bone crest when the ovariectomy was combined with alcohol consumption. ALONSO

et al. (Alonso et al., 2016) found an increase in the severity of the inflammatory process and disorientation of the periodontal ligament fibers when ovariectomy and alcohol consumption were combined. It is interesting to highlight that these results were observed even without experimental periodontitis induction, in the abovementioned studies (Alonso et al., 2016; Marchini et al., 2012). In the present study, the animals also were not subjected to experimental periodontitis induction.

Although the animals with the alcoholic diet had free access to solid food, they ate significantly less compared to animals with the ad libitum diet. Alcohol is a substance with high caloric value (7.1 Kcal/g), which usually causes the animal to replace nutritionally important foods with alcohol (Lieber, 2000; Marchini et al., 2012, 2014). In the present experiment, to control the influence of this variable, we used an isocaloric diet, in which the animals received a similar amount of calories as the alcoholic diet. One must consider that proper nutrition is important for maintaining bone quality (Kueper, Beyth, Liebergall, Kaplan, & Schroeder, 2015). Furthermore, lower food consumption is also related to lower incidence of masticatory forces with the isocaloric and alcoholic diets (compared to the ad libitum diet) and, theoretically, mechanical forces generated by masticatory forces could locally alter the metabolism of alveolar bone (Mavropoulos, Kiliaridis, Rizzoli, & Ammann, 2014). However, no statistically significant differences were observed in the present study regarding immunostaining for RANKL and OPG when the ad libitum and isocaloric diets were compared. Therefore, it is not possible to affirm that immunohistochemical results were due to differences in food consumption or the influence of masticatory forces.

Results showed that over 50% of daily calories were coming from alcohol. It has been suggested that the consumption of alcohol in amounts exceeding 10% of the daily energy requirements can be considered excessive consumption (Gaddini et al., 2016). Based on this assumption, the animals in the present study consumed alcohol excessively. However, this statement should be viewed with caution, as it will be discussed in the limitations of this study.

All cell types evaluated in this study (bone cells, inflammatory cells, and fibroblasts) showed immunostaining for RANKL and OPG.

Regarding bone cells, it was observed that uninuclear cells in close contact with the alveolar bone crest (considered to be osteoblasts) were those that exhibited higher amounts of immunostaining. These cells are important sources of RANKL and OPG, not only because of the frequency with which they express these proteins, but also by their physical proximity to the bone tissue, which emphasizes the importance of these cells in regulating bone metabolism (Kearns, Khosla, & Kostenuik, 2008).

Osteocytes were also found immunostained for RANKL and OPG. Currently, osteocytes are no longer considered as the final destination of osteoblasts that were trapped in the bone matrix. It is known that osteocytes can play an important role in the regulation of bone metabolism. The expression of RANKL and OPG is one of the multiple ways osteocytes can regulate bone metabolism (Bellido, 2014).

Some multinuclear cells (considered as osteoclasts) were visualized immunostained for RANKL and OPG. RANKL expression on osteoclasts can be readily explained by its affinity for RANK receptors (present on the surface of osteoclast progenitor cells) (Lerner, 2006a; Walsh & Choi, 2014). On the other hand, OPG expression in osteoclasts could be more difficult to understand, as OPG is known for having a protective role against bone loss while osteoclasts are cells responsible for bone resorption. A possible explanation could be related to an increase in apoptosis rates of osteoclasts and osteoclast precursor cells in the presence of OPG (Kang et al., 2014; Liu et al., 2015). It would help to understand the protective role of OPG against bone loss, even when observed in osteoclasts.

Fibroblasts, especially those present in the periodontal ligament, were frequently immunostained for OPG and RANKL. Periodontal ligament fibroblasts can play an important role in the remodeling of the

alveolar bone, and the RANK / RANKL / OPG pathway is considered very important in this process (Sokos, Everts, & de Vries, 2015).

In this study, immunostaining for inflammatory cells was found more often for RANKL as compared to OPG. The expression of RANKL in inflammatory cells deserves special attention since inflammatory cells (B and T lymphocytes) were considered the primary sources of RANKL in bone resorptive lesions associated with periodontal disease (Kawai et al., 2006).

One of the analyses performed in this study (percentage of immunostaining) used a computer program which provided a good estimation of the amount of immunostaining. However, this technique has some limitations since it can be difficult to distinguish immunostained cells from non-immunostained cells with absolute precision using this method.

This study has other limitations. Based on the analysis of alcohol consumption, animals consumed alcohol excessively, as previously discussed. However, analysis of blood alcohol concentration would be needed to confirm this assumption. It is also important to note that rats consumed alcoholic solution by a self-administration process, and the amount of alcohol consumed was calculated from the amount offered and remaining solution, which did not allow control for any losses of alcoholic solution during the intake process.

In this study, the cells considered osteoclasts were detected by simple visual inspection as multinuclear cells in close contact with the alveolar bone crest as previously described (Marchini et al., 2014). However, this type of method has limitations and this is not considered a very accurate method to detect osteoclasts. One example of a more accurate method to detect osteoclasts is the staining for tartrate-resistant acid phosphatase (TRAP). This method could be considered in future studies.

5. Conclusion

The estrogen deficiency in conjunction with an alcoholic diet, as well as the estrogen deficiency (analyzed independently of the type of diet), decreased the immunostaining for OPG and increased the immunostaining for RANKL in the apex of the alveolar bone crest and adjacent tissues in rats.

ETHICAL APPROVAL

This research was approved by the Ethics Committee of the Institute of Science and Technology, UNESP - Univ Estadual Paulista, São José dos Campos, São Paulo, Brazil (protocol # 05/2013-PA/CEP).

Conflicts of interest

The authors declare they have no conflict of interest related to this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.archoralbio.2018.07.006>.

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