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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 immunoexpression 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 novergicus 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, Jacareí, SP, Brasil) and ketamine chloride 1.16 g/10 ml (Dopalen – Vetbrands, Jacareí, 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, Pentabiótico 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%