Assessing the Action of Phytoestrogens on Alveolar Bone Tissue of Female of Ovariectomized Rats with Induced Periodontal Disease

Carolina de Cassia Boamorte
Master's degree in Biosciences and Health
State University of Western Parana (UNIOESTE)
Cascavel, Parana, Brazil.

Laís Fernanda Pasqualotto
Academic of course in Dentistry
State University of Western Parana (UNIOESTE)
Cascavel, Parana, Brazil.

Rose Meire Costa Brancalhão
Adjunct Professor
Center of Biological Science and Health and Graduate Program in Bioscience and Health
State University of Western Parana (UNIOESTE),
Cascavel, Parana, Brazil.

Lucinéia de Fátima Chasko Ribeiro
Adjunct Professor
Course in Center of Biological Science and Health and Graduate Program in Bioscience and Health,
State University of Western Parana (UNIOESTE)
Cascavel, Parana, Brazil.

Patricia Oehlmeyer Nassar
Associate Professor of course in
Periodontics and Graduate Programs in Bioscience, Health and Dentistry
State University of Western Parana (UNIOESTE)
Cascavel, Parana, Brazil

Sara Cristina Sagae
Adjunct Professor
Course in Center of Medical and Pharmaceutical Sciences
State University of Western Parana (UNIOESTE
Cascavel, Parana, Brazil.

Carlos Augusto Nassar
Associate Professor of course in Periodontics
Graduate Programs in Bioscience, Health and Dentistry
State University of Western Parana (UNIOESTE)
Cascavel, Parana, Brazil.

Abstract

Osteoporosis can be a risk factor for periodontal disease. One of the alternatives for preventing bone loss under these conditions is hormone replacement therapy with isoflavone, a phyto-hormone similar to estrogen. The objective of this paper was to assess the action of isoflavone on alveolar bone tissue of female ovariectomized rats with induced periodontal disease.

Sixty female rats were randomly divided into 6 groups: Control (CON); Ligature (LIG); Ovariectomy (OVX); Ovariectomy and Isoflavone (OVX+ISO); Ovariectomy and Ligature (OVX+LIG); Ovariectomy, Isoflavone and Ligature (OVX+ISO+LIG); all animals were treated with a standard diet and water ad libitum. Experimental periodontitis was induced when the animals were 70 days old and then submitted to ovariectomy surgery after 79 days. The rats were euthanized when they were 100 days old and the mandibles dissected for macroscopic, microscopic and radiographic analysis. The result of the analysis showed that the groups OVX+ISO and OVX+ISO+LIG presented less alveolar bone loss than the groups that did not receive isoflavone therapy (OVX+LIG and LIG) ($p< 0.05$). Therefore, isoflavone presents a protection effect on alveolar bone loss and progression of periodontal disease enhanced by osteoporosis.

Key-words: periodontitis, post-menopause, osteoporosis, isoflavone.

1. Introduction

Menopause, referred to as the last menstruation, is a period of inactive reproduction, which causes a decrease in hormone production in women, mainly estradiol, preventing endothelium growth. Such cycle of the female reproduction system, also known as the climacteric period, has biological, psychological and social consequences. Hypoestrogenism after menopause can cause physiological changes over the course of years (Wise et al., 1999). During post-menopause, biological consequences cause diseases due to hormone changes, among them, those responsible for the decrease in bone mass, making women susceptible to developing osteoporosis. This bone metabolic disease, considered a systemic dysfunction, changes the micro architecture of the bones, including the face ones, which can influence, among other diseases, the progression of periodontal diseases (Lerner, 2006). Another disease that greatly affects a large part of the population is periodontitis. Due to its poly microbial nature, it orchestrates a complex mechanism of inflammation that is characterized by the destruction of collagen fibers, among other components of the periodontal ligature matrix and the alveolar bone, together with the formation of periodontal pocket (Rivera et al., 2013; Sakalauskiene et al., 2014). It is predominantly caused by bacteria that release endotoxins that activate pro-inflammatory cytokines (interleukin 1 - IL1; alfa tumoral necrosis factor - TNF-α; among others) that affect the tooth-supporting tissues (Nishimura & Murayama, 2001; Yakob et al., 2012).

Osteoporosis as well as periodontal disease cause bone loss, and the relationship between the two diseases is the interaction of the systemic factors of osteoporosis with the periodontal factors, intensifying the alveolar bone loss pattern. The literature indicates that the relationship of postmenopausal osteoporosis with the progression of periodontal disease has been found to be positive (Mohammad & Brunsvold, 1996). Hormone replacement therapy with estrogen has been used for women on the post-menopause, although studies have shown the negative side effects of such replacement, such as the risk for developing cardiac diseases and breast cancer. The practice of hormone replacement can bring some benefits though, including osteoporosis control. (Chlebowski et al., 2013; Amatykul et al., 2007). Studies such as the Women's Health Initiative Randomized Trial (WHI), at 2003, (Chlebowski et al., 2013) showed that the combined use of estrogen and progesterone could stimulate the development of breast cancer. Thus, other therapies have been considered since similar compounds to estrogen have not shown many side effects. Those therapies include the use of soy isoflavone, a phytoestrogen that has been pointed out to be effective and well-accepted for such objective (Colditz et al., 1995; Brandi, 1997; Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Amatykul et al., 2007).

In a longitudinal study (Aquino et al., 2016) in Brazil with 3.281 women from the southern, northwest and southwest regions, surveyed which women still used the hormone replacement therapy (HRT) and found that 27.4% of women used this therapy. In a study conducted by the North Association of Menopause for Clinical Care for middle-aged women, recommended hormonal therapy was as follows: the use of HRT could be appropriate for symptomatic women or to prevent osteoporosis (Shifren et al., 2014). Evidences showed that isoflavone protects against several chronic diseases, prevents postmenopausal bone loss and osteoporosis. Isoflavone would act on the bone cells as it can present a suppressor effect on the number of osteoclasts (Gao & Yamaguchi, 2000; Tanaka et al., 2008). The relationship between osteoporosis and post-menopause has been considered a risk factor for periodontal disease but the mechanism of action have not been clearly described (Tanaka et al., 2008). Therefore, the objective of this paper was to assess the action of isoflavone on alveolar bone tissue of female ovariectomized rats with induced periodontitis.

2. Methodology

This quantitative and experimental research was conducted at the Vivarium of the Biological Sciences and Health Department (CCBS - Biotério Central do Centro de Ciências Biológicas e da Saúde) of the State University of Western of Parana, in the city of Cascavel, Parana, Brazil.

2.1. Animals

A total of 60 Wistar rats (body weight between 180-350 g) obtained from the Central Vivarium of the Biological Sciences and Health Department of the State University of Western of Parana (UNIOESTE) were used in the study. They were kept in the laboratory of Physiology and Biophysics at CCBS in individual cages and exposed to a light-dark cycle (lights on at 7 A.M and off at 7 P.M) and controlled temperature, and received food and water ad libitum. They were separated into individual cages in groups of 3-5 animals in each cage (41 cm length X 34 cm width X 17 cm height). The project was approved by the Animal Ethics Committee (CEUA) at UNIOESTE, in accordance with the ethical principles for animal experimentation adopted by the National Board for animal experimentation (CONCEA).

2.2. Experimental group

Animals were divided into 6 groups, 10 rats in each group, number based on a previous study conducted by the research group (Nassar et al., 2009), totaling 60 rats for the experiment.

1- Control Group (CON);
2- Ligature Group (LIG): ligature-induced periodontal disease;
3- Ovariectomized Group (OVX): female rats that underwent ovariectomy surgery;
4- Ovariectomy and Isoflavone Group (OVX+ISO): female rats that underwent ovariectomy and hormone replacement with isoflavone;
5- Ligature and Ovariectomy Group (OVX+LIG): rats that underwent ovariectomy surgery and ligature-induced periodontitis.
6- Ovariectomized, Isoflavone and Ligature Group (OVX+ISO+LIG): rats that underwent ovariectomy surgery and hormone replacement with isoflavone and ligature-induced periodontitis.

After the groups were randomly divided, the animals were submitted to ligature-induced periodontal disease, totaling 30 rats in the LIG group. A total of 40 animals underwent surgery for ovary removal for menopause induction (OVX Group). A dose of isoflavone was administered to 20 rats, as hormone replacement therapy after menopause induction, in the ISO Group.

2.3. Periodontal disease induction

The animals were anesthetized at the age of 70 days (xylazine 0,04mL/100g and ketamine 0,08mL/100g) and placed on the operating table for optimal mouth opening of the rats, facilitating access to the teeth in the posterior jaw region. With the support of a modified pinch and an explorer probe, cotton ligatures number 40 were placed around the right mandibular first molar. Irritation by the ligature of the gingival margin remained for 30 days, causing the accumulation of bacterial plaque, and consequently the development of periodontal disease (Nassar et al., 2009).

2.4. Ovariectomy and hormone replacement

The animals were anesthetized with hydrochloride of ketamine (Dopalen, 100 mg, kg body weight, ip) and xylazine (Anadesan, 14 mg/kg body weight, ip) at the age of 79 days and a skin incision between the last rib and the hind leg was performed. A ligature was placed under the uterine tube and the ovary was isolated and removed. The muscular and skin layers were sutured separately. The ovary on the opposite side was removed following the same process. After surgery, the female rats were treated with pentabiotic (Fort Dodge, 0.1mL/kg, im) and analgesics (megluminaFluxinin, Banamine; Schering-Plough, 2.5 mg/kg, sc) and placed in individual cages. Hormone replacement in groups OVX+ISO and OVX+ISO+LIG was performed with isoflavone, diluted and applied subcutaneously (0.25 mg / kg / day, SC) for 21 days (Chow et al., 1992).

2.5. Descalcification, histological process and inclusion in paraffin blocks

After the animals were euthanized, the hemimandibles on the right side were fixed in 10% formalin solution for 24 hours. After this period, they were washed under running water for an hour and immersed in trichloroacetic acid solution, prepared as follows: 100mL of distilled water for 5 ml of acid. The samples were kept in the decalcification solution for approximately 20 days.

The decalcification level was verified daily and the trichloroacetic acid solution was changed every 5 days. After this time, the samples were placed in a 5% sodium sulfate solution for three hours and a half to neutralize the acid; last, the samples were washed again under running water for twelve hours and dehydrated and diaphonized. After that, the samples were embedded in paraffin and 600 blocks were obtained. Those were selected by a semi-automatic micro atoms, each section measuring 7μm. Samples were made for each group, meaning that the sample represented 4 serial sections, totaling 40 sections for each group. The sections were stained with hematoxylin and eosin.

2.6. Microscopic observations

Microscopic observation was performed by a single examination of the histological stained slices. The sections were analyzed with the aid of a light microscope (Leica Microsystems, Switzerland) for morphological observation of gingival tissue as well as the alveolar process and osteoblast, osteocyte and osteoclast count in the hemimandibles.

2.7. Gingival morphometric measurements

Morphometric measurements were performed on the right buccal and lingual marginal gingival area in all groups using a light microscope with 10x objective lens and imaging software for analysis, with breaks of 10 sections between the counts in the serial sections (about 70 μm). Measurements were performed using the pre-determined morphological points on the marginal gingiva, as shown in Image 1. The results are expressed in μm.

2.8. Bone morphometrics

The osteoblast, osteocyte and osteoclast count presented in five consecutive areas of the buccal alveolar bone crest, from the highest point of the crest, was performed. For the observation, we used a 100 x microscope immersion objective lenses. Two observations were performed in the field, obtaining an average of the values found for each animal and each group. The measurement of the alveolar bone crest was performed using a microscope attached to a computer, which permitted the images to be captured using the LazEz® software. A measurement at a shorter distance between the top of the buccal alveolar crest and the cemento-enamel junction was performed. The measurements were repeated once a day, for three different days, obtaining an average of the values.

2.9. Radiographic analysis

Soon after the animals were euthanized, the hemimandible on the left side of each animal was removed and fixed in a buffered formaldehyde solution (pH 7.2) for 48 hours. This entailed placing the hemimandibles with the lingual side on periapical X-ray film (AGFA DENTUS®, Ultraspeed) and positioned so that the buccal and lingual cusps of the first molars stayed in the same vertical plane. The X-ray machine X GE – 1000 was used, adjusted to 15mA, 65Vp, 18 impulses, and focus/film distance of 50 centimeters with x-ray incidence perpendicular to the sections. The processing of the films was performed using the Kodak® developer and fixer in the respective processing time/temperature and the images were scanned through a scanner for slides (Polaroid Sprint Scan 35 Plus, Polaroid). The digitized images were analyzed through 3 measurements using the Image Tools 3.0 program (University of Texas Health Science Center, San Antonio, TX, USA) and an average among them was obtained through a linear measurement, which was based on a distance of the cemento-enamel junction up to the alveolar bone crest on the mesial side of the lower first left molar of the rat, with values in pixels.

2.10. Statistical analysis

For the statistical analysis, all numeric numbers were expressed as an average of ± standard deviation. At first, the Shapiro-Wilk test was used to assess the distribution of the data normality. After that, ANOVA tests, followed by Tukey's test, $p<0.05$, were used to assess the differences among the groups.

3. Results

3.1. Radiographic and histomorphometric analyses of the alveolar bone

The radiographic and histomorphometric analyses of the alveolar bone of the lower first molars (Table 1) showed that significant alveolar bone loss occurred in the groups with ligature-induced periodontal disease ($p<0.05$), confirming the action of induced periodontitis on the bone tissue. The OVX+LIG group also showed that alveolar bone loss was more statistically significant ($p<0.05$) than the in second OVX+ISO group, as well as the group that was given isoflavone treatment, presented less bone loss than the groups without treatment.

We could also observe that in those groups in which the periodontitis was not induced, but submitted to the ovariectomy (OVX+ISO and OVX), significant bone loss was found when compared with the control group, which could suggest induction of osteoporosis. The group that was received isoflavone treatment presented less bone loss when compared with the groups without treatment ($p<0.05$), suggesting the hypothesis that isoflavone protects against bone loss.

3.2. Histomorphometric analysis of gingival tissue and morphometric bone analysis

The results of all morphometric gingival measurements showed that no statically significant differences were found among the groups with experimental periodontitis induction ($p>0.05$) (Table 2). Relating to the groups submitted to experimental periodontitis, all of them presented significant increased measurements ($p<0.05$) in comparison with the other groups. However, no statistical difference was found among them ($p>0.05$) considering the average of oral epithelium tissue. Although over the other measurements, the groups LIG and OVX+LIG presented averages significantly higher than the group OVX+ISO+LIG ($p<0.05$), suggesting again that there is a protective action upon the inflammation of the gingival tissue in rats submitted to experimental periodontitis. Bone histomorphometry (Table 3) of the groups OVX and OVX+LIG, followed by the LIG (($p<0.05$), presented a higher number of osteoclasts in the cell count, which could also suggest an increase in osteoclastogenesis. We observed a decrease in the OVX+FIT+LIG group, as well as when comparing the OVX+ISO group with the OVX group, in which the number of osteoclasts was lower in the ISO group ($p<0.05$) with the greater presence of osteoblasts and osteocytes, suggesting a positive action of isoflavone.

3.3. Histological plate description

Control Group

In the control group, the assessment of the parameters showed normality of the oral epithelium, junctional, sulcular and connective tissues, without any inflammatory process. The alveolar bone was intact, compact and regular and the bone crest presented a height proportional to the third cervical area of the root. We observed the presence of osteoblasts, osteocytes and osteoclasts, incremental lines in the normality pattern of bone remodeling. The cemento-enamel, cementum and periodontal ligament didn"t show normally alterations (Images 2 and 3).

Ligature groups

In the groups in which the periodontal disease was induced by ligature, we noticed morphological abnormality in the oral epithelium, junctional, sulcular tissues, with the migration to the apical area and the connective tissue with dominance of acute inflammatory state. Bone crest retraction was observed in the ligature groups (Images 4, 6), but less in those groups that received isoflavone. The bone crest also was irregular, with extensive alveolar bone loss and exposure of the cervical third (Image 5). A greater number of osteoclasts were present in the ligature (Image 7) and ligature-ovariectomy groups, revealing greater activity of bone resorption. In the ligature-ovariectomy-isoflavone group, bone resorption was less accentuated than in the other groups. Moreover, changes with significant loss of tissues were observed in the cemento and periodontal ligature.

Ovariectomy groups

In the groups submitted to ovariectomy, we observed a pattern of bone retraction, even in the groups without the ligature induction, which could suggest the onset of osteoporosis (Image 8). Groups that received isoflavone treatment presented less accentuated bone resorption, but an increase in the resorption pattern was found in the ligature groups. A higher concentration of osteoclasts was found in the bone cells in the ovariectomized groups without isoflavone, suggesting greater bone resorption, different from the isoflavone treatment that presented a greater concentration of osteoclasts and higher osteoblasts/osteocytes, suggesting its protective effect on the bone tissue (Image 11).

4. Discussion

Menopause causes biopsychosocial consequences and it is part of the female aging process. Not only considered a disease, the signs and symptoms associated with this period can be treated with specific therapeutic procedures to mitigate these signs. The signs and symptoms of menopause mainly consist of the decrease in the estrogen hormone and, consequently the development of late diseases, among them, cardiovascular diseases and osteoporosis. Therefore, the present research searched for a natural alternative therapy, such as isoflavone, that could be used to protect against the alveolar bone loss caused by the development of periodontal disease (WHO, 2004) Hypoestrogenism is directly related to the increase of bone resorption, including the mandibles bones.

Estrogen action on the bone seems to be related to the reduction of precursory osteoclast cells and expression of estrogen receptors in the osteoblast cells (Eriksen et al., 1998). Bone changes caused by osteoporosis can be a risk factor for the progression of periodontal disease. Reduced bone mass density in osteoporosis can facilitate alveolar bone resorption in periodontal disease (Tezal et al., 2000). Therefore, as shown in Table 1, bone loss was significant in the ovariectomy and ligature groups, which might suggest that the periodontal disease could be related to osteoporosis.

In our study of surgically induced menopause, using the ovariectomy technique, an effective decrease in estrogen occurred, which led to a significant increase in alveolar bone loss in the ovariectomized group when compared with the control group, which was even greater in the ovariectomized rats and in the groups that did not receive hormone replacement with isoflavone. Hormonal changes in women, such as during menopause, could be an indicator of periodontal tissue inflammation, particularly of the gingiva, when the progesterone interaction with estrogen and constant clinical and hormonal changes occur in the periodontium, as the cells are the tissue target of steroid hormones for the reduction of estrogen, which could promote greater trabecular bone loss than cortical loss (Genco & Borgnakke, 2013). Our results showed (Table 1) that the OVX and OVX+LIG groups presented more significant alveolar bone loss than the OVX+ISO and OVX+FIT+LIG groups, which showed greater bone retraction in the ligature and/or ovariectomized groups without the isoflavone action (Image 4 and 7).

The results in Table 3 show bone cell count, higher in relation to bone loss, in the OVX+LIG group, followed by the LIG group, and in the groups that received isoflavone. We observed less bone loss in the OVX+ISO group and the isoflavone group also presented less bone loss, suggesting the protective action of isoflavone on bone mass loss. In a study of ovariectomized rats with induced periodontitis, Xu et al. (2015) showed that the estrogen deficiency causes alveolar bone mass loss and reduction in the alveolar crest height; therefore, postmenopausal osteoporosis can influence the progression of periodontal disease. A study conducted by Mohammad & Brunsvold (1996) indicated that there was a relationship between osteoporosis and the progression of the periodontal disease over the studied women, which they are in agreement with our results. The alternative hormone therapy presented in this study aims to establish a trial for the maintenance of the bone mass density with the isoflavone, given that it's chemical structure is similar to the 17β-estradiol and has affinity to link to estrogen receptors of typeβ (ERβ), a receptor that is present in the stroma cells or osteoblasts, measurement of osteoclastogenesis. (Zhang et al., 1995).

The most suggested mechanism to explain the positive effects of soy isoflavone on the bone tissue, which can help prevent the development of osteoporosis, was proposed by Williams et al. (1998). Genistein combines with estrogen receptors and acts on the same mechanism of this hormone. Genistein inhibits topoisomerase II, interfering with the cell cycle progression, or, yet, genistein activates the peptides receptors linked to the membrane, initiating an independent effect of estrogen. For example, osteoclasts are different from the tyrosine kinase activity (PTK), so the PTK inhibitors are candidates for osteoporosis prevention. Genistein and daidzein, natural isoflavones, are PTK inhibitors and could act through their mechanism (Williams et al., 1998), suggesting the hypothesis of the results of our study in which the isoflavone therapy presented positive results, as shown in Table 1 and 3. Ishida et al. (1998) studied the genistein and daidzein action in ovariectomized rats and on poor calcium diets and found positive results related to the assessment of bone loss prevention and bone metabolism, supposing the turn-over bone suppressor action, caused by derived isoflavone.

Other studies are in accordance with these findings, since the use of isoflavone in ovariectomized rats showed reduced bone loss after ovariectomy (Arjmandi et al., 1998a; Picherit et al., 2001). Conversely, Arjmandi et al. (1998b) carried out a study in ovariectomized rats with and without the hormone replacement treatment and the results pointed out that groups of rats that received a prolonged diet with soy protein (isoflavone) presented an effective protective action against the bone loss, when compared with ovariectomized rats without the treatment, which is in accordance with our study (Table 1).

These evidences with isoflavone can occur also in women. The study of Tanaka et al. (2008) showed that a rich isoflavone diet presented a positive result regarding bone loss protection and the progression of periodontal disease, as the intake dose is directly associated with the decrease in the prevalence of periodontal disease. Alekel et al. (2000), in a study with postmenopausal women and control diet groups who received a rich soy diet, poor soy diet and isolated protein, found that isoflavone accentuated bone mass loss in the spinal cord of women who received a rich soy diet.

However, in a bibliographic review study, Yu et al. (2016) points out that isoflavone can present an anti-inflammatory action. Ganai et al. (2015) suggests that pro-inflammatory cytokines suppress the action of isoflavone. In accordance with the results shown in Table 2, the groups that received isoflavone presented less connective infiltrated tissue, decreasing in height and length.

6. Conclusion

Therefore, we can suggest that isoflavone can grant a protective effect against alveolar bone loss and the progression of periodontal disease. The decrease in the use of hormonal therapy, due to the suspicion of cancer, has contributed to the search for alternative treatments to prevent osteoporosis bone loss. Although the choice and initiative to use isoflavone or not must be an option for women and doctors, the welfare and quality of life of each patient is imperative.

7. Acknowledgements and Funding

The authors would like to thank CAPES, the Araucaria Foundation and the State University of Western Paraná (UNIOESTE) for financial support. We would also like thank the Laboratories of Endocrine Physiology and Metabolism, and Cell Biology at (UNIOESTE) for structural support. The authors are grateful to Celeste da Rocha Paiva, technician at the Laboratory of Cell Biology, for her technical assistance in this study.

8. Competing interests

The authors declare that there are no conflicts of interest in this study.

8. References

Alekel DL, Germain AS, Peterson CT, Hanson KB, Stewart JW, Toda T (2000). Isoflavone-rich soy spares lumbar spine bone. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenipausal women. Am. J. Clin. Nut.; 72: 844-852.

Amatykul S, Kasiyaphat A, Patumraj S, Chotipaibulpana S, Siriviriyakul P(2007). Effects of genistein supplementation on gingival blood flow disturbances after menopause: in vivo study using ovarectomized rats. Asian Biomed.; 1:421-424.

Arjmandi BH, Getliger MJ, Goyal NV, Alekel L, Hasler CM, Juma S (1998 a). Role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian hormone defience in rats. Am. J. Clin. Nut.; 68: 1358S-1363S.

Arjmandi B, Birnbaum R, Goyal NV, Getlinger MJ, Juma S, Alekel L , Hasler CM, Drum ML, Hollis BW, Kukreja SC (1998b). Bone-sparing effect of soy protein in ovarian hormone-deficient rats is related to its isoflavone content. Am. J. Clin. Nut.; 68: 1364S-1368S

Aquino EM, Almeida MC, Menezes GM, de Figueiredo RC, Bensenor IM, Menque SS, da Fonseca MJ, Gabrielli L(2016). Postmenopausal hormone therapy in the brazilian longitudinal study os adult health (ELSA-Brasil): who still uses it? Pharmacoepidemiol. Drug Safe.; 25 :609-617.

Brandi ML (1997). Natural and Synthetic Isoflavones in the prevention and treatment of chronic diseases. Calcif. Tissue Int.; 61:S5-S8.

Chlebowski RT, Hendrix SL, Stefanick ML, Gass M, Lane D, Rodabough RJ, Gilligan MA, Cyr MG, Thomson CA, Khandekar J, Petrovitch H,McTiernan A, WHI Investigators (2003). Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women. J. Am. Med. Assoc.; 289: 3243-3253.

Chow J, Tobias JH, Colston KW, Chamber TJ(1992). Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. J. Clin. Invest.; 89:74-78.

Colditz G, Hankinson SE, Hunter DJ, Willett WC, Manson JE, Stampfer MJ, Hennekens C, Rosner B, Speizer FE(1995). The use of estrogens and progestin and the risk of breast cancer in postmenopausal women. New Engl. J. Med.; 332:1589-1593.

Collaborative Group on Hormonal Factors in Breast Cancer (1997). Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52, 705 women with breast cancer and 108, 411 women without breast cancer. Lancet; 350:1047–1059.

Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC(1998). Evidence of estrogen receptors in normal human osteoblas-like cells. Science; 241:84-86.

Ganai AA, Faroogi H(2015). Bioactivity of genistein: A review of in vitro and in vivo studies. Biom. Pharm.; 76:30-38.

Gao YH, Yamaguchi M(2000). Suppressive effect of genistein on rat bone osteoclast: involvement of protein kinase inhibition and protein tyrosine phosphatase activation. Int. J. Mol. Med.; 5:261-267.

Genco RJ, Borgnakke WS(2013). Risk factors for periodontal disease. Periodontol. 2000; 62:59–94.

Ishida H, Uesugi T, Hirai K, Toda T, Nukaya H, Yokotsuka K, Tsuji K(1998). Preventive effects of the plant isoflavones, daidzin and genistin, on bone loss in ovariectomized rats fed a calcium-deficient diet. Biol. Pharmacy Bulletts; 21:62-66.

Lerner UH(2006). Inflammation-induced bone remodeling in periodontal disease and the influence of post-menopausal osteoporosis. J. Dent. Res.;. 85:596-607.

Mohammad AR, Brunsvold M(1996). The strength of association between systemic postmenopausal osteoporosis and periodontal disease. Int. J. Prosthod.; 9:479-483.

Nassar PO, Nassar CA, Guimarães MR, Aquino SG, Andia DC, Muscara MN, Spolidorio DM, Rossa C Jr, Spolidorio LC(2009). Simvastatin therapy in cyclosporine A- induced alveolar bone loss in rats. J. Period. Res.; 44:479-488.

Nishimura F, Murayama Y(2001). Periodontal inflammation and insulin resistance – lesson from obesity. J. Dent. Res.; 80:1690-1694.

Picherit C, Chanteranne B, Bennetau-Pelissero C, Davicco MJ, Lebecque P, Barlet JP(2001). Dose dependent bone-sparing effects dietary isoflavones in the ovariectomized rat. Brit. J. Nut.; 85:307-316.

Rivera MF, Lee JY, Aneja M, Goswami V, Liu L, Velsko IM, Chukkapalli SS, Bhattacharyya I, Chen H, Lucas AR(2013). Polimicrobial infection with major periodontal pathogens induced disease and aortic atherosclerosis in hyperlipidemicapoe null mice. Plos One; 8:1-10.

Sakalauskiene J, Giedrimiene D, Kubilius R, Gleiznys A, Vitkauskiene A, Ivanauskiene E, Puskunigyte K(2014). Cytokine production by leukocytes in patients with periodontitis. Cent Europ. J. Med.; 99: 821-829.

Shifren JL, Gass MLS,, NAMS Recommendations for Clinical Care of Midlife Women Working Group The North American Menopause Society Recommendations for Clinical Care of Midlife Women (2014). Menopause: J. North Am. Menopause Soc.; 21:1038-1062.

Tanaka K, Sasaki S, Murakami K, Okubo H, Takahashi Y, Miyake Y, and for the Freshmen in Dietetic Courses Study II Group (2008).. Relationship between soy and isoflavone intake and periodontal disease: the freshmen in dietetic courses study II. BMC Public Health; 29, 8:39

Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ(2000). The relationship between bone mineral density and periodontitis in postmenopausal women. J. Periodontol.; 71:1492–1498.

Williams JP, Jordan SE, Barnes S, Blair HC (1998). Tyrosine kinase innibitor effects on avian osteoclastic acid transport. Am. J. Clin. Nut.; 68 (supl):1369S-1374S

Wise PM, Smith MJ, Dubal DB, Wilson ME, Krajnak KM, Rosewell KL(1999). Neuroendocrine Influences and Repercussions of the Menopause. Endo. Rev.; 20:243-248.

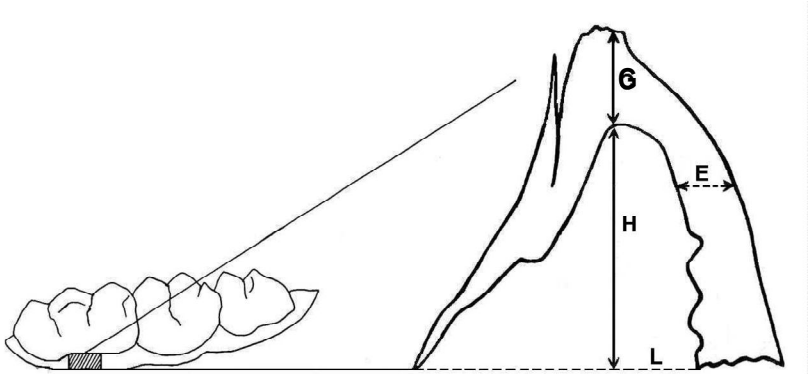
World Health Organization (2004). Scientific group on the assessment of osteoporosis at primary health care level. Sumary Meeting Report Brussels, Belgium;5-7.

Xu XC, Chen H, Zhang ZJ, Liu XQ, Zheng XY, Zhang J, Qin A, Lu EY(2015). Effects of estrogen deficiency on the alveolar bone of rats with experimental periodontitis. Mol. Med. Reports; 12:3494-3502.

Yakob M, Meurman JH, Jogestrand T, Novak J, Söder PO, Söder B(2012). C-reative protein in relation to early atherosclerosis and periodontitis. Clin. Oral Invest.; 16:259-265.

Figures and Legends

Figure 1: Scheme of marginal gingiva of rat, showing the reference points used for morphometric measurements of the oral epithelium, epithelial crest and connective tissue.



G - Height of the epithelium of the gingival crest
E - Width of the buccal epithelium
H - Height of connective tissue in the middle region
L - Width of connective tissue in the basal region

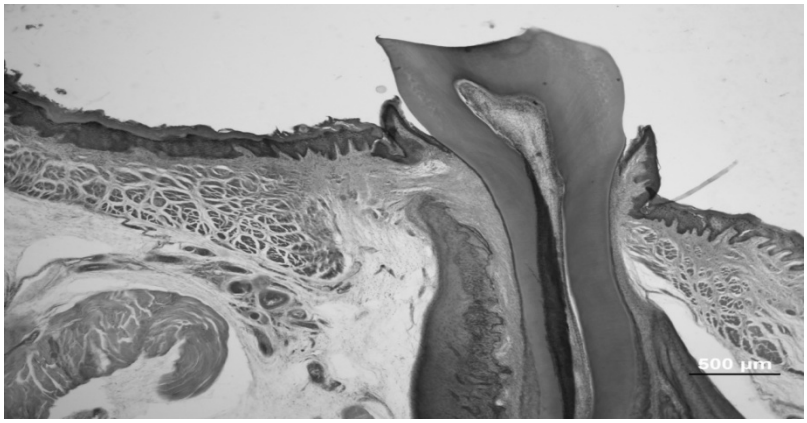


Figure 2: Representative photomicrographs of an animal in the Control group. The following are shown: EJ, junctional epithelium; ES sulcular epithelium; COA, alveolar bone crest. Hematoxylin and eosin, 4x.

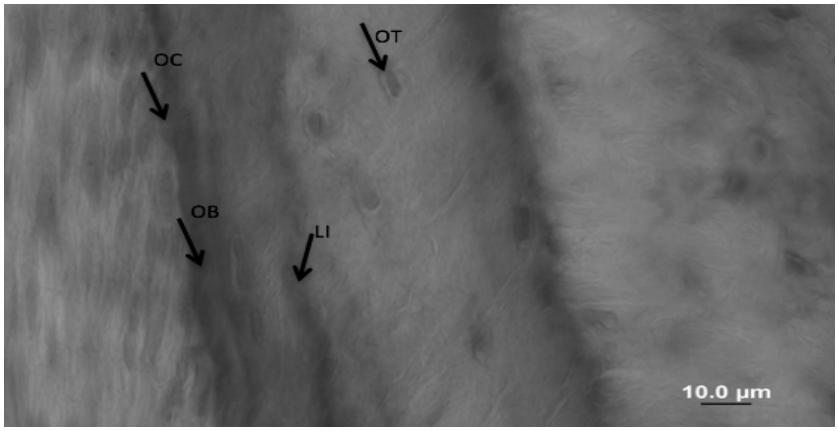


Figure 3: Representative photomicrographs of an animal in the Control group. The following are shown: OC, osteoclast; OB, osteoblast; OT, osteocyte; LI, incremental lines. Hematoxylin and eosin, 1000x

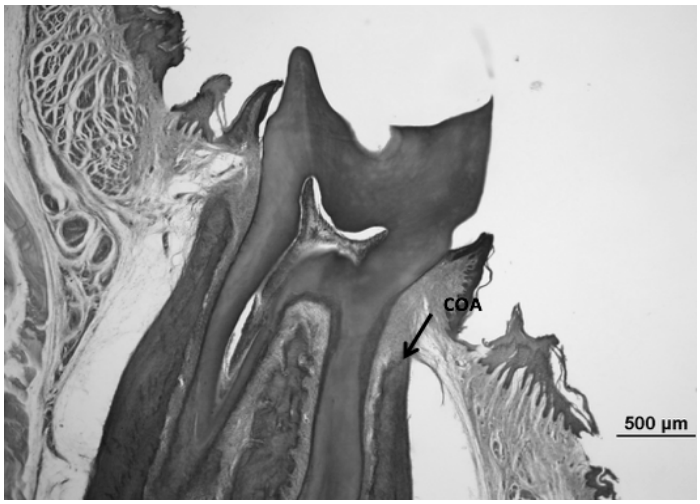


Figure 4: Representative photomicrographs of an animal in the ligature group. The image shows alveolar bone loss. COA, alveolar bone crest. Hematoxylin and eosin, 4x

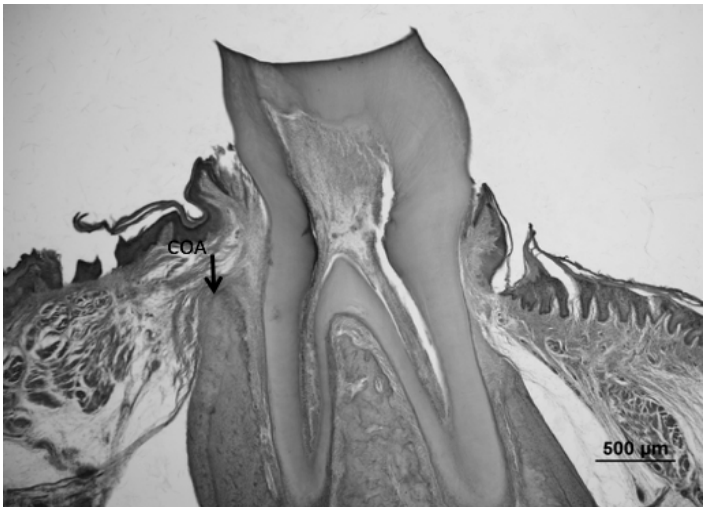


Figure 5: Representative photomicrographs of an animal in the OVX+LIG+FIT group. The image shows alveolar bone reabsorption with isoflavone influence . COA, alveolar bone crest. Hematoxylin and eosin, 4x

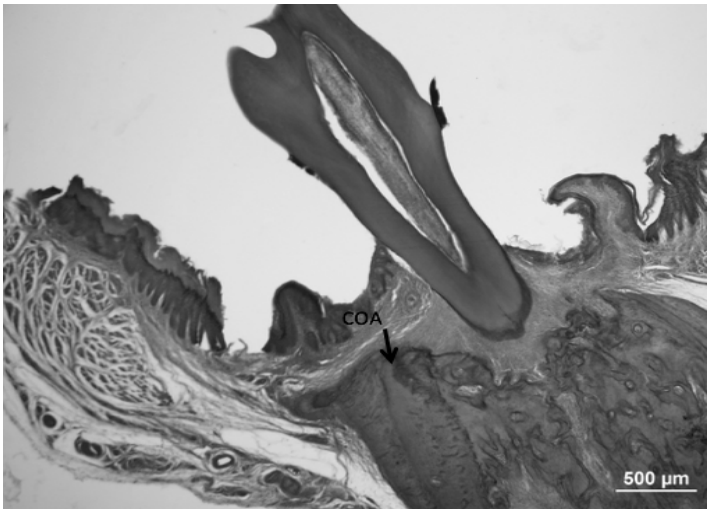


Figure 6: Representative photomicrographs of an animal in the OVX+LIG group. The image shows alveolar bone reabsorption in the groups with periodontal disease induced . COA, alveolar bone crest. Hematoxylin and eosin, 4x

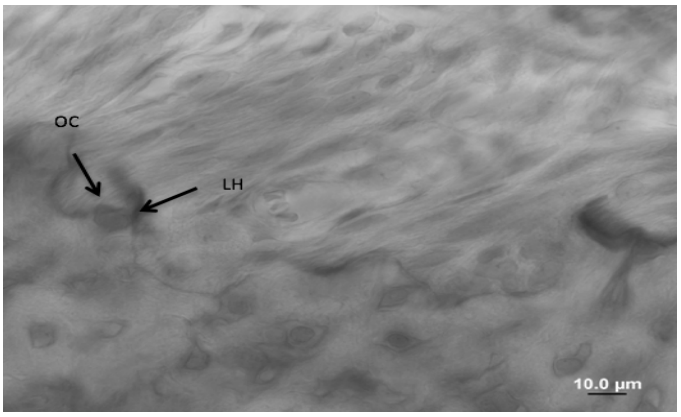


Figure 7: Representative photomicrographs of an animal in the Ligature group. The image shows alveolar bone with reabsorption evidence of the experimental groups where the periodontal disease was induced . Osteoclast in the Howship Lacune shows bone reabsorption area. LH Howship lacune; OC, Osteoclast. Hematoxylin and eosin, 1000x

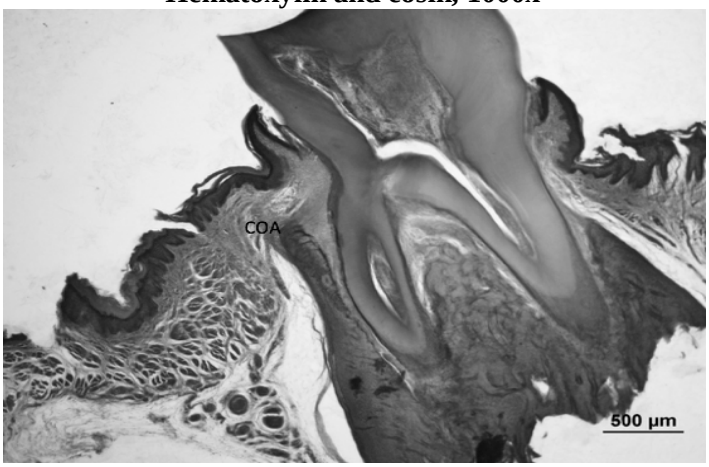


Figure 8: Representative photomicrographs of an animal in the OVX group. The image shows alveolar bone reabsortion in the ovariectomy groups. COA, alveolar bone crest. Hematoxylin and eosin, 4x

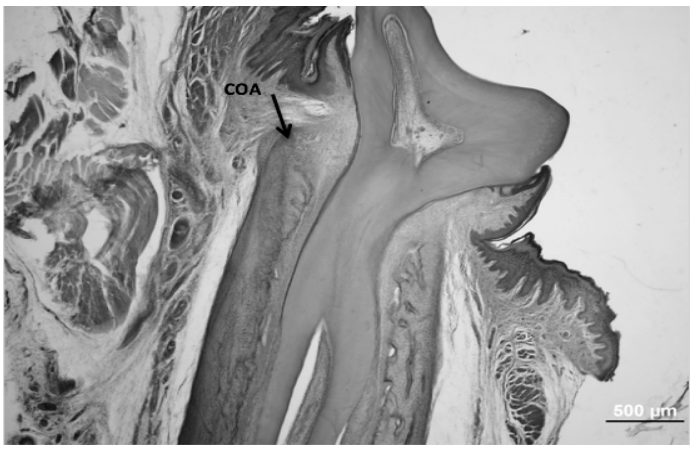


Figure 9: Representative photomicrographs of an animal in the OVX+ FIT group. The image shows alveolar bone reabsorption in the ovariectomy groups with isoflavone treatment. COA, alveolar bone crest. Hematoxylin and eosin, 4x

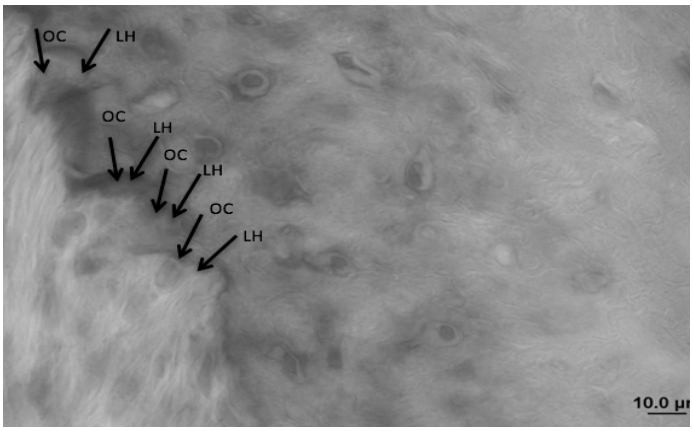


Figure 10: Representative photomicrographs of an animal in the OVX+ LIG group. The image shows alveolar bone with bone rebsorption evidence. LU, Howship lacune; OC, osteoclast. Hematoxylin and eosin, 1000x

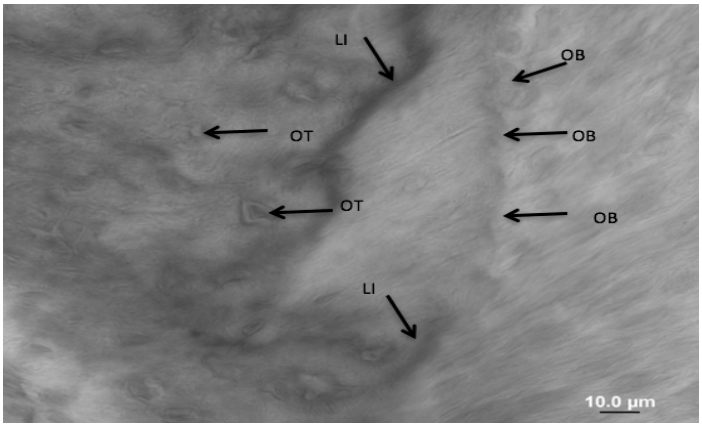


Figure 11: Representative photomicrographs of an animal in the OVX+FIT group. The image shows alveolar bone with bone formation evidence, with osteoblast and incremental lines. isoflavone treatment. LI, incremental lines; OB, osteoblast; OT, osteocyte. Hematoxylin and eosin, 1000x

Tables

Table 1: Morphometric Analysis of right hemimandible of rats measuring the distance from the crest alveolar bone to enamel-cemente junction and radiographic analysis of distance from enamel–cement junction until the alveolar bone crest of the left lower first molar mesial side of all the groups' rats. The values represent mean standard deviation and are expressed in pixels.

Groups	Analysis	
	Radiographic	Morphometric
CON	6.70±0.49 A	312.70±5.07 A
LIG	10.21±0.11 B	348.62±13.84 B
OVX+ISO+LIG	10.03±0.18 C	336.87±4.96 C
OVX+ISO	9.01±0.67 D	320.69±5.38 D
OVX	9.83±0.1 E	330.91±4.92 E
OVX+LIG	11.02±0.15 F	367.93 ±8.21 F

Different Letters, mean that the data are statistically different the same parameter at $p<0.05$ and the tests were ANOVA and TUKEY Test.

Table 2: Histomorphometric analysis of gingiva of right hemimandible of rats in all the groups. Values represent mean ± standard deviation and are expressed in µm.

Groups	Morphometrical measurements			
	G	**H**	**E**	**L**
CON	32.45±0.36 A	224.21±5.80A	29.20±2.12 A	84.96±6.93 A
LIG	37.47±1.23 B	241.07±1.27B	33.22±1.27B	132.01±10.57B
OVX+ISO+LIG	35.58±0.33 C	234.53±5.30 C	33.11±0.26B	122.10±2.24 C
OVX+ISO	32.34±0.86 A	222.03±6.19A	28.50±0.89A	82.21±9.75 A
OVX	32.28±0.57 A	223.56±4.79A	29.00±3.29A	84.27±3.47 A
OVX+LIG	37.34±0.21B	240.92±3.13B	34.13±2.51B	126.45±5.91B

Different Letters. mean that the data are statistically different the same parameter at $p<0.05$ and the tests were ANOVA and TUKEY Test. G - Height of the epithelium of the gingival crest; E - Width of the buccal epithelium; H - Height of connective tissue in the middle region ; L - Width of connective tissue in the basal region

Table 3: Histomorphometric analysis of right hemimandible of rats of all the groups, for the quantification of osteoblasts, osteocytes and osteoclasts. Values represent mean ± standard deviation and are expressed in units.

Groups	Quantification		
	Osteocytes	Osteoblasts	Osteoclasts
CON	389.87±7.02A	72.09±1.22A	9.87±1.22A
LIG	354.72±12.97B	64.60±1.57B	13.40±1.09B
OVX+ISO+LIG	367.25±11.51C	67.45±1.47C	12.36±1.10C
OVX+ISO	382.85±7.58D	69.30±1.75D	11.00±1.01D
OVX	328.62±10.27E	59.33±2.68E	15.00±1.35E
OVX+LIG	317.00±12.22F	56.78±2.47F	16.85 ±1.67F

Different Letters. Mean that the data are statistically different the same parameter at $p<0.05$ and the tests were ANOVA and TUKEY Test