

Genistein acts in bone metabolism and improves peri-implant healing in rats with estrogen deficiency

Genisteína atua no metabolismo ósseo e melhora o reparo peri-implantar em ratas com deficiência de estrógeno

La genisteína actúa sobre el metabolismo óseo y mejora la reparación periimplantaria en ratas con deficiencia de estrógenos

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Abstract

The present study aimed to evaluate the peri-implantar bone healing in the presence of genistein treatment in ovariectomized rats. Thirty female rats with 4 months old were divided into 3 groups according to the experimental condition and the drug treatment: SHAM (rats submitted to the fictional surgery and gavage with 0.9% saline solution); OVX (rats submitted to bilateral ovariectomy and gavage with 0.9% saline solution); OVX GEN (rats submitted to bilateral ovariectomy and gavage with 1mg/day of genistein). 60 implants were installed, with two implants in each animal. The calcified group was subjected microcomputerized tomography and the parameters analysed was bone volume per tissue volume (BV/TV) and connective density (Cnn.Dn). The decalcified samples were evaluated through immunolabeling analysis, in order to detect the presence of RUNX2, Alkaline Phosphatase, Osteocalcin, Osteopontin and TRAP. All the quantitative data were submitted to the normality curve to determine the most adequate test. The significance level of $p < 0.05$ was considered for all tests. The morphometric analysis of the OVX GEN group showed higher percentage of bone volume and lower connective density when compared with

OVX. Immunohistochemical analysis favors expression. For the markers that positively label osteoblastic activity. This study shows that genistein therapy improves peri-implant bone healing in ovariectomized rats.

Keywords: Bone; Osteoporosis; Genistein; Dental implants.

Resumo

O presente estudo teve como objetivo avaliar a consolidação óssea peri-implantar na presença de tratamento com genisteína em ratas ovariectomizadas. Trinta ratas com 4 meses de idade foram divididas em 3 grupos de acordo com a condição experimental e o tratamento medicamentoso: SHAM (ratas submetidas à cirurgia fictícia e gavagem com solução fisiológica 0,9%); OVX (ratas submetidas a ovariectomia bilateral e gavagem com solução salina 0,9%); OVX GEN (ratas submetidas a ovariectomia bilateral e gavagem com 1mg / dia de genisteína). Foram instalados 60 implantes, com dois implantes em cada animal. O grupo calcificado foi submetido a tomografia microcomputarizada e os parâmetros analisados foram volume ósseo por volume de tecido (VB / VT) e densidade conectiva (Cnn.Dn). As amostras descalcificadas foram avaliadas por meio de análise de imunomarcagem, a fim de detectar a presença de RUNX2, Fosfatase Alcalina, Osteocalcina, Osteopontina e TRAP. Todos os dados quantitativos foram submetidos à curva de normalidade para determinar o teste mais adequado. O nível de significância de $p < 0,05$ foi considerado para todos os testes. A análise morfológica do grupo OVX GEN mostrou maior porcentagem de volume ósseo e menor densidade conectiva quando comparada com OVX. A análise imunohistoquímica favorece a expressão dos marcadores que atuam positivamente na atividade osteoblástica. Este estudo mostra que a terapia com genisteína melhora a cicatrização óssea peri-implantar em ratas ovariectomizadas.

Palavras-chave: Osso; Osteoporoze; Genisteína; Implantes dentários.

Resumen

El presente estudio tuvo como objetivo evaluar la cicatrización ósea periimplantar en presencia de tratamiento con genisteína en ratas ovariectomizadas. Treinta ratas hembras de 4 meses de edad se dividieron en 3 grupos según la condición experimental y el tratamiento farmacológico: SHAM (ratas sometidas a la cirugía ficticia y alimentación por sonda con solución salina al 0,9%); OVX (ratas sometidas a ovariectomía bilateral y alimentación por sonda con solución salina al 0,9%); OVX GEN (ratas sometidas a ovariectomía bilateral y sonda con 1 mg / día de genisteína). Se instalaron 60 implantes, con dos implantes en cada animal. El grupo calcificado fue sometido a tomografía microcomputarizada y los parámetros analizados fueron volumen óseo por volumen tisular (BV / TV) y densidad conectiva (Cnn.Dn). Las muestras descalcificadas se evaluaron mediante análisis de inmunomarcaje, con el fin de detectar la presencia de RUNX2, Fosfatasa Alcalina, Osteocalcina, Osteopontina y TRAP. Todos los datos cuantitativos se sometieron a la curva de normalidad para determinar la prueba más adecuada. Se consideró el nivel de significancia de $p < 0.05$ para todas las pruebas. El análisis morfológico del grupo OVX GEN mostró un mayor porcentaje de volumen óseo y una menor densidad conectiva en comparación con OVX. El análisis inmunohistoquímico favorece la expresión de los marcadores que actúan positivamente sobre la actividad osteoblástica. Este estudio muestra que la terapia con genisteína mejora la curación ósea periimplantaria en ratas ovariectomizadas.

Palabras clave: Hueso; Osteoporosis; Genisteína; Implantes dentales.

1. Introduction

Bone is a mineralized connective tissue that it is always remodeling through balance between the osteoblasts, osteocytes and osteoclasts cells. Moreover, bone is regulated by several systemic hormones (e.g., estradiol, testosterone, parathyroid hormone, 1,25-dihydroxyvitamin D3 and calcitonin) and local factors (e.g., nitric oxide, prostaglandins, growth factors and cytokines). When the imbalance of this process occurs, systemic diseases like osteoporosis can develop diseases such as osteoporosis (An, et al. 2016). Osteoporosis is a bone metabolic disorder mainly of postmenopausal women, with consequent reduction of estrogen levels. It induces progressive bone loss and structural modifications of trabecular bone. Moreover, causes imbalance of bone remodeling and bone microarchitecture deterioration. Finally, it reduces the bone strength and rises the susceptibility to fractures of long bones (Harvey, et al. 2010; Drake, et al. 2012; Khosla, et al. 2008). Long and skull bones show a reduced bone mass in patients with osteoporosis (Oliveira, et al. 2017; Lirani-Galvão, et al. 2010). There is a decrease of bone density, especially in the posterior region of maxillary bones. This systemic interference decreases the primary stability (Merheb, et al. 2016) and increases the marginal bone loss of implants (Giro, et al. 2015). Both factors are directly crucial to the success of the implant treatment (Oliveira, et al. 2017; Drage, et al. 2007; Shapurian, et al. 2006).

Phytoestrogens have been used as natural therapy for prevention the bone loss in the postmenopausal. There are three

subtypes of phytoestrogens: isoflavones, lignans and coumestans. Genistein is the most abundant isoflavone. This substance acts as a natural selective modulator of estrogen receptors (ER) (Fu, et al. 2014), due the similarity structural with estradiol, it is able to have agonist or antagonist activity. Genistein has high affinity with ER β . It has been reported that genistein modulate signaling pathways of cell growth and proliferation through of the activation of membrane G-protein coupled estrogen receptors (GPERs) (Cepeda, et al. 2017). The action mechanism involves estrogen receptor and nitric oxide pathways, thus genistein improves osteoblastogenesis and promotes osteoclasts differentiation (Cepeda, et al. 2020). In vitro studies indicate the role of genistein in the formation of mineralization nodules (Nishide, et al. 2015) and the increase of osteocalcin expression, extracellular collagen deposition, and alkaline phosphatase activity (Cepeda, et al. 2017). The hypothesis is supported by the low rates of osteoporosis in Asian populations where the diet is rich in soy.

Peri-implant bone healing happens due to various cellular events such as protein synthesis involved in bone formation and resorption (Zhang, et al. 2003). A successful oral rehabilitation requires adequate bone quality and quantitate for the implant stability, especially in the early stages of the osseointegration (Oliveira, et al. 2017; Von Wowern, et al. 1992). The increase of elderly population and life expectancy increases patient demand with systemic interferences such as osteoporosis for dental implant installation. According to the work developed by our research group the osteoporosis can affect bone quality and quantitate around the implants because this interference causes delay in the initial stages of repair process (Faverani, et al. 2018; Yogui, et al. 2018; Ramalho-Ferreira, et al. 2015).

Considering the evidences about genistein and its action in the bone, we considering necessary the study about genistein effect in peri-implant bone healing to best characterization this process.

2. Materials and Methods

2.1 Study design and ethics

The principles of laboratory animal care and national laws on animal use have complied within the present study, the Ethics Committee in the Use of Animals of Araçatuba Dental School (CEUA: 00511-2016) approved this study. Thirty female wistar rats (*Rattus norvegicus*, *Albinus*) weighting about 250 g were provided from the Central Vivarium of the Aracatuba Dental School UNESP. They were maintained at a temperature of 22°C, in a 12-h light/12-h dark cycle, with balanced feed (Ração Mogiana Alimentos SA, Campinas, Brazil). The rats were selected for the experiment after confirmation of their regular estrous cycle. Rats were ovx or sham-operated under general anesthesia with xylazine (0,03 ml/100 g bw/ip–Dopaser® Laboratories Calier S.A., Barcelona, Spain) and ketamine (0.07 ml/100 g bw/ip–Fort Dodge Saúde Animal Ltda, Brazil). Bilateral incisions were performed to remove the ovaries (OVX) and for sham operations, in which ovaries were only exposed without ablation (SHAM). Rats were kept in individual cages to daily evaluate the estrous cycle (Evans, H. M et al. 1922). Animals were randomized into three groups with 10 animals in each group: SHAM, OVX (gavage administration of saline), OVX GEN (gavage administration of genistein– 1mg/day) (Fu, S. W et al. 2014). Administrations started after 30 days after ovariectomy. Genistein was administrated until euthanasia.

2.2 Genistein treatment

Genistein was prepared in solvent Dimethyl sulfoxide (DMSO) (Aphoticario Manipulation Pharmacy, Araçatuba SP, Brazil). The OVX animals received through gavage genistein (1mg/day;) (Fu, et al. 2014). For genistein administration, the rats were immobilized, and then an adequate cannula was introduced orally. All treatments began thirty days after the ovariectomy and lasted for 120 days, that is, until the time of euthanasia.

2.3 Implant placement

Ninety days after ovariectomy surgery, animals were anesthetized (50 mg/kg of ketamine intramuscularly and 5 mg/kg xylazine (mepivacaine; 0.3 ml/kg 2%, adrenaline 1:100,000, Septodont, Saint-Maur-des Fossés, France), followed by antisepsis (polyvinylpyrrolidone iodide; Indústria Química e Farmacêutica Rioquímica Ltda, Brazil). Surgical access was performed with approximately 1.5 cm long in the tibial metaphysis region and then the soft tissue was divided in full-thickness and removed with the aid of periosteal detachers exposing the bone to receive the implants. Grade 4 titanium implants with 1.5 mm diameter and 5 mm length with an acid-etched surface (Emfills, Itu, São Paulo, Brazil) were installed bilaterally in each tibia, with bicortical stabilization. The suture was made by plans with Vycril (Poliglactina 910, Ethicon, Johnson & Johnson Prod, São José dos Campos, Brazil). In the immediate postoperative period, each animal received pentabiotic (0.1 ml/kg; Fort Dodge Saúde Animal Ltda, Campinas, São Paulo, Brazil) and sodic dipyrone (1 mg/kg; Ariston, Indústrias Químicas e Farmacêuticas Ltda, São Paulo, Brazil). Sixty days after implant placement animals received a lethal dose of thiopental (150 mg/kg body weight; Cristália, Ltda., Itapira, SP, Brazil). All implant placement followed previous studies (Oliveira D et al. 2017).

2.4 Microcomputerized tomography (μ CT)

For the three-dimensional analysis of animals from the SHAM, OVX and OVX GEN groups after euthanasia at the 60-day after implants installation. The right tibias were separated to microcomputerized tomography. For this analysis the implants were kept in position. The samples were fixed in 10% buffered formalin (Reagentes Analíticos®, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) for 48 hours, washed in running water for 24 hours, and stored in 70% alcohol. These pieces were scanned in the longitudinal plane with a SkyScan 1,172 (Bruker microCT, Aartselaar, Belgium) at 70 kV/114 mA with an integration time of 1 x 380 ms in a standard configuration (tube current: 165 μ A, image pixel size: 9.92 μ m, filter for beam hardening aluminum–copper: 0.5 mm, frame averaging: 4, rotation step: 0.6°). The images obtained by the projection of X-rays on the samples were stored and reconstituted to determine the area of interest by the NRecon software (SkyScan 2011, Version 1.6.6.0), with an artifact ring correction of 8, beam hardening correction of 24% and the image conversion varied from 0.0 to 0.14. Using the Data Viewer software (SkyScan, Version 1.4.4, 64-bit), reconstructed and observed in the images in three planes (transversal, longitudinal and sagittal). Then, the CTAnalyser-CTAn software (2003-11SkyScan, 2012 BrukerMicroCT Version 1.12.4.0) was determined region of interest (ROI) that corresponding to trabecular bone of valleys located between the second and fifth loop, the ROI was delimited 0,5 mm around implant in the area determined. Moreover, through CTAnalyser-CTAn software was determined bone volume. Finally, the three-dimensional reconstruction was performed using the CTvox software (SkyScan, Version 2.7). The parameter analysed was bone volume per tissue volume (BV/TV) and connectivity density (Cnn.Dn) (Dempster, et al. 2013).

2.5 Immunohistochemical analysis

The left tibias were fixed in 10% buffered formalin (Reagentes Analíticos®, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) for 48 hours, washed in running water for 24 hours. After the left tibias were descalcified in 10% ethylenediaminetetraacetic acid (EDTA) (Merck, Darmstadt, Germany) for 2 months. Subsequently, the samples were washed in running water for 24 hours, dehydrated, diaphonized and paraffin embedded. In moment of inclusion in paraffin the implants were removed. The pieces were sliced along the longitudinal axis of the implant until to obtain 5 micrometers. The slices were put at histological slides. In immunohistochemical analysis the primary antibodies were used as following: runt-related transcription factor 2 (RUNX2, goat anti-RUNX2; Santa Cruz Biotechnology), alkaline phosphatase (ALP, goat anti-ALP; Santa Cruz Biotechnology), osteopontin (OPN, goat anti- OPN; Santa Cruz Biotechnology), osteocalcin (OCN, goat anti-OCN;

Santa Cruz Biotechnology), tartrate-resistant acid phosphatase (TRAP, goat anti-TRAP; Santa Cruz Biotechnology). As a secondary antibody, a biotinylated donkey anti-goat antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used. The immunohistochemical reaction was amplified with an avidin-biotin system (Kit ABC-Vectastain Elite ABC–peroxidase standard, reagent A and B only–PK6100; Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (Sigma, Saint Louis, MO, USA) was used as chromogen. Immunohistochemical reactions were controlled to evaluate the specificity of the labels. Hematoxylin was used for counter-staining. Data analysis was performed in a qualitative manner, with scores ranging from “0” for absence of marking and “1, 2, and 3” for little, medium, and intense, respectively, according to previous published studies (dos Santos, et al. 2013; Queiroz, et al. 2008; Luvizuto, et al. 2010)

2.6 Statistics

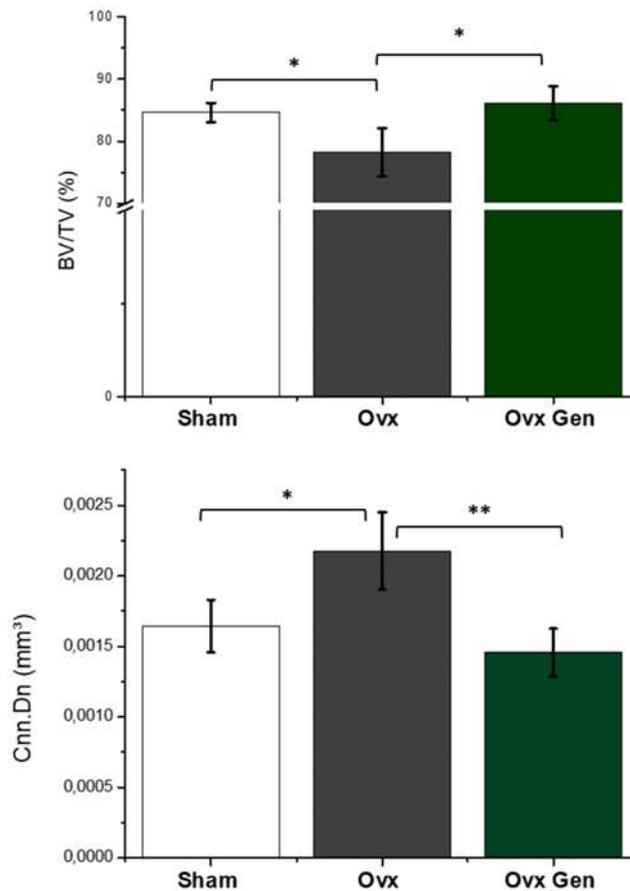
The statistical tests were performed in the GraphPad Prism 7 program (GraphPad Software; La Jolla; USA). For the quantitative parameter: μ CT (BV/TV and Cnn.Dn), normality and homoscedasticity tests were applied to verify the distribution in the normality curve. The Shapiro-Wilk test was computed to check for normality. Based on this, two-way ANOVA followed by Tukey tests.

3. Results and Discussion

3.1 Microtomographic analysis

The computerized microtomography analysis to the bone volume percentage parameter the average of the referenced values were in SHAM 84,62%, in OVX 78,23%, and 86,09% in OVX GEN. There was a statistical difference between SHAM and OVX and between the OVX and OVX GEN groups ($p < 0,05$). For connective density the values were in SHAM 0,00164 mm³, in OVX 0,00218 mm³ and in OVX GEN 0,00146 mm³. There were statistical differences between SHAM and OVX and between OVX and OVX GEN groups ($p < 0,05$). [Figure 1]

Figure 1 – Microtomographic analysis of the medullary peri implant bone. Morphological parameter was calculated and reported as bone volume per tissue volume (BV/TV) and connective density (Cnn.Dn) for SHAM, OVX and OVX GEN groups at 60 days. The * indicates significant statistical difference ($p < 0.05$).



Source: Authors.

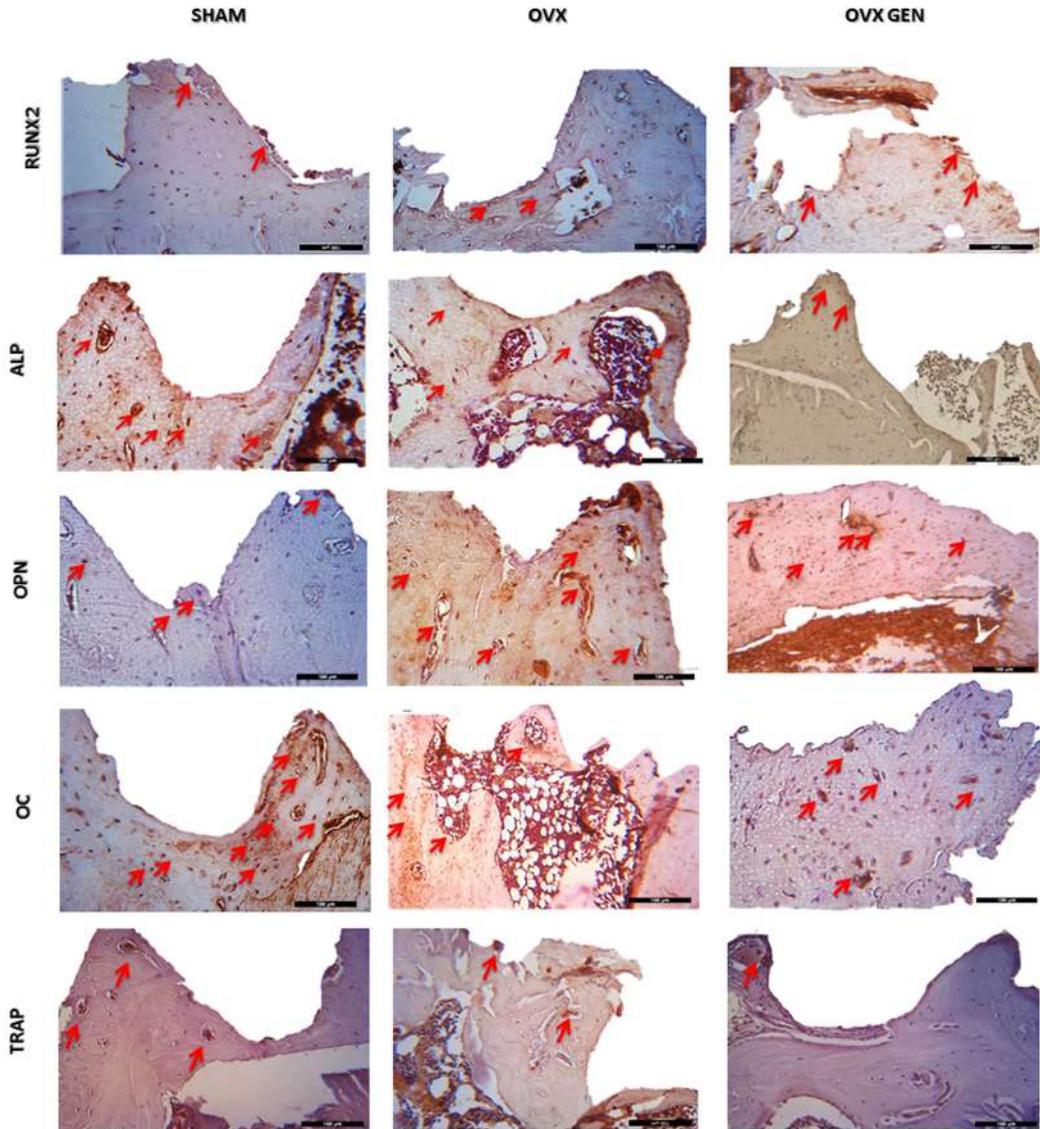
3.2 Immunolabeling Analysis

With the object to indicate osteoblast differentiation steps and the role of extracellular bone matrix in the peri-implant bone tissue, we performed immunohistochemical analysis. Therefore, the transcription factor RUNX2 and the proteins alkaline phosphatase, osteopontin, and osteocalcin were immunolabeled and the area close to the implant's threads were considered as the region of interest. RUNX2 showed positive immunolabeling all the groups. In the SHAM group had little marking in osteoblastic lineage cells around the bone tissue formed near the implants. In the OVX group showed less marking in bone tissue. While for OVX GEN observed medium marking to RUNX2 makings of young osteoblastic lineage cells in the peri-implant bone tissue.

Alkaline phosphatase showed positive marking all the groups. In the Sham group had medium to intense marking with important marking of osteoblasts present in peri implant-bone tissue. In OVX showed little to medium marking to alkaline phosphatase. The OVX GEN group had medium marking in the osteoblasts presents in the tissue. Osteopontin showed medium in SHAM and OVX GEN group while the OVX group had little marking in the peri-implant bone tissue. Osteocalcin showed in the SHAM group marking mainly in osteocytes with medium to intense marking. The marked osteocytes point the degree of maturation of the bone near the implant. OVX group had little marking while in the OVX GEN group showed medium

marking in osteocytes similar to the sham group. TRAP showed in SHAM and OVX group marking in osteoclasts with little to medium marking. While in OVX GEN group showed little marking. [Figure 2] [Table 1]

Figure 2 – Immunolabeling on the bone healing peri-implant from experimental groups (SHAM, OVX, OVX GEN) at 60 days. In immunohistochemical analysis were evaluated the proteins Runx2, ALP, OPN, OC, TRAP.



Source: Authors.

Table 1 - Scores observed in immunohistochemical analysis on the bone healing peri-implant from experimental groups (SHAM, OVX, OVX GEN) at 60 days. The proteins evaluated were Runx2, Alkaline Phosphatase, Osteopontin, Osteocalcin and TRAP.

	SHAM	OVX	OVX GEN
RUNX2	1	1	2
ALP	2-3	1-2	2
OPN	2	1	2
OC	1-2	1	2
TRAP	1-2	1-2	1

Source: Authors.

4. Discussion

Genistein is the most abundant isoflavone that can be found in soy. Studies show that genistein acts as a natural selective modulator of estrogen receptors preventing bone loss. However, this effect in the peri-implant bone repair has not been evaluated (Fanti, P et al. 1998; Wei, H et al. 2003; Polkowski, K. et al. 2000). Besides it is expected that genistein may have a positive effect on bone metabolism, improving the perimplantar repair process, it is important to highlight that in this study, different aspects of repair process are characterized. Therefore, parameters related to bone microarchitecture, like percentual of trabecular bone and the structure of connection between the trabecular show that this aspect may be stimulated by genistein treatment. Other point that is import to highlight is related to bone matrix protein that seems to be directly involved in this process. Genistein and its activity on the stimulation of bone formation positively affects expression of bone formation markers. The data set of the microtomographic parameters BV/TV and Cnn.Dn and protein immunostaining (RUNX2, ALP, OPN, OC, TRAP), it became evident that the OVX GEN group rats showed superior results regarding repair patterns having greater bone tissue maturation, higher bone volume, and consequently higher bone turnover compared to the OVX positive control group. The morphometric results are similar between OVX GEN and SHAM group showing the capacity of genistein to act in bone tissue to improve peri-implant bone healing in estrogen deficiency conditions. The evaluation of extracellular bone matrix proteins assure that the repair bone formed close to the implant's loops is characterized by an important cellular viability, with osteoblasts in intense synthesis activity what is highly desirable when bone quality is considered for the implant's longevity.

The morphometric analysis of the parameter evaluated in the present study shows a tendency for the formation of better quality and higher reparative bone tissue in the SHAM and OVX GEN animals, confirming the immunolabeling observations. In osteoporosis, bone loss is the result of an imbalance between bone formation and resorption, with the predominance of resorption significantly interfering with the maintenance of bone volume percentage and its microarchitecture, ranging from a normal bone to the most porous bone (Weitzmann, et al. 2006; Harada, et al. 2003; Parfitt, et al. 1987). The values for bone volume percentage were similar for SHAM and OVX GEN groups, showing that genistein was able to reverse bone volume loss, which was statistically reduced in OVX SAL group. While computerized microtomography is related with bone morphometry, immunohistochemical with cell responses that happens during bone healing. The characterization of proteins present in perimplantar bone indicates that the improvement of OVX GEN, with responses that are similar to SHAM groups may be related with the differentiation of osteoblastic lineage cells that starts a mineralization process in these both groups previously than on OVX animals.

Immunohistochemical analysis showed that bone tissue formation was close to the implant loops, with better formation patterns in the SHAM group followed by OVX GEN. Regarding immunostaining, it is noteworthy that the proteins

evaluated were moderately expressed in the OVX GEN group, differing from the OVX group which showed slight immunostaining for them. This result indicates the performance of genistein treatment positively on the expression of osteoblastic phenotype markers favoring peri-implant bone repair. According Cepeda et al. genistein has signaling pathways involved as estrogen receptor and nitric oxide pathways (Cepeda, et al. 2020), its vasodilator action improves osteoblastogenesis and osteoclasts differentiation, therefore genistein is able to act in bone tissue improving the bone healing repair in estrogen deficiency conditions. Corroborating the morphometric analysis, the immunohistochemistry showed the formation of bone tissue near the turns of the implants, with better formation patterns in the OVX GEN group followed by SHAM.

5. Conclusion

Genistein showed positive results in bone tissue, while its side effects are unknown in the literature, which has hitherto been shown to be a safe substance to administrate in estrogen deficiency conditions. The set of results obtained in this study demonstrates that genistein was able to act in peri-implant bone tissue of ovariectomized rats, improving the peri-implant bone healing process. The evaluated parameters show that genistein was able to act in the perimplantar reparative bone tissue providing a similar repair between treated and healthy group.

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