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Original Article

Evaluation of strontium ranelate in the repair of standardized intrabuccal bone defects in a rat model

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Abstract: The treatment of intrabuccal bone defects and the search for new agents to optimize regeneration procedures are extremely valuable. The present experimental study aimed to evaluate the effects of orally administered Strontium Ranelate (SrR), in the repair of intrabuccal bone defects in rats. Twenty Lewis rats, divided in 4 groups (2 control and 2 test groups) were used and evaluated at 14 and 42 days. Standardized bone defects in the distal alveolar region of the first superior molar were created in all animals. Test groups received a daily dose of SrR (625 mg/kg), and control groups received a placebo. Bone neoformation within the defects were evaluated through histological and morphometric analysis. At 14 days, histological analysis revealed similar healing patterns between groups. However, at 42 days, the test group presented healing patterns with better tissue organization, compatible with slightly advanced bone maturation. At 14 days, morphometric analysis revealed a higher rate of bone deposition in the test group when compared to the control group ($P<0.05$). At 42 days, no significant differences between groups were observed in relation to morphometric parameters. SrR seemed to accelerate the process of bone neoformation.

Keywords: Bone mineralization, bone regeneration, intrabuccal bone defects, strontium ranelate

Introduction

Bone tissue is one of the body structures that keep the regenerative capacity during adult life. This unique characteristic allows the periodic remodeling of bone skeleton, fracture healing, and bone defects repair [1].

Bone reconstruction is crucial to the functional and aesthetic oral rehabilitation, especially in the correction of alterations by trauma or atrophic changes of the alveolar bone [2, 3]. Recent techniques for treatment of bone defects associated with periodontal diseases, dental implants, or even skeletal fractures consists of replacing the lost bone tissue by bone particles or synthetic compounds for adequate filling of the defects [4].

Currently, autogenous bone grafts are considered the gold standard in grafting materials [5]. Despite the advantages this therapeutic modality offers, some unfavorable points should be considerate: the need for a second surgical site, and the low availability of viable donor areas. These disadvantages have encouraged the search for other forms of treatment, such as the use of biomaterials. In addition, bone therapies also use the administration of osteogenic molecules or drugs that promote bone deposition. Therefore, several agents have been proposed; BMPs [6], PDGF factor [7], hyaluronic acid [8], among others. These are some active molecules that are generally applied directly on the site of interest. Many other drugs can be used systemically for the stimulation of bone metabolism. Among these drugs, the Strontium Ranelate (SrR) has received special attention from researchers in the past few years [9-14].

The SrR is a novel compound currently used in the treatment of osteoporosis in post-menopausal women with the aim of reducing the risk of bone fractures [9, 15, 16]. The SrR mechanism of action on the bone tissue still remains poorly understood. Some *in vitro* studies suggested that SrR may promote the prolifera-

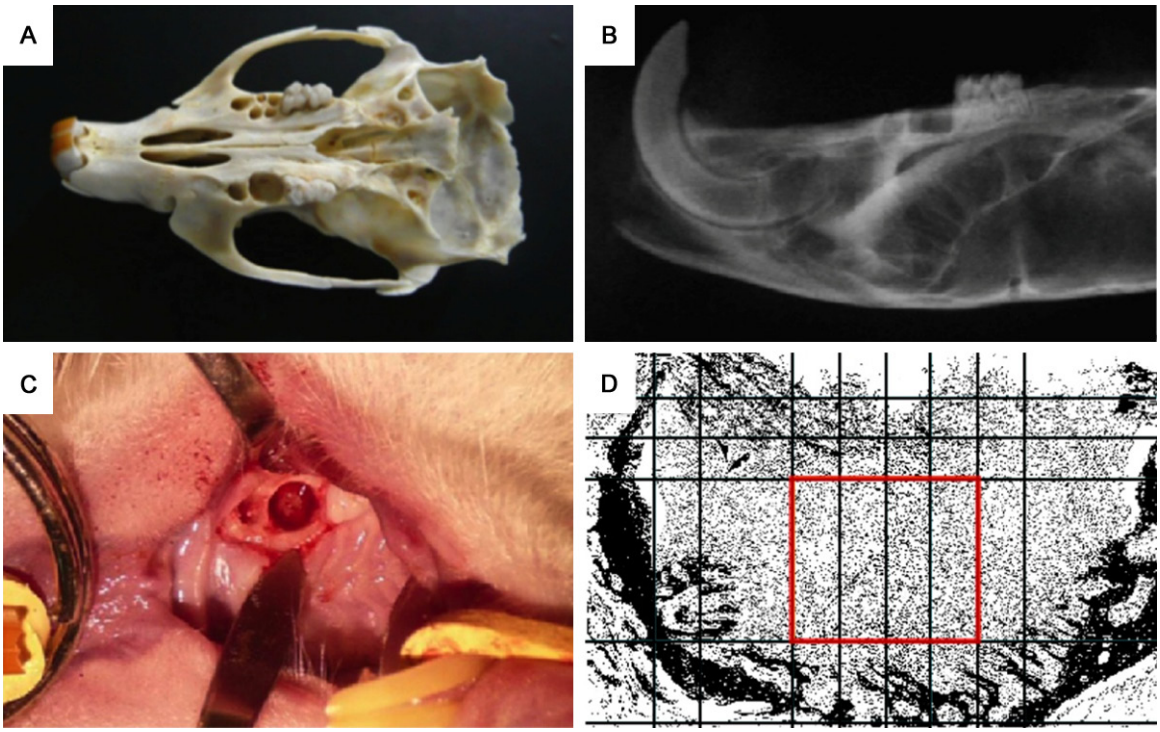

Figure 1. Illustrative photographs of bone defect. A. Superior view of rat skull with the bone defect created by osteotomy of the distal alveolar roots. Note the preservation of the septum between medial and distal roots. B. Lateral radiographic image of rat skull showing the limits of the standardized bone defect in the area of the distal roots of the superior first molar. C. Superior view during surgery procedure. Note the preservation of the defect floor and bone septum. D. Definition of the reading area. Histological sections staining with Gomori trichrome were select to the channel color with the greatest contrast to collagen deposition. The red square identifies the area to be read for the software.

tion and differentiation of osteoblasts [17, 18] and may increase osteogenic differentiation of bone marrow cells [19, 20]. There is some evidence that SrR has a dual mode of action, simultaneously inhibiting osteoclast activity and stimulating osteoblastic maturation and replication [21]. Although SrR has been widely studied and used in different segments of medicine [9, 15, 22], studies in the dental field are still scarce [11, 13, 23].

Ever since bone regeneration is an important issue in dentistry, the evaluation of SrR effects on bone repair and remodeling is valuable and deserve additional investigation. Thus, the present study aimed to evaluate the effects of SrR, orally administered, on the repair of intrabuccal bone defects in a rat model.

Materials and methods

Experimental model

Twenty, 10-week-old, male Lewis rats (*Rattus norvegicus albinus*), from the vivarium of the Department of Biochemistry and Immunology from the Institute of Biological Sciences of the Federal University of Minas Gerais were used. Sample size was determined through a sample size calculation using the primary outcome (bone volume to total volume) from a previous study [24].

Animals were randomly divided into 4 groups (n = 5): experimental groups (T14 and T42) and control groups (C14 and C42). T14 and C14 animals were euthanized after 14 days and T42 and C42, after 42 days of surgical procedures.

Animals were maintained in appropriate animal cages with controlled temperature and luminosity according to the guidelines of the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA/UFMG). Animals had *ad libitum* access to water and food, except during the period of 5 days after surgery, when only water and pasty diet were offered. It is important to emphasize that during the postoperative period, animals were

kept isolate one per cage and weight were monitored during all period.

The present study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA/UFMG) (Protocol nº. 303/2013). When applicable, the present study followed the ARRIVE (Animal Research Reporting *in Vivo* Experiments) checklist guidelines.

Surgical procedures

Animals were anesthetized with an intramuscular injection of a mixture of 2% xylazine (Ronpum®, Bayer, São Paulo, Brazil) and 10% ketamine (Dopalen®, Vetbrands, São Paulo, Brazil) 1:1, 0.1 ml/100 g body weight, i.m. Prior to surgical procedures, animals received a dose of the anti-inflammatory flunixin-meglumine for veterinary use (Banamine® injectable PET, Schering-Plough Animal Health, São Paulo, Brazil) 1.1 mg/kg. Animals were placed on a surgical table in supine position and then subjected to the extraction of the upper right first molar through the alveolar pathway. The standardized full-thickness circular bone defect (2.5 mm diameter × 2.5 mm deep) was created in the alveolar area with a cylindrical diamond bur #2094 (KG Sorensen® ISO, São Paulo, Brazil). The distal alveolar septum was removed and the mesial alveolar septum was always preserved (**Figure 1**). Control of bleeding and secretions were performed by vacuum aspiration.

After confirming the integrity of the defect walls, the mucosal were sutured with silk 6.0 (Ethicon®, Jonhson & Jonhson, São Paulo, Brazil). Animals received subcutaneous doses of oxytetracycline antibiotic (Terramycin® Injectable solution, Pfizer, São Paulo, Brazil), 10 mg/kg once every 24 hours for 3 days. All over the postoperative period, no wood shavings were used on cages in order to prevent intraoral residues that could contaminate or damage the surgical site. The sutures were removed on the 14th day after surgery. After 14 and 42 days of clinical procedures, the animals were euthanized by decapitation under anesthesia with 10% ketamine and 2% xylazine (1:1, 0.1 mL/100 g body weight, i.m.).

Administration of strontium ranelate

SrR administration schedule was initiated right after the surgical procedure. Animals in the test groups received 625 mg/kg of SrR (Protos®, Laboratory Servier, Rio de Janeiro, Brazil) added to 2 grams of pasty food, 7 days per week. This dose was determined to be similar to human regimen of 2 g/day [25, 26]. In order to control drug intake, the regular food of the animals were daily suspended for two hours and replaced by SrR pasty food for drug administration. After confirmation of drug intake, regular food was reestablished. Animals in the control groups received the same amount of pasty food (2 grams/day).

Histological and morphometric evaluations

Animals were euthanized after 14 and 42 days of surgical procedure. The upper maxillaries were immediately fixed in neutral 10% buffered formalin for 72 hours at ambient temperature and then demineralized with formic acid, hydrochloric acid, and aluminum chloride (Plank-Rychlo's Solution, HC World Life Science Products & Services, Woodstock, USA). A preliminary slice in the central area of the defect enabled the standardization of the microtome area and consequently the uniformity of the region to be evaluated in all groups. This preliminary slice was made 1 mm away from mesial surface of the 2nd molar crown. After demineralization, samples were processed for histology as routine protocol.

110 serial sections of 6-microns thickness of each sample were set in 13 sequential histological slides previously treated with 2% Silane. The slides were then stained with Gomori trichrome for analysis.

The presence of hair, wood shavings, food or any external fragment in the defect area were considered as exclusion criteria, especially at 14 days period, when the new and fragile mucous membrane could allow the entered of strange particles into the wound area. In addition to these criteria, it was also excluded samples with images of necrotic bone, especially those visible at 42 days for animals whose bone remodeling resulted in the disruption of one of the defect walls (buccal or palatal).

For morphometric analysis, 3 histological sections were selected as follows: cut 1-corresponding to the center of the defect; cut 3-corresponding to the distal end of the defect; and cut 2-corresponding to the midpoint between cuts 1 and 3. Images were captured with a 4 × objective Q-colour 3 camera, attached to a light microscope (Olympus BX-41, Olympus Corpor-

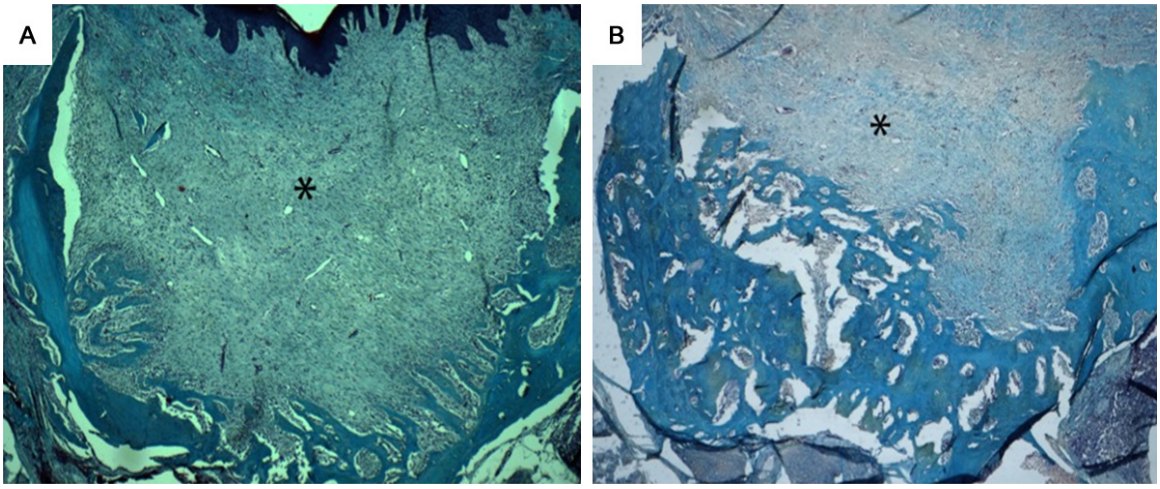

Figure 2. Illustrative photomicrographs of bone repair at 14 days. (A) Control group (C14) and (B) treated group (T14). In both groups, it was possible to note a repaired oral mucosa with stratified keratinized epithelium covering connective tissue area. Mild inflammatory process (asterisks), without infiltration, was observed in the connective tissue in both groups. The new bone formation level, observed by trabecular bone stained in green, was lower in the control group (A), restricted to the apical 1/3 bone defect. In the SrR treated group (B), however, the trabecular bone exceeded the apical 1/3, reaching the middle 1/3 of the bone defect. Original magnification 2 ×. Gomori's thricome staining.

Table 1. Comparison between test (T14 and T42) and control (C14 and C42) groups in regard to the percent of bone formation

Groups	Percent of bone formation			P*
	Mean (± s.d.)	Minimum	Maximum	
C14	19.948 ± 6.876	10.666	26.777	<0.01
T14	41.484 ± 8.648	34.003	54.693	
C42	77.465 ± 2.580	80.324	74.262	>0.05
T42	79.096 ± 9.089	67.452	90.007	

*ANOVA with Bonferroni correction.

ation, Center Valley, PA, USA). Then, images were transferred to a computer and evaluated with morphometric software (Image J®, National Institutes of Health, Bethesta, Maryland, USA). The central region of the defect (**Figure 1**) was selected to evaluate the areas stained in green by Gomori trichrome (type I collagen) corresponding to bone formation, thereby providing the percentage of neoformation in the total area selected.

For the definition of the most representative cuts, a preliminary study of images obtained was conducted considering the maximum and minimum healing levels of wound.

Statistical analysis

Data was tested for normality and homoscedasticity through Liliefors and Bartlett tests, respectively. Groups were compared through One-way ANOVA with Bonferroni's correction. All data was analyzed by means of statistical software (Statistical Package for Social Sciences-SPSS version 17, IBM, Chicago, IL, USA). Differences between groups were considered significant if $P<0.05$.

Results

Histological evaluation revealed patterns of tissue healing quite similar between the control and test groups within 14 days. In both groups, the wound was completely closed, covered with stratified squamous keratinized epithelium and underlying connective tissue presenting healthy histological appearance, with no inflammatory infiltrate. At 14 days, new bone formation was observed particularly in the apical and middle third of the defect in both groups (**Figure 2**).

Histological findings also showed osteoblast proliferation in regions of newly formed bone trabeculae. These aspects occurred in both groups (T14 and C14). Quantitative comparison of bone formation between T14 and C14 groups showed a higher collagen deposition in the test group. Group T14 presented a defect filling area of bone neoformation significantly higher ($P<0.05$) (**Table 1**).

Although it was not the scope of the present study to evaluate osteoclast proliferation, it

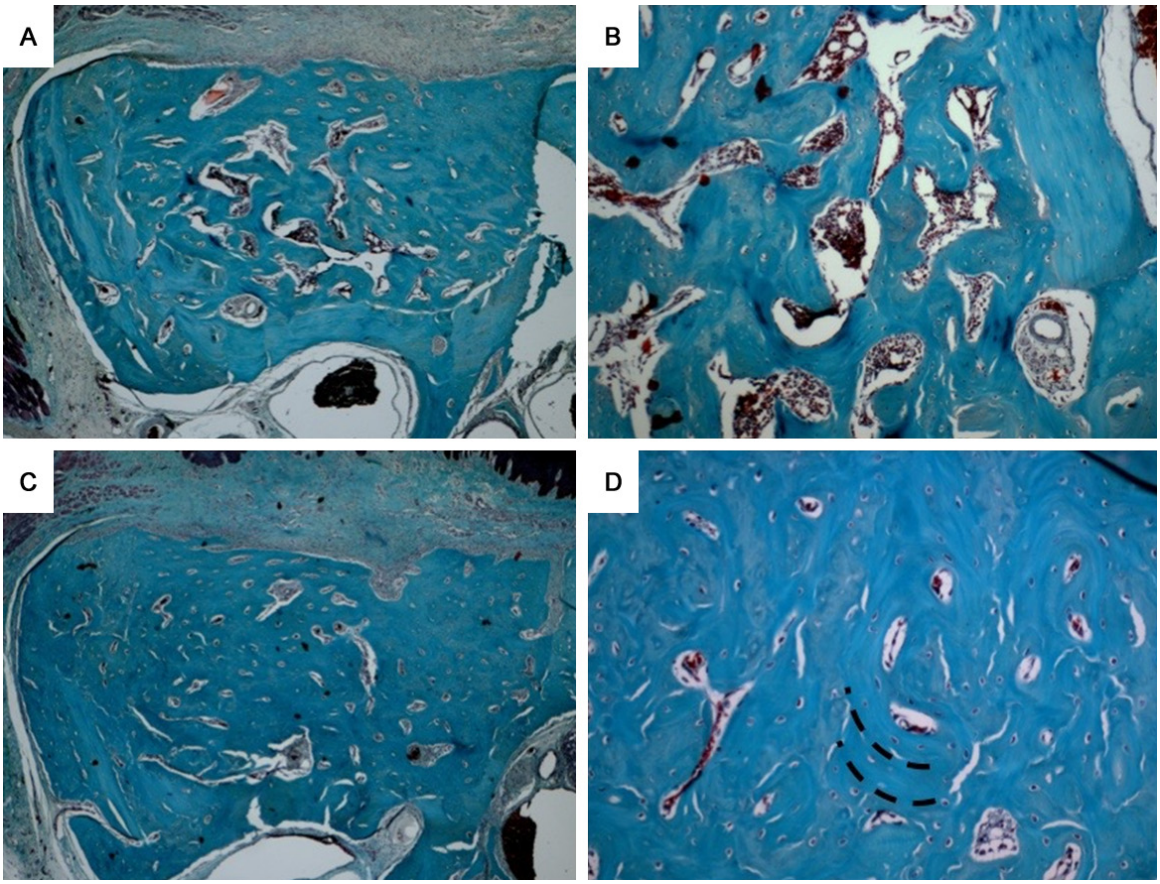

Figure 3. Illustrative photomicrographs of bone repair at 42 days. (A, B) Control group (C42) and (C-D) treated group (T42). Both groups presented the defect area completely filled with new bone tissue. Image magnification (B and D), however, revealed different levels of tissue organization. In the control group (B), marrow spaces were found to be larger, while the osteocytes were found as disorganized cells, suggesting a structure of immature bone. In the treated group (D), trabeculae was thicker, marrow spaces were smaller, and the osteocytes were found as lined up cells, suggesting the organization of haversian systems (dashed lines). These aspects indicate the remodeling of newly formed bone into mature bone. Original magnification 4 × (A and C), 20 × (B and D). Gomori's thricome staining.

was evident at 14 days, in both groups, the typical bone remodeling process. The cuts revealed the simultaneous activity of osteoclasts in the process of resorption of bone walls, as well as osteoblasts in the process of synthesis or deposition of collagen matrix. Apparently, in the 3 points of histological evaluation, no sign of imbalance between osteoclasts and osteoblasts cells was observed in favor of the control or test groups within 14 days.

Within 42 days, the operated sites were covered with keratinized stratified squamous epithelium in both groups. The area of the defect was filled with bone tissue characterized by thick trabecular bone fused with few marrow spaces in the central area. Histologically, it was noticed a better organization of the bone matrix with some osteocytes already outlining organization patterns of Havers system in the samples of treated animals (T42) (**Figure 3**).

There were no statistically significant differences in the quantitative comparison of bone formation between the C42 and T42 groups ($P>0.05$) (**Table 1**).

Discussion

Results from the present study showed a positive effect of SrR on bone metabolism during the healing process of oral bone defects. Although C42 and T42 groups had similar bone repair, T14 group showed to be advance on repair process compared to 14 days control group. Early bone deposition in bone defects regions is extremely important to faster and complete re-establish of masticatory function,

aesthetics and phonetics. Thus, the SrR can be a promising drug in bone regeneration therapies, since rehabilitations time is precious.

A great variety of in vitro and in vivo studies have been conducted in order to test new therapies and bone osteogenic strategies [27-29]. In vivo analyses of bone repair are mostly evaluated after creation of bone defects in animal models [7, 30]. Traditionally, these defects are created in extra-oral sites [31]. However, for better dental research, it would be interesting to use a bone defect model with similar characteristics to the maxillofacial bone complex repair. Therefore, the post extraction dental sockets have been a commonly model used to evaluate factors that may accelerate or delay the process of bone repair [32, 33]. Although dental alveoli is a viable model for the analysis of bone reconstruction of small cavities, some disadvantages should be mentioned, such as differences in the alveolar size, the presence of inter-root septum, and the different amounts of the remaining periodontal ligament, which may modified the interpretation of the results.

In order to control these variables, a standardized intraoral defect in a rat model was used in the present study. This model added the benefits of standardization techniques of extra oral defects with characteristics found in the oral cavity, such as microorganisms, humidity conditions, and masticatory trauma. Moreover, it is important to emphasize dimensions of the defect and the fact that it can be considered a critical-size bone defect. The importance of a critical bone defect to study bone regeneration techniques was previously discussed [34, 35].

In view of the possibility of new therapeutic strategies for improving bone regeneration, some drugs have been studied. SrR has been investigated due to its double mechanism of action, favoring bone metabolism through inhibition of osteoclasts and stimulation of osteoblasts [9, 15, 16]. Therefore, SrR was chosen to be tested as an inductor of bone formation in the present study. Until now, according to our knowledge, this is the first study evaluating the effect of this drug in intraoral defects.

The 14-days period was selected to analyze bone deposition for the reason it represents the initial phase of mineral deposition on the sides and bottom of the defect. Thus, any bone induction could be best observed in this period. A previous study of similar nature using post-extraction sockets as a model, suggested after 42 days the bone repair process is complete [36]. Clinical and histological findings from the present study showed that after 42 days, osteogenic cells were still active on surface edge of the new bone. Although the bone defect was filled with a minority of bone marrow spaces, these findings indicate the incomplete bone remodeling. Furthermore, it was observed an immature appearance in most of the newly formed bone in both groups. The discrepancies between these data and the results of the literature possibly derive from the bone defect model, which traditionally are dental post extraction sockets.

Previous in vitro studies revealed that SrR has osteoinductive properties, within reduction of osteoclast cell number and increase of osteoblasts differentiation [17, 18, 20, 37]. In our study, it was not possible to identify a clear difference in the balance between these cells, since it was not used specific techniques of cell labeling. Future studies should be conducted to confirm these findings.

Histomorphometric results revealed a higher percentage of new bone matrix (collagen I stained by Gomori trichrome) by the total area assessed in the C14 group during the repair of the defect. These results come in agreement with some findings in the literature. Previous studies have reported that the systemic use of strontium in animals and patients was effective on improving the quality of bone tissue, increasing its strength and reducing the risk of fractures [10, 22, 25, 38-40].

Some studies have shown conflicting results when compared to those found in the present study [41, 42]. Discrepancies may exist due to methodological differences, such as type of bone defect, evaluation period, and evaluation methods. In our experiment, it was used a new bone defect model allowing the evaluation of bone deposition during the repair process, while in other studies the evaluation was performed in mature bone or osteoporosis process.

Another important finding was the presence of a better bone matrix organization in T42 group when compared to C42, signaling a possible advantage over bone maturation stage.

The results found in this study can be considered as an important research when added to those previously published in the literature. It reinforces the advantage of using SrR in situations of physiological changes in bone metabolism. Thus, the application of SrR as a new therapeutic strategy can bring great benefits in different areas of dentistry, such as periodontology, implant dentistry, and oral maxillofacial surgery. Conditions of fast bone formation in humans represents less number of surgical procedures, less morbidity, quicker therapy and consequently lower costs.

In this manner, the promising findings from the present animal study points to the relevance of future studies in order to explore the potential effects of SrR on the repair of human oral bone defects. Moreover, future studies could provide a better understanding of the molecular and cellular action of this drug on bone tissue, as well as the potential therapeutic strategies. Future studies should also focus on different types of defects, different methods of SrR administration and evaluation of specific markers of bone metabolism.

In summary, this study provides preliminary information of the SrR effects on bone formation. We demonstrated that SrR anticipates the process of neoformation and bone maturation in an animal model. These results suggest a possible benefit in the use of oral SrR to accelerate the intraoral bone repair.

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Disclosure of conflict of interest

None.

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