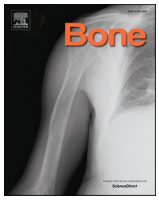
Bone 187 (2024) 117211

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone



Full Length Article

Preventing bisphosphonate induced osteonecrosis of the jaw with a polyguanidine conjugate (GuaDex): A promising new approach

Arquímedes Cantorán-Castillo [a], Belinda Beltrán-Salinas [a], Jorge M. Antúnez-Treviño [a], Ricardo Martínez-Pedraza [a], Rodolfo Franco-Márquez [b], Mario A. Guzmán-García [c], Ricardo M. Cerda-Flores [d], Raúl V. Perales-Pérez [e], Christian Zakian [f], Jesús Ancer-Rodriguez [d], Marcela Márquez-Méndez [d,*]

[a] *Faculty of Dentistry, Autonomous University of Nuevo Leon, Dr. Eduardo Aguirre Pequeno, Mitras Centro, 64460 Monterrey, NL, Mexico*
[b] *Department of Pathology and Cytopathology, Hospital Universitario, Autonomous University of Nuevo León, Av. Dr. J. Eleuterio Gonzalez S/N, Mitras Centro, 64460 Monterrey, NL, Mexico*
[c] *Faculty of Veterinary Medicine and Zootechnics, Autonomous University of Nuevo Leon, 66054 Gral. Escobedo, NL, Mexico*
[d] *Center for Research and Development on Health Science, Autonomous University of Nuevo Leon, Dr. J. Eluterio Gonzalez/Dr. Carlos Canseco, Mitras Centro, 64460 Monterrey, NL, Mexico*
[e] *Odontología Avanzada Laser, Calle Juarez 109 Sur, Centro, 67500 Montemorelos, NL, Mexico*
[f] *Kevork Instruments, Palacio de Justicia #888, Col. Anahuac, 66450 San Nicolas De Los Garza, NL, Mexico*

ARTICLE INFO

Keywords:
Polyguanidine
Bisphosphonates
Osteonecrosis
Rat model
Bone regeneration
Bone injury healing

ABSTRACT

Osteonecrosis of the jaw (ONJ) is a relatively rare side effect after prolonged use of bisphosphonates, which are drugs used to treat bone resorption in osteoporosis and certain cancers. This study introduces a novel ONJ model in rats by combining exposure to bisphosphonates, oral surgery, and bacterial inoculation. Potential ONJ preventive effects of polyguanidine (GuaDex) or antibiotics were evaluated.

The study consisted of twenty-four male Wistar rats were divided into four groups. Groups 1 to 3 were given weekly doses of i.v. Zoledronic acid (ZA), four weeks before and two weeks after an osteotomy procedure on their left mandibular first molar. Group 4 was a negative control. *Streptococcus gordonii* bacteria were introduced into the osteotomy pulp chamber and via the food for seven days. On day eight, the rats were given different treatments. Group 1 was given a GuaDex injection into the osteotomy socket, Group 2 was given an intramuscular (i.m.) injection of clindamycin, Group 3 (positive control) was given an i.m. injection of saline, and Group 4 was given an i.m. injection of saline. Blood samples were taken two weeks after the osteotomy procedure, after which the rats were euthanized. Bone healing, bone mineral density, histology, and blood status were analyzed.

The results showed that Group 1 (GuaDex) had no ONJ, extensive ongoing bone regeneration, active healing activity, vascularization, and no presence of bacteria. Group 2 (clindamycin) showed early stages of ONJ, avascular areas, and bacteria. Group 3 showed stages of ONJ, inflammatory infiltrates, defective healing, and bacterial presence, and Group 4 had normal healing activity and no bacterial presence.

Conclusion: ZA treatment and bacterial inoculation after tooth extraction inhibited bone remodeling/healing and induced ONJ characteristic lesions in the rats. Only GuaDex apparently prevented ONJ development, stimulated bone remodeling, and provided an antimicrobial effect.

1. Introduction

Bisphosphonates (BPs) have been used for several decades to reduce bone resorption in various diseases such as osteoporosis, Paget's disease, bone metastases from breast/prostate cancer, and multiple myeloma. Bisphosphonate (BP) treatment reduces skeletal-related events (SRE), such as hypercalcemia, and has an analgesic effect [1]. Prolonged BP treatment, there is a certain risk of developing osteonecrosis of the jaw (ONJ). The risk increases together with oral lesions, e.g., after dental surgery, ill-fitting dentures, etc. The incidence of ONJ increases in

* Corresponding author at: Autonomous University of Nuevo León, Center for Research and Development in Health Sciences, Av José Eleuterio Gonzalez, Mitras centro, 64460 Monterrey, NL, Mexico.
E-mail address: holmbergmarcela@gmail.com (M. Márquez-Méndez).

https://doi.org/10.1016/j.bone.2024.117211
Received 24 April 2024; Received in revised form 22 July 2024; Accepted 22 July 2024
Available online 23 July 2024

malignancies affecting bone. In multiple myeloma, the incidence of ONJ after prolonged BP medication is approximately 12 %, while the corresponding incidence in breast and prostate cancer is approximately 1–5 % [2].

ONJ leads to bone exposure, inflammation, suppuration, and sequestration [3], which can have devastating implications on the patient's quality of life, causing pain, discomfort while eating, anxiety, and depression [4,5]. The etiology of BPs-induced ONJ depends on several factors, for example, inhibition of bone resorption by suppression of osteoclast activity [6], decreased vascular supply, inhibition of the growth and migratory capacity of oral fibroblasts and down-regulation of the transcription of type 1 collagen, necessary for the deposition of granulation tissue and re-epithelization [7].

Bacteria are also involved in the development of ONJ, particularly the microflora distinct to the oral cavity, which has been considered a potential trigger or stimulating factor [8]. ONJ lesions are challenging to treat, and there is a consensus on the importance of prevention [9]. Treatment recommendations include effective oral hygiene, debridement of necrotic bone, pain control, infection management, antimicrobial oral rinses, and temporally withdrawing BPs at the treating physician's discretion [10]. Without effective ONJ treatment, prevention is essential. Therefore, new preventive therapies that can reduce the incidence of ONJ are highly desirable. However, depending on the stage of ONJ, the response to surgical treatment of ONJ appears successful [9].

In previous studies, polyguanidine (GuaDex) has shown interesting properties, such as potent tumor cell toxicity [11,12] and antimicrobial activity demonstrated in oral bacteria, *S. mutants* UA130, *P. gingivalis* W83, and *P. endodontalis* [13,14]. Escamilla et al. showed that GuaDex had only mild side effects on normal dental pulp stem cells and was significantly less toxic than chlorhexidine while maintaining the morphology and integrity without evidence of cellular damage in fibroblasts [13]. GuaDex showed no toxicity to fibroblast even after prolonged exposure [15].

Based on these findings, a Wistar rat model for BP-related ONJ was developed. This model was instrumental in further evaluating GuaDex's antibacterial properties and potential to prevent ONJ.

Wistar rats were chosen for their physiological similarities to humans, particularly in bone metabolism and BP response. Their larger size allows for ease of surgical procedures and sample collection, and their established protocols make them ideal for large-scale research.

The BP-induced ONJ model in the present study depends on principal factors associated with ONJ, i.e., bacterial presence and trauma after dental surgery.

Bacteria are known to be associated with ONJ and periodontal diseases. Bacterial infection causes wound inflammation and hinders angiogenesis, which complicates wound healing [16]. *Streptococcus gordonii* has been found in ONJ lesions [17,18], and may induce bone resorption by increasing osteoclast differentiation [19]. The inflammatory response triggered by *S. gordonii* may impair blood supply, osteoblast function and exacerbate bone resorption, crucial factors in osteonecrosis.

Zoledronic acid (ZA) is a potent amino-bisphosphonate frequently used in animal models of ONJ [20]. In the present model, weekly i.v. doses of ZA were used, as potent BPs administered i.v. are associated with ONJ.

The mean molecular weight (MW) of GuaDex is approximately 60kD and the high MW results in only slow intravasation. It will stay at the location of the lesion, and even the single local dose of GuaDex used in the present study would have a prolonged effect.

Clindamycin, commonly used for jawbone infections, was employed as a control due to its ability to distribute throughout the body, including bones, when administered intramuscularly (i.m.) [21]. However, local (topical) administration of clindamycin to the affected area is problematic, as the drug has difficulty reaching the affected bone [22].

The study was finalized when the ONJ in the positive control group was evident, and the rats were euthanized.

In this pilot study, an ONJ rat model was developed to assess the potential of GuaDex, a new polyguanidine compound, in preventing ONJ. The guanidine moiety appears in many drugs and exhibits various properties, including antimicrobial and anti-inflammatory effects [23].

2. Materials and methods

2.1. Polyguanidine (GuaDex)

GuaDex was synthesized as described previously [11], briefly; aminoguanidine was coupled to oxidized dextran followed by reductive amination. The conjugate was purified by gel filtration on a PD − 10 disposable Sephadex G-25 column (GE Healthcare, Uppsala, Sweden).

2.2. Bacterial culture

Streptococcus gordonii (ATCC®12396) was a kind gift from Dr. Myriam A. de la Garza-Ramos at the Center for Research and Development in Health Sciences (CIDICS), Autonomous University of Nuevo León (UANL).

2.3. Animals

A sample of twenty-four clinically heathy, male Wistar rats, eight weeks old, were fed at 20 g/day until they reached a mean weight of 280 g (250–300 g) and were housed for two weeks for adaptation under pathogen-free conditions at the biological model unit from the CIDICS, UANL, following the institutional guidelines.

2.4. Ethical approval

The UANL Ethics Committee approved the experimental animal procedure. Protocol 05.2014. The animal study complies with the ARRIVE guidelines 2.0, and in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals in Mexico, NOM-062-ZOO-1999.

2.5. Animal model

The rat model was developed using three factors associated with ONJ development: exposure to BPs, oral surgery, and bacterial presence. It facilitated to study whether the induction of ONJ could be prevented by a single administration of GuaDex or clindamycin.

Twenty-four rats were randomized into four groups ($N = 6$/group). Four weeks before dental surgery, Groups 1 to 3 received ZA once per week, caudal vein i.v. 1 mg/kg, (ZA) (Aclasta®, Novartis), in phosphate-buffered saline pH 7.4. After four weeks, day 0, ~100 μg *S. gordonii* bacterium was injected into the planned surgical area. On day 1, the rats were given one dose of muscle relaxant xylazine, i.m. (Procin®-PiSA Agropecuaria, Mexico), followed by intraperitoneal anesthesia with pentobarbital 25–40 mg/kg (Pisabental®-PiSA Agropecuaria, Mexico). A left-side mandibular osteotomy surgery was performed, resulting in pulp exposure by debriding, luxation, and tooth extraction. After tooth extraction, the *S. gordonii* bacterium was inoculated with a micropipette into the pulp chamber (~100 μg). Groups 1–3 received daily bacterial inoculations from day 2 to day 8. On day 8, Group 1 received, into the pulp chamber, one single dose, ~6.5 mg (23 mg/kg), of GuaDex, Group 2 received one single dose, intramuscular, ~6 mg (21 mg/kg), of clindamycin (Indamid PiSA® Farmacéutica, Mexico), Group 3 (positive control). Group 4 (negative control) was injected i.m. with 0.2 mL saline. Groups 1, 2, and 3 received ZA i.v. once per week for two weeks after osteotomy.

All the rats were fed standard food during the study. Groups 1–3 received *S. gordonii* bacteria added to the food (see Fig. 1). On day 15, blood samples were collected from all the rats. On day 16, osteonecrosis was evident in the positive control group, and then all the groups of rats

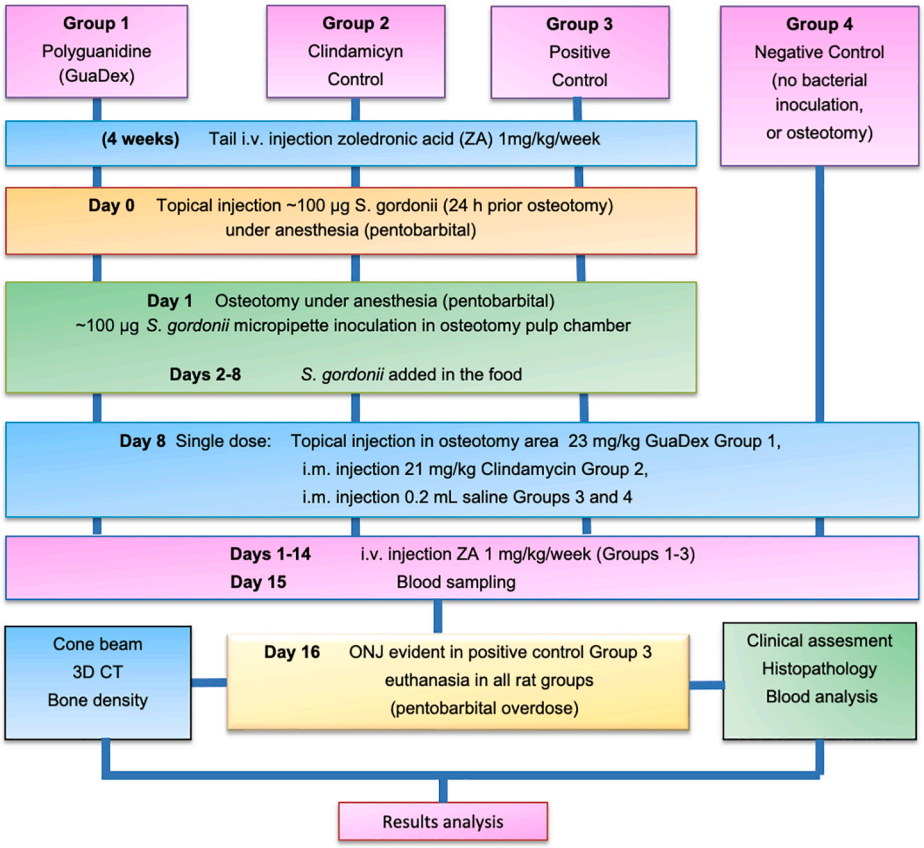


Fig. 1. The ONJ rat model, schedule of procedures.

were euthanized by an overdose of intraperitoneal pentobarbital (Pisabental PiSA® Farmacéutica, Mexico). All procedures with the rats were supervised by a veterinarian ensuring correct management of the animals.

All the rats underwent macroscopical oral assessment, bone mineral density, histological analysis, and blood analysis. The bone mineral density structure was visualized using cone-beam computer tomography (CBCT). The experimental procedures and schedules are illustrated in Fig. 1.

2.6. Macroscopic oral assessment

The rats were inspected intra-orally after osteotomy, accessing the cavity's healing ground. In addition, extraction sockets were examined, accessing mucosal ulceration, healing, the presence of abscess/fistula formation, and necrotic bone. The presence and extent of ONJ were recorded.

2.7. Blood sampling and analysis

As part of our comprehensive research, a crucial aspect was the blood analysis. This was undertaken to investigate the potential effects of the procedures and treatments on the related biochemical analytes.

Under anesthesia, blood samples (1 to 1.5 cc) were collected by amputating a small portion of the rat tail (about 0.2 cm) or by cardiac punction using a 5 mL syringe with a needle 23G through the xiphoid apophysis of the sternum. After this, it was punctured laterally in the craniovertebral direction at an angle of 30 degrees using negative pressure until the desired blood content was obtained. The blood samples were collected in EDTA tubes.

Hematological analysis and enzymes associated with bone metabolism were analyzed.

Since ZA is myelotoxic, a complete hematological examination was performed. The following nine parameters were accessed: total leucocytes, granulocytes, eosinophils, monocytes, platelets, lymphocytes, erythrocytes, total hemoglobin concentration, and hematocrit index.

Biochemical analysis was done as nine parameters: total protein, glucose, blood urea nitrogen (BUN), albumin, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), phosphatase alkaline (ALP), calcium, phosphor, cholesterol, sodium, potassium, bilirubin, and amylase. The high-level ALP bone biomarkers demonstrate bone disorder. The reference range of ALP is 30 to 130 IU/L in rats [24].

2.8. Bone mineral density

Despite the relatively short study time, bone density measurements were done to investigate whether the treatments would affect the rats' bone density.

After euthanasia, the bone mineral density of both sides of the hemimandibles of each rat group was evaluated. The cone-beam tomography high-contrast images of the left and right jaws were used to evaluate the bone lesions using three unique three-dimensional measurements. First, the rats were placed in position, and the axial, sagittal, and coronal jaw cuts were made, respectively. Then, the rat jaws were placed on the base of the tomography apparatus, and capture was made with four perimetral point measurements of the osteotomy areas.

2.8.1. Hounsfield units (HU)

The bone sample data was acquired with a high-resolution *i-CAT*® Cone-Beam 3D dental imaging system (Imaging Sciences International, Hatfield, PA, USA). The following parameters were used for the scan: 120 kVp tube voltage, 5 mA current intensity, 160 × 131 mm field of view, 400 µm voxel, 0.004 s duration, 2 mm thickness measures, and a

bone filter of high resolution. The DICOM data obtained were analyzed with *i-CAT vision* software version 1.9.3.1.4 to reconstruct in multiple plane views. Then, the left jaw (lesion) was statistically compared with the negative control (right jaw).

2.9. Histological analysis

The jaws of the disarticulated rats measured between 1.5 and 2.0 cm and were processed completely. They were drained in 10 % formaldehyde at room temperature, and after 24 h, they were placed in 80 % trichloroacetic acid for 24 to 36 h (demineralizing). The jaws were embedded in paraffin, and the anteroposterior resected 3 μm cut thickness. Finally, the sections were mounted on microscope slides for staining in hematoxylin and eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA).

The stained sections were examined under optic light microscopy (Carl Zeiss) with 20× and 40× magnification objective lenses, and images were obtained using a digital camera.

Histologic proof of the presence of ONJ and the effects in the different groups were accessed, estimating necrotic alveolar bone, empty lacunae of osteoclasts, bone neoformation, presence of bacteria, and infiltration of inflammatory cells. The area of osteonecrosis was defined as a bone region with empty lacunae >500 μm^2, according to Allen and Burr [25].

Two oral pathologists independently did the histology analysis.

2.10. Statistical analysis

Means with standard deviation (SD) and *t*-tests were used to describe the cone beam results. In addition, the blood analysis was presented as a One-way ANOVA test to reveal significant differences, and the Student-Neuman-Keuls (SNK) test was used to identify sample means that are significantly different between the control negative (right jaw), control ONJ positive (left jaw), and GuaDex group variables. The sample size of each group was determined using the one-way ANOVA test with a reliability of 80 %. All *p*-values <0.05 were considered statistically significant. Analyses were conducted using the SPSS for Windows software (version 21.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Summary

The total number of rats was 24, divided into four groups with six rats in each group. Out of these, 22 were evaluated/treated, while two died during the anesthesia (one in the clindamycin group and one in the negative control group).

3.2. Macroscopic oral evaluation

The positive control Group 3 showed that by combining ZA and *S. gordonii* inoculation induced epithelial wounds and ONJ characteristic lesions. The lesions resulted from delayed or incomplete healing, slight exposure of bone tissue, inflammation, and slight epithelization in the periphery of the osteotomy. The lesions are compatible with human ONJ induced by BPs, according to the definition by the American Association for Oral and Maxillofacial Surgery (AAOMS) (Fig. 2A and B).

The GuaDex treatment Group 1 showed gingival epithelial healing with complete re-epithelialization of the wounds in all the rats. The clindamycin-treated Group 2 showed incomplete extraction socket healing. The negative control Group 4 showed normal gingival epithelium.

3.3. Hematological and biochemical analysis

The ANOVA blood count analysis showed significant differences between leucocytes and monocytes between control negative, control positive, and Guadex groups. The Student-Neuman-Keuls analysis blood count analysis of the three groups showed that GuaDex single treatment increases the production of leucocytes. The control positive (ONJ Group 3) induced the production of monocytes.

The ANOVA statistical blood biochemistry analysis showed significant differences in the control positive (Group 3), control negative (Group 4), and GuaDex in the albumin, glucose, creatinine, ALT, ALP, calcium, phosphorous, cholesterol, sodium, and amylase values.

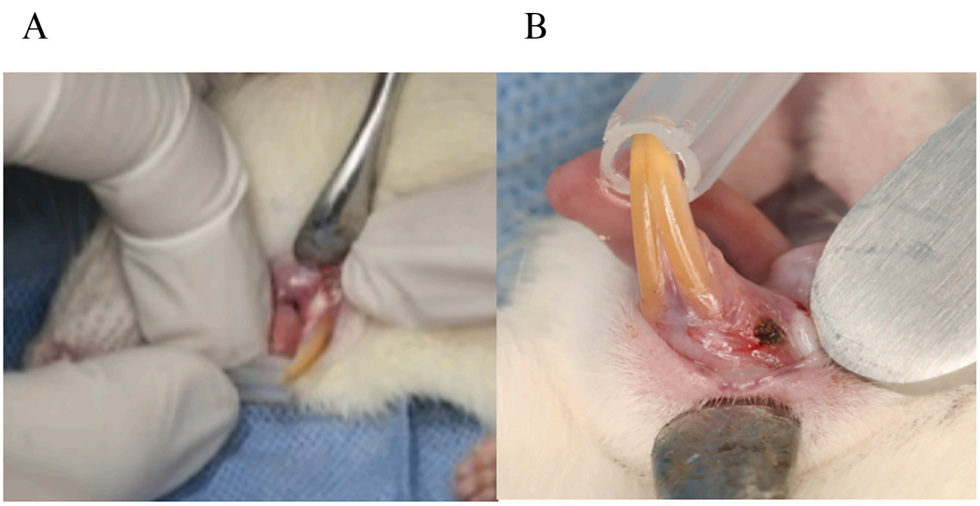
The SNK of the three Groups showed that the GuaDex treatment had lower glucose and sodium values than the control positive (Group 3) and negative (Group 4). GuaDex (Group 1) had lower albumin, ALT, ALP, phosphorus, and amylase values than the control positive (ONJ group 3). Conversely, the GuaDex Group 1 had higher creatinine and calcium values.

The control positive or ONJ group 3 and control negative Group 4 showed higher albumin, ALT, ALP, phosphorous, and amylase and lower values in calcium and cholesterol.

The missing biochemical analysis values had no significant differences among the groups, Table 1A and B.

3.4. Bone mineral density analysis

The statistical analysis of the left and right jaw in all the rat groups



Figs. 2. A) and B) Positive control rat left jaw with healing delay after three weeks osteotomy.

Table 1
A) and B) One-way ANOVA with Student-Newman-Keuls multiple comparisons test at control negative, control positive (ONJ), and GuaDex between and within groups. A) Hematological and B) Biochemical analysis. A value of $p < 0.05$ was considered significant for all tests.

A

Blood count	ANOVA		Student-Neuman-Keuls		
	F	Significance	Group	1	2
Leucocytes	8,108	0,0008	Control +	7,10	
			Control -	7,42	
			GuaDex		10,66
Monocytes	6,378	0,016	Control +		2,00
			Control -	0,20	
			GuaDex	1,00	1,00

B

Variable	ANOVA		Student-Neuman-Keuls			
	F	Significance	Groups	1	2	3
Albumin	4,43	0,036	GuaDex	4,06		
			Control -	4,28	4,28	
			Control +		4,76	
Creatinine	6,29	0,014	Control -	0,24		
			Control +	0,34	0,34	
			GuaDex		0,50	
ALT	9,44	0,003	Control -	23,40		
			GuaDex	23,48		
			Control +		30,40	
ALP	58,64	0,000	Control -	72,60		
			GuaDex		110,52	
			Control +			126,20
Calcium	56,63	0,000	Control +	7,00		
			Control -		8,16	
			GuaDex			10,26
Phosphor	115,60	0,000	Control -	5,64		
			GuaDex		7,18	
			Control +			10,26
Cholesterol	16,02	0,000	Control +	72,00		
			GuaDex		87,00	
			Control -		93,20	
Sodium	17,25	0,000	GuaDex	123,40		
			Control +		138,00	
			Control -		140,20	
Amylase	31,57	0,000	Control -	155,60		
			GuaDex		238,80	
			Control -			351,60
Glucose	11,76	0,001	GuaDex	84,40		
			Control -		98,80	
			Control +		108,20	

Abbreviations: ALT; alanine transaminase, ALP; alkaline phosphatase, +; positive control, -; negative control.

showed no significant differences between negative, positive control, clindamycin, and Guadex, Table 2.

3.5. Histological analysis

Group 1: All the rats showed ongoing bone regeneration characterized by the presence of numerous bone cells, including osteoblasts, osteocytes, and well-formed osteons. Abundant fibroblasts demonstrated fibroblastic activity. The medullar spaces displayed high cellularity and pronounced osteoblastic activity, which contributed to forming a new matrix. Vascularization was evident in all samples, and there was no bacterial presence.

Group 2: In this group, the onset of ONJ was observed in 80 % of the rats with a lack of fibroblastic and osteoblastic activity. Avascular regions were noted in 60 % of the subjects, with one rat exhibiting moderate neovascularization. Bacterial presence was identified in 60 % of the rats, highlighting a potential association with the development of ONJ.

Group 3: Most of the rats (83 %) showed onset of ONJ, with one rat with manifest ONJ. Histology showed frequent inflammatory infiltrates, and the absence of collagen and hyaluronic acid in 83 % of the rats. One rat showed avascular areas. All the rats had ulcerations of gingival mucosa, reduced medullar spaces, presence of osteocytes and osteoclasts, loss of the organic part, compression of bone structure, and cellular disorganization with bacterial presence.

Group 4: Two out of five rats displayed high cellularity with prominent osteocytes and osteoblasts, while all rats exhibited normal histology. All the samples had no inflammatory infiltrates, indicating a lack of pathological changes.

Table 2
Statistical differences between the left (socket lesion) and the right (control) jaws in all the rat groups. The measurements are in Hounsfield units (HU). Values are presented as mean (SD). A value of $p < 0.05$ was considered significant for the tests.

Group	Left jaw (osteotomy)	Right jaw (control)	t value	*p* value
GuaDex	744 (476)	1084 (165)	-1,995	0,103
Clindamycin	694 (483)	998 (334)	-0,962	0,390
Positive control	1009 (401)	1145 (128)	-0,830	0,444
Negative control	923 (333)	1163 (108)	-1,553	0,195

Histologic images are shown in Fig. 3.

The histological analysis of the two oral pathologists was 100 % in concordance.

A) 20×: Vast proliferation of the cellular matrix (mesenchymal) between spicules of osteoids beginning to mineralize. 40×: The trabeculae are surrounded by reactive osteoblasts depositing osteoid, indicating bone proliferation.
B) 20×: Mature bone and scant proliferating matrix. Hazard disposition of periodontal bone. There is no new bone formation, only some areas of pre-existing bone tissue. 40×: Scant mesenchymal and or bone proliferation. There are no reactive osteoblasts, only fibroblasts in a state of latency.
C) 20×: Pre-existing bone tissue in latency (similar to B). 40×: Scattered fibroblasts in a reactive state. No bone or osteoid formation.
D) 20× Mature bone tissue without evident histological alterations. 40×: Quiescent stroma and fibroblasts with mature bone without osteogenic activity.

4. Discussion

In an ONJ rat model using metformin treatment as prevention (metformin is a bi-guanidine), the treatment showed a protective effect against ONJ [26]. Metformin regulates genes via AMP-activated protein kinase (AMPK). AMPK increases the expression of osteogenic genes such as osteocalcin and alkaline phosphatase, stimulating osteoblast differentiation. Also, the AMPK activation protects against oxidative stress-induced apoptosis of osteocytes [27,28].

GuaDex is, by definition, a kind of polyamine having multiple guanidine side groups with amine groups. Polyamines are known immunomodulators that suppress macrophage production of inflammatory cytokines [29,30]. Furthermore, polyamines and efferocytosis have each been shown to suppress the transcription of pro-inflammatory cytokines, including IL-1β, tumor necrosis factor-alpha (TNFα), and IL-6 [30–34]. In addition, during efferocytosis, phagocytic cells remove apoptotic cells [35] to help resolve inflammatory responses and modulate immune balance [36].

ODX is a sister compound of GuaDex, having an additional BP ligand that yields an affinity to bone minerals. It has shown potent antitumoral efficacy and with primary inhibition of bone resorption markers (C-terminal telopeptide of type 1 collagen, CTX) with secondary suppression of osteoblast markers (clinical phase I and II studies in patients with castration-resistant prostate cancer) [37,38]. Previous studies on GuaDex and ODX might explain some of the findings in the present study, i.e., antimicrobial effects and its stimulation of bone regeneration.

Experimental rat models of ONJ intend to simulate the relationship between BP medication, oral surgery/oral lesions, and the development of ONJ.

According to a proteomic study, alendronate treatment causes significant alterations in the RIPK3/Wnt/GSK3/β-catenin signaling pathway, leading to abnormal angiogenesis, inflammation, anabolism, remodeling, and mineralization in bone cells when studied in an in vitro cell culture system [39].

In previous experimental models of ONJ, the time for ZA medication was usually four to six weeks before tooth extraction [40]. ZA medication at the time of tooth extraction/oral surgery appears to be an essential factor for the subsequent development of ONJ [40,41].

Additionally, the alveolar bone proteins and gingival tissue crosstalk are affected following a tooth extraction. The proteins affected are associated with the ATP5B mitochondrial ATP synthase and fibronectin networks. ATP5B is involved in nucleic acid metabolism, and fibronectin 1 is involved in cellular assembly, organization, and maintenance. Both networks are necessary for bone regeneration or healing [42].

ONJ-related bone sequestration has shown the presence of *Fusobacterium*, *Eikenella*, *Bacillus*, *Actinomyces*, *Staphylococcus*, and *Streptococcus* [9]. Microscopic identification of *Actinomyces* (~70 %) and *Streptococcus* (~55 %) have been confirmed in ONJ lesions [43]. Microorganisms related to biofilm cause increased circulation of cytokines and dysfunction of matrix proteases, which may lead to a chronic inflammatory response [44].

In the current study, ZA medication was administered four weeks prior to and two weeks after the osteotomy. One crucial component was the inclusion of a periodontal pathogen, *S. gordonii*. The role of oral bacteria in the development of ONJ in rats has also been studied with Pamidronate, 1 mg/kg/week, four doses before tooth extraction, followed by oral inoculation of *Fusobacterium nucleatum* for two weeks [8].

Rat bone metabolism in the mandible is much faster than in human bone metabolism. Consequently, no ONJ rat model can completely mimic ONJ development in human subjects. In humans, the meantime to ONJ diagnosis is quite long. After ZA treatment, 1.8 years and with a minimum of 10 months treatment, with Pamidronate, 2.8 years and a minimum of 1.5 years treatment. With oral bisphosphonates, treatment to onset of ONJ was more prolonged, 4.6 years, and with a minimum of three years of treatment [45].

Experimental models may elucidate risk factors that contribute to the development of ONJ.

This study showed that GuaDex, by inhibiting the growth of *S. gordonii* prevented infection and osteonecrosis in this ZA-induced ONJ rat model. GuaDex apparently reduced oral bacteria more effectively than clindamycin, which was administered systemically (i.m.), while GuaDex was administered locally directly into the extraction cavity. Both routes of administration have advantages, such as sustained

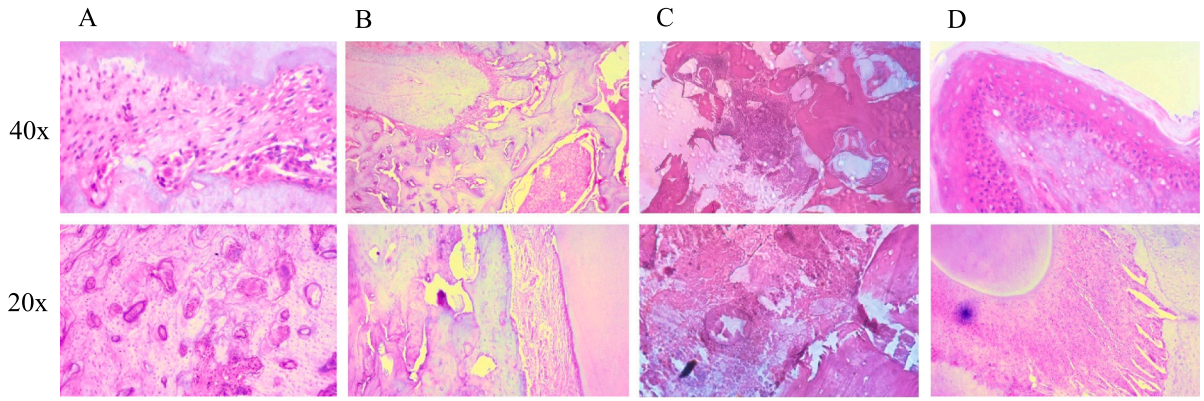


Fig. 3. Representative histological sections of gingival mucosa (stain, hematoxylin-eosin, H&E). A) GuaDex treatment, B) Clindamycin treatment, C) Positive control, and D) Negative control. The stained sections were examined under optic light microscopy (Carl Zeiss) with 20× and 40× magnification objective lenses, and the images were obtained using a digital camera.

delivery (clindamycin) compared to a direct local effect (GuaDex). However, the observed differences between groups 1 and 2 are probably not associated with the difference in administration routes but with the local effects of the polyguanidine. GuaDex apparently functions as an antiseptic without negatively altering the cellular structure of the cell membrane of fibroblasts [15] and, possibly, the bone tissues. The local topical administration of GuaDex into the osteotomy cavity stimulated bone formation. GuaDex is a polymer (mean MW 60kD) with a strong electropositive charge, probably delayed intravasation, yielding a prolonged antiseptic effect in the osteotomy cavity. When GuaDex accumulates in the exposed bone in the osteotomy cavity, the cationic electrostatic charge potential at the bone surface interface might be analogous to electric polarization in fracture healing, an old technique for healing complicated fractures in orthopedics [46]. The effective bacterial killing mechanism of GuaDex is likely due to its strong positive charge from its multiple guanidinium side groups interacting with the bacterial cell membranes and eventually eliminating the bacteria.

The role of bacteria in ONJ development highlights the importance of addressing microbial factors in prevention and treatment strategies. However, wound healing is crucial in managing and preventing ONJ lesions.

Wound healing is a complex process that includes inflammation, homeostasis, granulation by cell proliferation, matrix deposition, and tissue remodeling. This process involves various factors and interactions between cells. Chemokines, cytokines, growth factors, and chemical mediators regulate this process [47]. Polyamines, like spermidine, are critical wound-healing components for endothelial cell proliferation and angiogenesis [48]. Studies have shown that topical treatment of spermidine, a polyamine, can increase pro-inflammatory cytokine production, which may help accelerate wound closure [49].

This study explores GuaDex's antimicrobial and anti-inflammatory properties, which are crucial for effective wound healing and ONJ prevention.

Our observations after accumulation in the lesion area, GuaDex acts as polyamine and might be associated with the suppression of inflammatory cytokines and control of the bacterial infection without exuberant inflammation by a hypusination, posttranslational modification of eIF5A, a eukaryotic translation factor [50].

Polyamines disrupt the differentiation and maturation of osteoclasts, rather than affecting osteoblasts, to prevent bone loss [51].

Furthermore, the observed effects of GuaDex may also be explained by improved osteoblast migration and their osteoconductive activities.

Polymers containing functional NH^{3+} groups (GuaDex with guanidinium side groups) increase electrostatic interactions, leading to stronger protein adsorption and structural rearrangements, exposing hidden binding sites for cell attachment [52].

Regarding the hemogram analysis, the GuaDex rat group stimulates the immune system by producing leucocytes.

The biochemical analysis showed an elevated serum ALP value (but still an average value) and decreased calcium, indicating the presence of ONJ in the positive control. On the contrary, in the GuaDex treatment group, the calcium value increased, and the ALP levels decreased, suggesting increased bone formation activity after the development of ONJ.

To date, no consistent conclusion exists on the association between serum T-ALP levels and bone mineral density [53].

The Guadex treatment decreased the serum glucose values, indicating that serum glucose and bone metabolism are associated [28,54], and was confirmed in the Nakagawa ONJ rat model study [26].

5. Conclusions

The current rat model, using ZA administration and *S. gordonii* after osteotomy, successfully induced lesions characteristic of ONJ.

Local treatment with GuaDex stimulated bone regeneration, promoting new bone formation. It also had antimicrobial effects and induced neovascularization. GuaDex prevented the development of ONJ. The results encourage further studies on GuaDex and the prevention of ONJ.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Arquímedes Cantorán-Castillo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Belinda Beltrán-Salinas:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Conceptualization. **Jorge M. Antúnez-Treviño:** Writing – original draft, Validation, Investigation. **Ricardo Martínez-Pedraza:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Rodolfo Franco-Márquez:** Writing – review & editing, Visualization, Investigation, Conceptualization. **Mario A. Guzmán-García:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Ricardo M. Cerda-Flores:** Writing – review & editing, Visualization, Validation, Software, Data curation, Conceptualization. **Raúl V. Perales-Pérez:** Writing – review & editing, Validation, Software, Resources, Conceptualization. **Christian Zakian:** Writing – review & editing, Software, Resources, Investigation, Conceptualization. **Jesús Ancer-Rodriguez:** Writing – review & editing, Resources, Project administration, Conceptualization. **Marcela Márquez-Méndez:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors stated that they have no conflict of interest.

Data availability

The article's data underlying this article will be shared with the corresponding author upon reasonable request.

References

[1] X.L. Xu, W.L. Gou, A.Y. Wang, et al., Basic research and clinical applications of bisphosphonates in bone disease: what have we learned over the last 40 years? J. Transl. Med. 11 (1) (2013) 303, https://doi.org/10.1186/1479-5876-11-303.
[2] B. Faiman, A.L.P.C. Pillai, A.G. Benghiac, Bisphosphonate-related osteonecrosis of the jaw: historical, ethical, and legal issues associated with prescribing, J. Adv. Pract. Oncol. 4 (1) (2013) 25–35.
[3] R.E. Marx, R. Tursun, Suppurative osteomyelitis, bisphosphonate induced osteonecrosis, osteoradionecrosis: a blinded histopathologic comparison and its implications for the mechanism of each disease, Int. J. Oral Maxillofac. Surg. 41 (2012) 283–289, https://doi.org/10.1016/j.ijom.2011.12.016.
[4] R.A. Miksad, K.C. Lai, T.B. Dodson, et al., Quality of life implications of bisphosphonate-associated osteonecrosis of the jaw, Oncologist 16 (2011) 121–132, https://doi.org/10.1634/theoncologist.2010-0183.
[5] Y. Bi, Y. Gao, D. Ehirchiou, et al., Bisphosphonates cause osteonecrosis of the jaw-like disease in mice, Amer J Pathol. 177 (2010) 280–290, https://doi.org/10.2353/ajpath.2010.090592.
[6] R. Fliefel, M. Tröltzsch, J. Kühnisch, et al., Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review, Int. J. Oral Maxillofac. Surg. 44 (2015) 568–585, https://doi.org/10.1016/j.ijom.2015.01.026.
[7] A.M. Hinson, E.R. Siegel, B.C. Stack Jr., Temporal correlation between bisphosphonate termination and symptom. Resolution in osteonecrosis of the jaw: a pooled case report analysis, J. Oral Maxillofac. Surg. 73 (2015) 53–62, https://doi.org/10.1016/j.ijoms.2014.07.012.
[8] H. Mawardi, G. Giro, M. Kajiya, et al., A role of oral bacteria in bisphosphonate-induced osteonecrosis of the jaw, J. Dent. Res. 90 (2011) 1339–1345, https://doi.org/10.1177/0022034511420430.
[9] B. Palla, E. Burian, A. Deek, et al., Comparing the surgical response of bisphosphonate-related versus denosumab-related osteonecrosis of the jaws, J. Oral Maxillofac. Surg. 79 (5) (2021) 1045–1052, https://doi.org/10.1016/j.joms.2020.11.017.

[10] N. Yarom, C.L. Shapiro, D.E. Peterson, et al., Medication-related osteonecrosis of the jaw: MASCC/ISOO/ASCO clinical practice guideline, J. Clin. Oncol. 37 (25) (2019) 2270–2290, https://doi.org/10.1200/JCO.19.01186.

[11] L. Meurling, M. Márquez, S. Nilsson, A.R. Holmberg, Polymer-conjugated guanidine is a potentially useful anti-tumor agent, Int. J. Oncol. 35 (2) (2009) 281–285, https://doi.org/10.3892/ijo_00000338.

[12] M. Márquez, S. Nilsson, L. Lennartsson, et al., Charge-dependent targeting: results in six tumor cell lines, Anticancer Res. 24 (2004) 1347–1352 (doi:ar.iiarjournals.org/content/24/3A/1347.long).

[13] E. Escamilla-García, A.G. Alcázar-Pizaña, J.C. Segoviano-Ramírez, et al., Antimicrobial activity of a cationic guanidine compound against two oral bacteria, Int J Microbiol. 2017 (2017) 5924717, https://doi.org/10.1155/2017/5924717.

[14] F.M. Carillo-Sosa Mara, I. Rodriguez-Delgado, J.J. Flores-Trevino, et al., *In vitro* antimicrobial activity of CatDex against *Porphyromonas endodontalis*, J Dent Oral Hygiene. 10 (2) (2018) 13–17, https://doi.org/10.5897/JDOH2018.0231.

[15] M. Márquez, K. Karl Holmberg-Olausson, S. Nilsson, A.R. Holmberg, Efficacy of a polyguanidine on glioma cell and stem cell lines, Invest New Drugs J. 40 (3) (2022) 565–575, https://doi.org/10.1007/s10637-022-01233-7.

[16] X. Xie, H. Lei, D. Fan, Antibacterial hydrogel with pH-responsive microcarriers of slow-release VEGF for bacterial infected wound repair, J. Mater. Sci. Technol. 144 (2023) 198–212, https://doi.org/10.1016/j.jmst.2022.09.062.

[17] E. Nastro, C. Musolino, A. Allegra, et al., Bisphosphonate-associated osteonecrosis of the jaw in patients with multiple myeloma and breast cancer, Acta Haematol. 117 (3) (2007) 181–187, https://doi.org/10.1159/000097876.

[18] L. De Bruyn, R. Coropciuc, W. Coucke, C. Politis, Microbial population changes in patients with medication-related osteonecrosis of the jaw treated with systemic antibiotics, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 125 (3) (2018) 268–275, https://doi.org/10.1016/j.oooo.2017.11.022.

[19] O.J. Park, J. Kim, H.Y. Kim, et al., *Streptococcus gordonii* induces bone resorption by increasing osteoclast differentiation and reducing osteoblast differentiation, Microb. Pathog. 126 (2019) 218–223, https://doi.org/10.1016/j.micpath.2018.11.005.

[20] P. Barba-Recreo, Del Castillo Pardo, J.L. de Vera, M. García-Arranz, et al., Zoledronic acid-related osteonecrosis of the jaws. Experimental model with dental extractions in rats, J. Craniomaxillofac. Surg. 42 (6) (2014) 744–750, https://doi.org/10.1016/j.jcms.2013.11.005.

[21] A. Bonnaire, V. Vernet-Garnier, D. Lebrun, et al., Clindamycin combination treatment for the treatment of bone and joint infections caused by clindamycin-susceptible, erythromycin-resistant Staphylococcus spp, Diagn. Microbiol. Infect. Dis. 99 (1) (2021) 115225, https://doi.org/10.1016/j.diagmicrobio.2020.115225.

[22] B.L. Ruggiero, S.P. Edwards, Chapter 8 - diabetes mellitus and its association with infection and osteonecrosis of the jaw, in: MengHee Tan (Ed.), Bones, Joints, and Hormones Series, Diabetes Mellitus, Academic Press, 2020, pp. 159–181, https://doi.org/10.1016/B978-0-12-820605-8.00008-5.

[23] S.H. Kim, D. Semenya, D. Castagnolo, Antimicrobial drugs bearing guanidine moieties: a review, Eur. J. Med. Chem. 216 (2021) 113293, https://doi.org/10.1016/j.ejmech.2021.113293.

[24] K.M.M. Hasan, N. Tamanna, M.A. Haque, Biochemical and histopathological profiling of Wistar rat treated with *Brassica napus* as a supplementary feed, Food Sci. Human Wellness 7 (2018) 77–82, https://doi.org/10.1016/j.fshw.2017.12.002.

[25] M.R. Allen, D.B. Burr, Mandible matrix necrosis in beagle dogs after 3 years of daily oral bisphosphonate treatment, J Oral Maxilofac Surg. 66 (2008) 987–994, https://doi.org/10.1016/j.joms.2008.01.038.

[26] T. Nakagawa, S. Tsuka, F. Aonuma, et al., Effects of metformin on the prevention of bisphosphonate-related osteonecrosis of the jaw-like lesions in rats, J. Prosthodont. Res. 65 (2021) 219–224, https://doi.org/10.2186/jpr.JPOR_2019_629.

[27] W.G. Jang, E.J. Kim, I.H. Bae, et al., Metformin induces osteoblast differentiation via orphan nuclear receptor SHP-mediated transactivation of Runx2, Bone 48 (2011) 885–893, https://doi.org/10.1016/j.bone.2010.12.003.

[28] I. Kanazawa, Interaction between bone and glucose metabolism (review), Endocr. J. 64 (2017) 1043–1053, https://doi.org/10.1507/endocrj.EJ17-0323.

[29] Y.L. Latour, A.P. Gobert, K.T. Wilson, The role of polyamines in the regulation of macrophage polarization and function, Amino Acids 52 (2020) 151–160, https://doi.org/10.1007/s00726-019-02719-0.

[30] M. Zhang, T. Caragine, H. Wang, et al., Spermine inhibits proinflammatory cytokines synthesis in human mononuclear cells: a counterregulatory mechanism that restrains the immune response, J. Exp. Med. 185 (1997) 1759–1768, https://doi.org/10.1084/jem.185.10.1759.

[31] D.M. Hardbower, M. Asim, P.B. Luis, et al., Ornithine decarboxylase regulates M1 macrophage activation and mucosal inflammation via histone modifications, Proc. Natl. Acad. Sci. USA 114 (2017) E751–E760, https://doi.org/10.1073/pnas.1614958114.

[32] K. Singh, L.A. Coburn, M. Asim, et al., Ornithine decarboxylase in macrophages exacerbates colitis and promotes colitis-associated colon carcinogenesis by impairing M1 immune responses, Cancer Res. 78 (15) (2018) 4303–4315, https://doi.org/10.1158/0008-5472.CAN-18-0116.

[33] L.P. Erwig, P.M. Henson, Immunological consequences of apoptotic cell phagocytosis, Am. J. Pathol. 171 (2007) 2–8, https://doi.org/10.2353/ajpath.2007.070135.

[34] R.E. Cocco, D.S. Ucker, Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure, Mol. Biol. Cell 12 (2001) 919–930, https://doi.org/10.1091/mbc.12.4.919.

[35] A.M. deCathelineau, P.M. Henson, The final step in programmed cell death: phagocytes carry apoptotic cells to the grave, Essays Biochem. 39 (2003) 105–117, https://doi.org/10.1042/bse0390105.

[36] Y. Ge, M. Huang, Y.M. Yao, Efferocytosis and its role in inflammatory disorders, Front. Cell Dev. Biol. 10 (2022) 839248, https://doi.org/10.3389/fcell.2022.839248.

[37] C. Thellenberg-Karlsson, C. Nyman, S. Nilsson, et al., Bone-targeted novel cytotoxic polybisphosphonate conjugate in castration-resistant prostate cancer: a multicenter phase 1 study, Anticancer Res. 36 (2016) 6499–6504, https://doi.org/10.21873/anticanres.11249.

[38] C. Thellenberg-Karlsson, E. Vjaters, M. Kase, et al., A randomized, double-blind, dose-finding, repeat dose phase II multicentre study of ODX for the treatment of patients with castration resistant prostate cancer (CRPC) and skeletal metastases, Eur. J. Cancer 181 (2023) 198–207, https://doi.org/10.1016/j.ejca.2022.12.006.

[39] J. Kim, A. Yeon, S.J. Parker, et al., Alendronate-induced perturbation of the bone proteome and microenvironmental pathophysiology, Int. J. Med. Sci. 18 (14) (2021) 3261–3270, https://doi.org/10.7150/ijms.61552.

[40] R. Yan, R. Jiang, L. Hu, Y. Deng, J. Wen, X. Jiang, Establishment and assessment of rodent models of medication-related osteonecrosis of the jaw (MRONJ), Int. J. Oral Sci. 14 (1) (2022) 41, https://doi.org/10.1038/s41368-022-00182-4.

[41] M.E. Kolpakova, A.A. Zuvareva, T.D. Artamonova, et al., Experimental model of osteonecrosis of the jaw in rats treated with zoledronic acid, Br. J. Oral Maxillofac. Surg. 55 (2017) 156–159, https://doi.org/10.1016/j.bjoms.2016.10.006.

[42] H.Y. Yang, J. Kwon, M.S. Kook, et al., Proteomic analysis of gingival tissue and alveolar bone during alveolar bone healing, Mol. Cell. Proteomics 12 (10) (2013) 2674–2688, https://doi.org/10.1074/mcp.M112.026740.

[43] A.M. Hinson, C.W. Smith, E.R. Siegel, B.C. Stack Jr., Is bisphosphonate-related osteonecrosis of the jaw an infection? A histological and microbiological ten-year summary, Int J Dent. 2014 (2014) 452737, https://doi.org/10.1155/2014/452737.

[44] M. Mozzati, G. Martinasso, M. Maggiora, et al., Oral mucosa produces cytokines and factors influencing osteoclast activity and endothelial cell proliferation, in patients with osteonecrosis of jaw after treatment with zoledronic acid, Clin. Oral Investig. 17 (2013) 1259–1266, https://doi.org/10.1007/s00784-012-0800-7.

[45] P.K. Palaska, V. Cartsos, A.I. Zavras, Bisphosphonates and time to osteonecrosis development, Oncologist 14 (11) (2009) 1154–1166, https://doi.org/10.1634/theoncologist.2009-0115.

[46] S. Itoh, S. Nakamura, T. Kobayashi, et al., The effects of electrically polarized hydroxyapatite on osteogenic cell activity and bone formation, Key Eng. Mater. 309-311 (2006) 153–156, https://doi.org/10.4028/www.scientific.net/KEM.309-311.153.

[47] S.A. Eming, P. Martin, M. Tomic-Canic, et al., Wound repair and regeneration: mechanisms, signaling, and translation, Sci. Transl. Med. 6 (265) (2014) 265sr6, https://doi.org/10.1126/scitranslmed.3009337.

[48] H. Li, C.J. Meininger, F.W. Bazer, G. Wu, Intracellular sources of ornithine for polyamine synthesis in endothelial cells, Amino Acids 48 (2016) 2401–2410, https://doi.org/10.1007/s00726-016-2256-6.

[49] D. Ito, H. Ito, T. Ideta, et al., Systemic and topical administration of spermidine accelerates skin wound healing, Cell Commun. Signal 19 (1) (2021) 36, https://doi.org/10.1186/s12964-021-00717-y.

[50] A.P. Gobert, J.L. Finley, Y.L. Latour, et al., Hypusination orchestrates the antimicrobial response of macrophages, Cell Rep. 33 (11) (2020) 108510, https://doi.org/10.1016/j.cel.rep.2020.108510.

[51] T. Yamamoto, E. Hinoi, H. Fujita, et al., The natural polyamines spermidine and spermine prevent bone loos through referential disruption of osteoclastic activation in ovariectomized mice, Br. J. Pharmacol. 166 (2012) 1084–1096, https://doi.org/10.1111/j.1476-5381.2012.01856.x.

[52] M.M. Stevens, Biomaterials for bone tissue engineering, Mater. Today 11 (5) (2008) 18–25, https://doi.org/10.1016/S1369-7021(08)70086-5.

[53] J. Shu, A. Tan, Y. Li, H. Huang, J. Yang, The correlation between serum total alkaline phosphatase and bone mineral density in young adults, BMC Musculoskelet. Disord. 23 (2022) 467, https://doi.org/10.1186/s12891-022-05438-y.

[54] C. Cipriani, L. Colangelo, R. Santori, et al., The interplay between bone and glucose metabolism, Front Endocrinol (Lausanne). 11 (2020) 122, https://doi.org/10.3389/fendo.2020.00122.