

# Photomodulation multiple sessions as a promising preventive therapy for medication-related osteonecrosis of the jaws after tooth extraction in rats



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## ABSTRACT

The aim of this study was to evaluate the effects of photobiomodulation (PBM) with multiple sessions of low-level laser on the alveolar repair process of rats with major risk factors for medication-related osteonecrosis of the jaws (MRONJ). Senile rats received 0.45 mL of vehicle (VEH and VEH-PBM) or 0.45 mL of 100 µg/kg zoledronate (ZOL and ZOL-PBM) administered intraperitoneally every two days during seven weeks. After three weeks of initiation of drug treatment the first lower left molar was extracted. No local treatment was performed in VEH and ZOL. VEH-PBM and ZOL-PBM were submitted to laser irradiation ( $660 \pm 10$  nm; 0.035 W; 2.1 J; 60 s) on the extraction site at 0, 2 and 4 days postoperatively. Euthanasia was performed 28 days after tooth extraction. Histological sections of the hemimandible were submitted to histopathological and histomorphometric analysis, as well as to histochemistry for collagen fiber maturation and immunohistochemistry for pro-inflammatory cytokines. In ZOL, general impairment of tissue repair, areas with osteonecrosis, lower newly formed bone tissue (NFBT), smaller amount of mature collagen fibers and increased immunoreactivity for TNF $\alpha$ , IL-1 $\beta$  and IL-6 were observed when compared to VEH and VEH-PBM. ZOL-PBM showed significant improvement in some parameters compared to ZOL, such as positive repair tissue, higher NFBT, greater amount of mature collagen fibers, besides TNF $\alpha$  and IL-1 $\beta$  immunoreactivity decrease. Zoledronate treatment severely compromised the tissue repair process of the tooth extraction site in rats with major risk factors for MRONJ. Based on parameters employed in the present study, PBM in multiple sessions can improve the alveolar repair process, constituting a promising preventive therapy to avoid the onset of post-extraction MRONJ.

## 1. Introduction

Bisphosphonates (BPs) are mainly used for the treatment of diseases with osteolytic activity and for inhibiting the progression of metastasis of malignant osteotropic tumors [1]. Such drugs exert an inhibitory action on bone resorption. BPs have a similar chemical structure to endogenous pyrophosphate, with two phosphate groups linked to a carbon atom and two side-chains, R1 and R2, responsible for the affinity to hydroxyapatite crystals and for the pharmacological properties of BPs, respectively. There are two classes of BPs, whose structures

differ for nitrogen presence in R2. Zoledronate is the most powerful nitrogen-containing BPs. This BP alters bone metabolism acting by inhibiting the mevalonate pathway that prevents the occurrence of numerous changes in the cytoskeletal dynamics of osteoclasts, which are essential for resorptive activity [2].

The progressive aging of the population causes an increase of osteolytic diseases. Thus, the use of antiresorptive drugs, namely BPs and RANKL inhibitor has risen in aged patients. Medication-related osteonecrosis of the jaws (MRONJ) is an adverse reaction caused by these types of drugs and still a challenge for dentistry. The American

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Association of Oral and Maxillofacial Surgery (AAOMS) defines MRONJ as the presence of exposed bone in the maxillofacial region for a period longer than eight weeks in patients under or submitted to prior treatment with antiresorptive drugs, without previous jaw radiotherapy [3,4]. MRONJ affects 1 to 12% patients under intravenous therapy with BPs and is less frequent in those under oral treatment. In addition, BPs have a cumulative effect, which may affect 21% patients after the third year of use [5].

Epidemiological studies show that MRONJ affects more often the female gender at an advanced age, who are or have made chronic use of intravenously administered BPs, specifically zoledronate. There is a tendency for occurrence in the premolar and molar region of the jaw, and tooth extraction has been identified as one of the main triggering factors [6,7]. Recently, clinical and experimental studies have attempted to the fact that teeth extraction with periodontal and/or periapical impairment is a great local risk factor; that is, prior presence of inflammation and local infection plays a crucial role in triggering post-extraction MRONJ [8–10].

Although the first reports of MRONJ have been published in 2003 [11], the etiopathogenesis of the disease is poorly understood, which greatly impairs its prevention and treatment. The supposed etiopathological factors identified as triggers of MRONJ include potent suppression of the osteoclasts resorptive activity, cytotoxic and anti-angiogenic effects, as well as infection susceptibility and dysfunction of local immune response induced by such drugs [3,4,12–14]. The investigation of the etiopathological factors of MRONJ in humans presents limitations. Therefore, experimental animal models are of great importance for directing clinical research, as well as for the assessment of curative and preventive therapies for this condition.

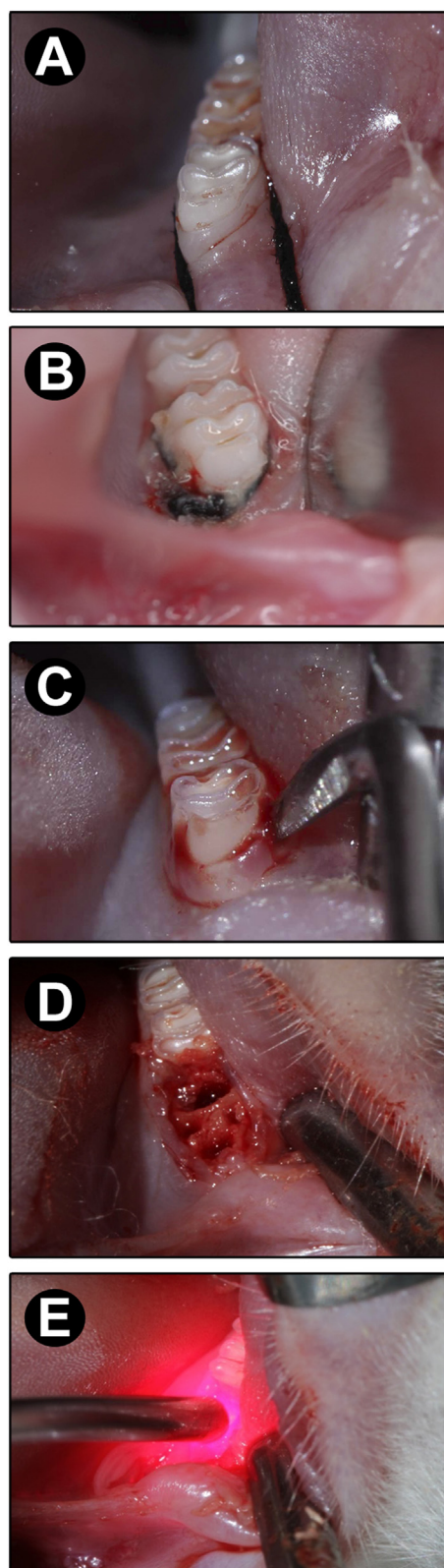
MRONJ significantly impairs patient quality of life and the treatment can be long, leave sequelae and sometimes be unsuccessful. There is no standard protocol for the treatment of MRONJ, which may be carried out with drug therapy and/or surgery. Drug therapy mainly consists of the prolonged use of antimicrobial agents. Surgical therapy ranges from conservative to aggressive, as well as from curettage and/or sequestrectomy to jaw resection. The use of pentoxifylline, tocopherol, teriparatide, in addition to ozone hyperbaric oxygen and low-level laser treatments have been suggested as adjuvant therapies [15]. However, the ideal clinical approach would be to employ therapies capable of preventing the onset of MRONJ.

Laser therapy has been widely used in healthcare, especially in oral disease conditions. Photobiomodulation (PBM) using low-level laser may constitute a preventive therapy for post-extraction MRONJ. Although the PBM action mechanism has not yet been fully elucidated, its effects are explained by primary reactions that occur especially at the mitochondrial level, where irradiation promotes the activation of photoreceptors involved in the electron-transport chain, increasing cell energy potential. In addition, secondary effects involving signal transduction to the cytoplasmic level occur, stimulating cell proliferation and/or differentiation [16].

Studies in animal models show that post-extraction PBM improves the repair process [17–19]. Similarly, clinical studies in humans also show that post-extraction PBM has positive effects on the main signs and symptoms that follow the repair process [20,21]. PBM in association with drug and/or surgical therapy has shown to be a promising strategy for osteonecrotic injury treatment [22–24]. However, few studies have investigated PBM as a MRONJ preventive treatment. In view of the benefits of laser therapy and given the need for safe and effective therapies to prevent the onset of post-extraction MRONJ, this study evaluated the effects of PBM multiple sessions on the alveolar repair process in an experimental model using rats with the main risk factors for MRONJ.

## 2. Material and Methods

The present study used twenty-eight female Wistar rats (*Rattus*



**Fig. 1.** Experimental procedures performed during the study. A: Installation of the ligature to induce experimental periodontitis. B: Ligature in position and experimental periodontitis installed. C: Surgical procedure for dental extraction. D: Dental alveolus after extraction. E: First PBM session.

*norvegicus*), aged twenty months and 350–450 g body weight. All measures to minimize the number of animals used and to prevent their suffering were taken. Procedures for experimental manipulation were

carried out according to the guidelines established by the “Guide for the Care and Use of Laboratory Animals” and the experimental protocol was approved by the Ethics Committee on Animal Use at FOA-UNESP (# 00581–2013).

## 2.1. Experimental Design

### 2.1.1. Anesthesia

Animals were anesthetized with ketamine (80 mg/kg, Francotar®, Virbac, SP, Brazil) and xylazine (10 mg/kg, Rompum® Bayer, RS, Brazil) for all surgical procedures (ligature placement, tooth extraction, PBM and euthanasia).

### 2.1.2. Experimental Periodontitis

One day before the beginning of the drug treatment plan, a cotton ligature (cotton thread #24, Coats Corrente®, SP, Brazil) was placed around the first lower left molar and kept for three weeks in order to induce experimental periodontitis (Fig. 1A and B).

### 2.1.3. Drug Treatment

The drug treatment plan lasted seven weeks and the administration of vehicle or zoledronate (Chemical® Sigma, St. Louis, MO, USA) was performed intraperitoneally, following an interval of two days between injections. The vehicle consisted of 0.45 mL of 0.9% sodium chloride solution. Zoledronate dose was 100 mg/kg, diluted in vehicle [25,26]. This dose and drug treatment plan consisted of the protocol currently used for complementation of oncologic therapy in humans, adapted to rats [25,26].

### 2.1.4. Tooth Extraction

After three weeks, animals were positioned on an operating table; the cotton ligature was removed, followed by antisepsis of the oral cavity, syndesmotomy, dislocation and extraction of the lower left first molar using adapted dental surgery tools (Fig. 1C and D).

### 2.1.5. Experimental Groups

Rats were randomly distributed in four experimental groups: VEH (n = 7), treated with vehicle and no local treatment post-extraction; VEH-PBM (n = 7), treated with vehicle and PBM on the extraction site; ZOL (n = 7), treated with zoledronate and no local treatment post-extraction; ZOL-PBM (n = 7), treated with zoledronate and PBM on the extraction site (Fig. 2).

### 2.1.6. PBM

Three PBM sessions were performed on the extraction site at 0, 2 and 4 days after extraction in groups VEH-PBM and ZOL-PBM. For PBM, an InGaAlP laser device ( $660 \pm 10$  nm; Thera Lase®, D.M.C. Equipamentos Ltda®, SP, Brazil) with spot size of  $0.0283\text{cm}^2$  was used, following the irradiation parameters: 35 mW power; continuous operation mode; energy point of 2.1 J/point, for 60 s; density energy of  $74.2\text{J}/\text{cm}^2$ ; power intensity of  $1.23\text{W}/\text{cm}^2$  [27]. Irradiation was

carried out at a single point in the center of the dental alveolus with the laser tip positioned parallel to the long axis and in contact with the treated area (Fig. 1E).

### 2.1.7. Euthanasia and Sample Collection

In all groups, euthanasia was performed 28 days post-extraction. For this, animals were deeply anesthetized and transcardially perfused with physiological saline added with 0.1% heparin (100 mL), followed by fixative solution (800 mL) consisting of 4% formaldehyde (Chemical® Sigma, St. Louis, MO, USA) in phosphate buffered saline (PBS, Chemical® Sigma), 0.1 M, 4 °C, pH 7.4. Subsequently, the hemi-mandibles were carefully dissected.

### 2.1.8. Histological Processing of Samples

The samples were post-fixed in the same fixative solution for 72 h and demineralized in 10% ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical®) diluted in PBS, 0.1 M, 4 °C, pH 7.4, during 60 days. The samples were submitted to conventional histological processing, embedded in paraffin, and sectioned in microtome at  $4\text{ }\mu\text{m}$  thick. Buccolingual histological sections of the alveolar portion previously occupied by the lower left first molar and overlying mucosa were collected in histological slides.

For histopathological and histomorphometric analysis of the tooth extraction sites and adjacencies, the histological sections were submitted to hematoxylin-eosin (HE) staining.

For histochemical analysis of the maturation level of collagen fibers, the histological sections were submitted to picosirius red staining.

For immunohistochemical analysis, the histological sections were submitted to indirect immunoperoxidase technique employing the following primary antibodies: anti-TNF $\alpha$  (SC-1348, Santa Cruz Biotechnology®), anti-IL-1 $\beta$  (SC-1252, Santa Cruz Biotechnology®) and anti-IL-6 (SC-1265, Santa Cruz Biotechnology®). The immunohistochemical processing followed the protocol described by Garcia et al. [28]. As negative control, the specimens were submitted to the same procedures, eliminating the use of primary antibody.

## 2.2. Analysis of the Results

### 2.2.1. Analysis of the General Health Condition and Intraoral Clinical Examination

The general health condition of the animals was observed throughout the experimental period and body weight was monitored weekly. Intraoral clinical examination was performed by a blinded examiner, consisting of a detailed visual inspection of the oral cavity, mainly of the tooth extraction site. The clinical aspect of the tooth extraction site and adjacencies was categorized according to the parameters contained in Table 1.

### 2.2.2. Microscopic Analyses

Three histological sections located on the vestibular, middle and lingual portion of the dental alveolus were used in the histopathological

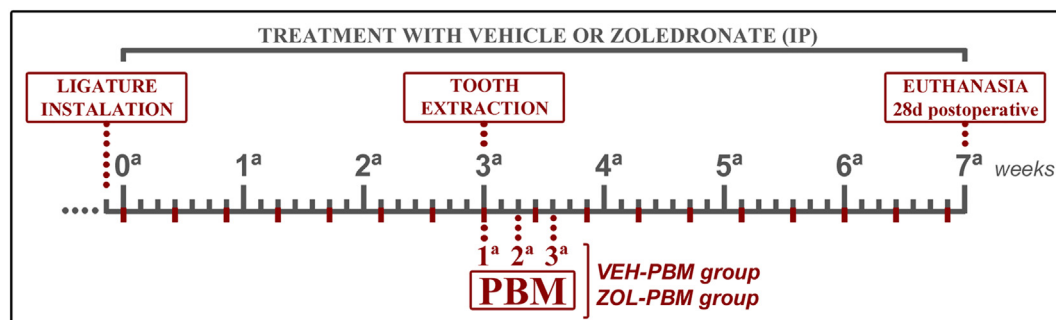


Fig. 2. Scheme illustrating experimental study delineation.



**Table 1**

Scores and distribution of specimens according to clinical analysis of tooth extraction site and adjacent sites in VEH, VEH-PBM, ZOL and ZOL-PBM groups at 28 postoperative days.

Clinical analysis				
Parameter and respective scores	Number of specimens			
	Experimental groups			
	VEH (n = 7)	VEH-PBM (n = 7)	ZOL (n = 7)	ZOL-PBM (n = 7)
Clinical aspect of tooth extraction site and adjacencies				
(1) absence of exposed bone and totally repaired mucous membrane	7	7	–	5
(2) absence of exposed bone and partially repaired mucous membrane	–	–	–	2
(3) MRONJ – large extraction site with small area of exposed bone (less than half of the alveolar space) and impairment of mucous membrane repair	–	–	3	–
(4) MRONJ – large extraction site with great area of exposed bone (more than half of the alveolar space) and impairment of mucous membrane repair	–	–	4	–
Median	1	1	4 <sup>a,b</sup>	1 <sup>c</sup>

<sup>a</sup> statistically significant difference in relation to VEH.

<sup>b</sup> statistically significant difference in relation to VEH-PBM.

<sup>c</sup> statistically significant difference in relation to ZOL.

and histomorphometric analysis. One histological section of the central portion of the alveolus was used for histochemical evaluation of the collagen fibers and three other sections for immunohistochemical analyzes. Images were captured using a digital camera (AxioCam® Carl Zeiss, Gottingen, Germany) coupled to an optical microscope (AxioLab® Carl Zeiss, Gottingen, Germany) and connected to a microcomputer. All analyzes were performed by a certified histologist (EE), calibrated and blinded to treatments.

### 2.2.3. Histopathological Analysis of the Tooth Extraction Site and Adjacent Areas

Histopathological analysis of the tooth extraction site and adjacent areas was performed in light microscopy evaluating the following parameters: 1) intensity of local inflammatory response; 2) extension of inflammatory process; 3) cellular pattern and epithelium tissue structure; 4) cellular pattern and connective tissue structure; 5) cellular pattern and bone tissue structure; 6) contamination pattern of tooth extraction site. Each of these parameters was categorized according to the histological aspects contained in Table 2.

### 2.2.4. Histometric Analysis of Newly Formed Bone Tissue

Images of the tooth alveolus adjacent areas were captured with the aid of an image analysis software (Axiovision 4.8.2® Carl Zeiss, Gottingen, Germany). The area occupied by newly formed bone tissue (NFBT) was measured and expressed in  $\mu\text{m}^2$  as mean  $\pm$  standard deviation in each experimental group.

### 2.2.5. Histochemical Analysis of the Maturation Level of Collagen Fibers

The histochemical analysis of the maturation level of collagen fibers in the lamina propria of the mucosa overlying the tooth extraction site was performed in polarized light microscopy with the aid of an image analysis software (Axiovision 4.8.2® Carl Zeiss, Gottingen, Germany). Two images ( $250\mu\text{m} \times 250\mu\text{m}$ ) located in the center of lamina propria at the level of site previously occupied by the mesial and distal roots of the first molar were analyzed. The area occupied by the different colors was measured, considering yellow-green staining as immature collagen fibers and orange-red staining as mature collagen fibers [29]. These values were expressed in percentages as mean  $\pm$  standard deviation in each experimental group.

### 2.2.6. Immunohistochemical Analysis

Images of the mucosal connective tissue overlying the tooth extraction site in histological sections immunolabeled with TNF $\alpha$ , IL-1 $\beta$

and IL-6 were obtained, as previously described. With the aid of an image analysis software (Axiovision 4.8.2® Carl Zeiss, Gottingen, Germany) the area corresponding to immunolabeling was delineated using the color threshold tool to obtain the immunolabeling optical density [30], expressed as random unit percentage of optical density (mean  $\pm$  standard deviation).

### 2.2.7. Statistical Analysis

For the statistical analysis of data, Bioestat 5.3 software program (Mamirauá Institute, Manaus, AM, Brazil) was used. The sample size (n = 7) was calculated to ensure statistical test power of 95% (p < 0.05). For clinical and histopathological analysis, Kruskal-Wallis analysis of variance test and Student-Newman-Keuls post-test were used. For histometric, histochemical and immunohistochemical analyses, Shapiro-Wilk test, analysis of variance (ANOVA) and Tukey's post-test were used. The significance level was set at 5% (p < 0.05).

## 3. Results

### 3.1. General Health Condition and Intraoral Clinical Examination

The general health condition of the animals used in this study remained constant throughout the experimental period. These animals tolerated the drug treatment and the surgical procedure of first molar extraction performed in all experimental groups, as well as the three PBM sessions in groups VEH-PBM and ZOL-PBM. There was no significant difference in the mean body weight of animals between groups during the experimental period (data not shown).

The clinical exam showed no macroscopic changes in the oral cavity or in the tooth extraction site in VEH, VEH-PBM and ZOL-PBM. In ZOL, the size of the surgical sites was higher compared to the other experimental groups, and in some rats, oral bone exposure was observed in the extraction site and surrounding areas (Table 1).

### 3.2. Histological Analysis of the Tissue Repair Process after Extraction

Groups VEH, VEH-PBM and ZOL-PBM showed a similar tissue repair process for the tooth extraction site and overlying mucosa. However, group ZOL had the tissue repair process severely compromised and consistent with an MRONJ. The parameters, scores and specimen distribution according to histological analysis in all experimental groups at 28 days postoperatively are presented in Table 2 and Fig. 3.

**Table 2**

Parameters, scores and distribution of specimens according to histopathological analysis during repair tissue after tooth extraction in VEH, VEH-PBM, ZOL and ZOL-PBM at 28 postoperative days.

Histopathological analysis				
Parameters and respective scores	Number of specimens			
	Experimental groups			
	VEH (n = 7)	VEH-PBM (n = 7)	ZOL (n = 7)	ZOL-PBM (n = 7)
Intensity of local inflammatory response				
(1) absence of inflammation	7	7	–	4
(2) mild quantity of inflammatory cells	–	–	–	3
(3) moderate quantity of inflammatory cells	–	–	3	–
(4) severe quantity of inflammatory cells	–	–	4	–
median	1	1	4 <sup>a,b</sup>	1
Inflammatory extensions				
(1) absence of inflammation	7	7	–	4
(2) partial extension of connective tissue	–	–	–	3
(3) entire extension of connective tissue, without reaching bone tissue	–	–	2	–
(4) entire extension of connective tissue and bone tissue	–	–	5	–
median	1	1	4 <sup>a,b</sup>	1
Cellular pattern and epithelial tissue structure				
(1) epithelial tissue with moderate thickness completely recovering extraction site	4	6	–	1
(2) epithelial tissue with thin thickness completely recovering extraction site	3	1	–	5
(3) thin layer of epithelial tissue only in edges of open surgical wound	–	–	4	1
(4) absence of epithelial tissue on open surgical wound	–	–	3	–
median	1	1	3 <sup>a,b</sup>	2 <sup>c</sup>
Cellular pattern and connective tissue structure				
(1) moderate quantity of fibroblasts and large quantity of collagen fibers	4	6	–	1
(2) moderate quantity of both fibroblasts and collagen fibers	3	1	–	5
(3) small quantity of both fibroblasts and collagen fibers	–	–	2	1
(4) severe tissue disorganization with necrosis areas	–	–	5	–
median	1	1	4 <sup>a,b</sup>	2 <sup>c</sup>
Cellular pattern and bone tissue structure				
(1) absence of non-vital bone in adjacencies of extraction site and trabecular bone filling more than half of dental alveolus	4	6	–	1
(2) absence of non-vital bone in adjacencies of extraction site and trabecular bone filling less than half of dental alveolus	3	1	–	5
(3) presence of few areas with non-vital bone in adjacencies of extraction site and trabecular bone filling less than a third of dental alveolus	–	–	2	1
(4) presence of many areas with non-vital bone in adjacencies of extraction site and trabecular bone filling less than a third of dental alveolus	–	–	5	–
median	1	1	4 <sup>a,b</sup>	2 <sup>c</sup>
Contamination pattern of tooth extraction site				
(1) presence of bacteria diffusely distributed in extraction site, typical of a normal condition	7	7	–	2
(2) presence of large colonies of bacteria in soft tissues over dental alveolus	–	–	–	4
(3) presence of large colonies of bacteria in surface of alveolar bone and in the interior of the dental alveolus	–	–	–	1
(4) presence of large colonies of bacteria involving necrosed bone and/or in medullar spaces and in adjacencies of dental alveolus	–	–	7	–
median	1	1	4 <sup>a,b</sup>	2 <sup>c</sup>

<sup>a</sup> statistically significant difference in relation to VEH.

<sup>b</sup> statistically significant difference in relation to VEH-PBM.

<sup>c</sup> statistically significant difference in relation to ZOL.

### 3.3. Newly Formed Bone Tissue in Tooth Extraction Site

NFBT was lower in ZOL and ZOL-PBM than in VEH and VEH-PBM. ZOL-PBM showed higher NFBT than ZOL. There was no statistically significant difference between VEH and VEH-PBM (Fig. 4).

### 3.4. Maturation Level of Collagen Fibers in the Mucosa Overlying the Tooth Extraction Site

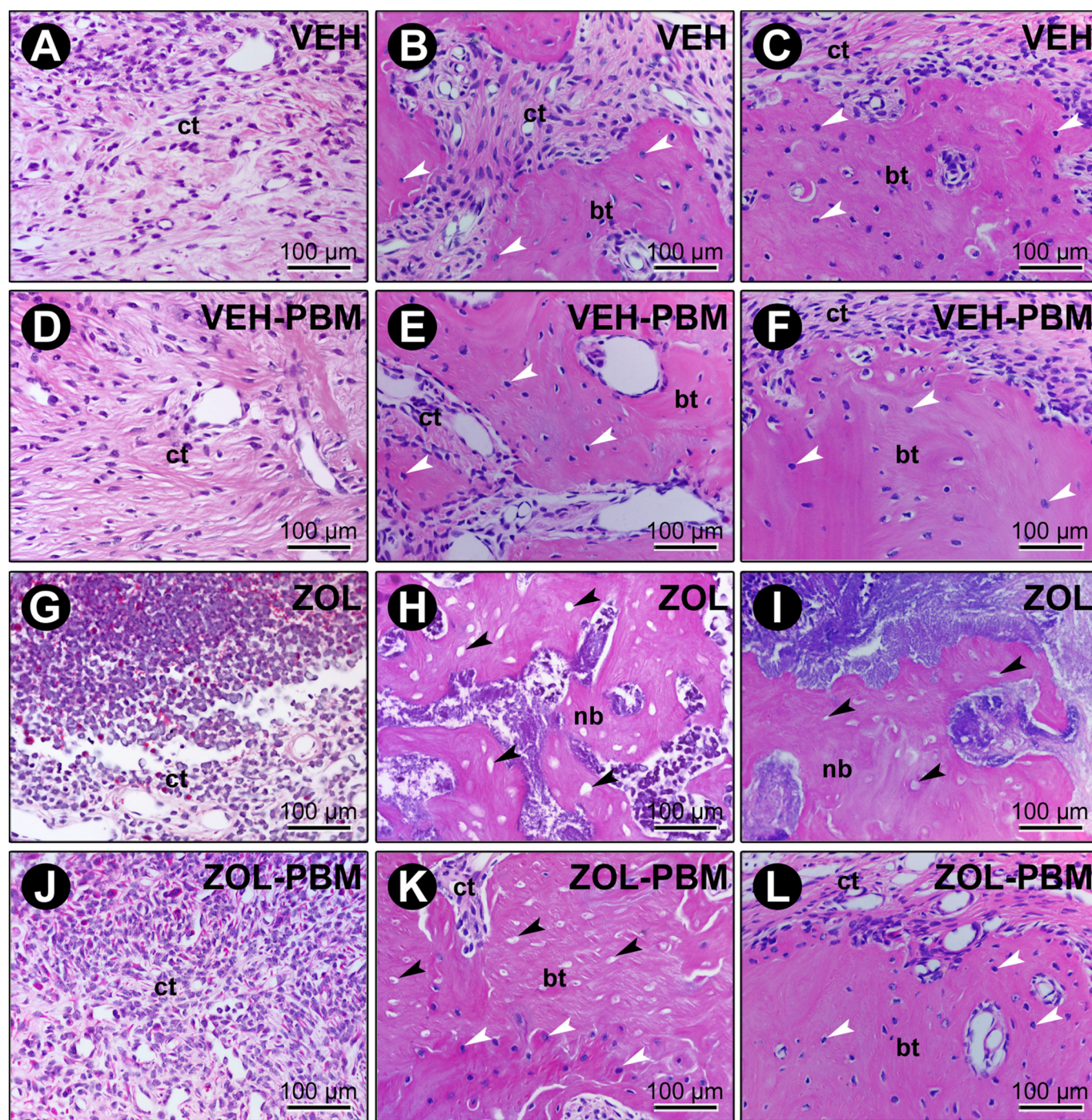
The percentage of mature collagen fibers was lower in ZOL than in VEH, VEH-PBM and ZOL-PBM. ZOL-PBM showed lower percentage of mature collagen fibers than in VEH and VEH-PBM. There was no statistically significant difference between VEH and VEH-PBM (Fig. 5).

### 3.5. Immunolabeling Pattern for TNF $\alpha$ , IL-1 $\beta$ and IL-6 in the Connective Tissue Overlying the Tooth Extraction Site

The immunohistochemical technique used for the detection of TNF $\alpha$ , IL-1 $\beta$  and IL-6 showed high specificity in the detection of such proteins, which was confirmed by the total absence of labeling in the negative control reactions. Immunoreactive cells showed dark brown staining confined to the cytoplasm of inflammatory cells and, to a lesser extent, to the extracellular matrix of connective tissue.

The immunolabeling optical density for TNF $\alpha$ , IL-1 $\beta$  and IL-6 did not differ between VEH and VEH-PBM. In ZOL, the immunolabeling pattern for pro-inflammatory cytokines was greater than in VEH and VEH-PBM. In ZOL-PBM, immunolabeling optical density for TNF $\alpha$  and IL-1 $\beta$  was lower than in ZOL; however, there was no significant difference in IL-6 labeling between these two experimental groups (Fig. 6).





**Fig. 3.** Histopathological aspects of dental extraction site and adjacent area. A-L: photomicrographs showing histological characteristics of connective and bone tissues in extraction site and adjacent area in VEH (A-C), VEH-PBM (D-F), ZOL (G-I) and ZOL-PBM (J-L) groups at 28 postoperative days. Observe in ZOL: persistence of inflammation in connective tissue overlying tooth extraction site (G); frequent presence of area occupied by non-vital bone (H, I); large sequestered bone in adjacencies of extraction site (I). In VEH and ZOL-PBM, histological characteristics were similar: absence of inflammation in connective tissue overlying tooth extraction site (A, J); absence of large areas of non-vital bone (B, C, K, L); presence of small quantity of empty lacunae (K) and; no sequestered bone in adjacencies of extraction site. Abbreviations and symbols: bt, bone tissue; ct, connective tissue; nb, necrotic bone; white arrows, osteocytes; black arrows, empty lacunae. Staining: HE. Original magnification: 400 $\times$ . Scale bars: 100  $\mu$ m.

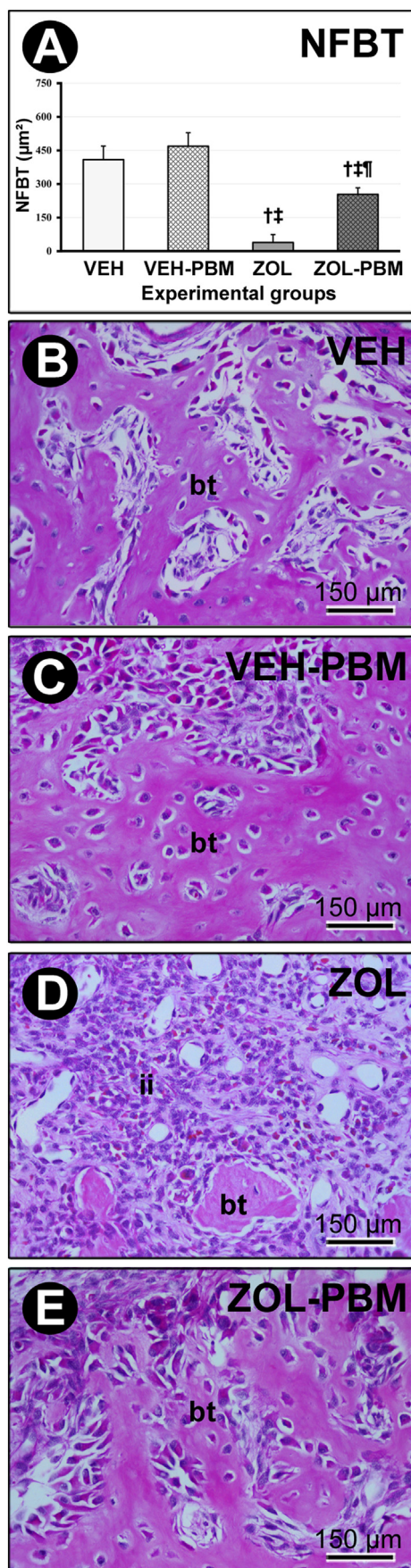
#### 4. Discussion

Zoledronate is used in the treatment of osteolytic diseases [1]. Since this drug is a potent bisphosphonate, its use has been associated with most cases of MRONJ [5]. The MRONJ pathogenesis is still poorly understood, complicating its prevention or treatment [3,4,12–14]. This study evaluated the effectiveness and safety of PBM multiple sessions in

an experimental model of rats with major risk factors for MRONJ. It was found that PBM minimized great part of the zoledronate negative effects on both soft and hard tissues of the tooth extraction site, particularly stimulating tissue repair capacity, placing it as a potentially able strategy to avoid the outbreak of post-extraction MRONJ.

The first reports of MRONJ were published in 2003 [11]. Over this period, studies built up and the epidemiological profile of the disease





**Fig. 4.** Newly formed bone in the tooth extraction site. **A:** Newly formed bone tissue (NFBT) in tooth extraction site in the different experimental groups at 28 postoperative days. **B-E:** Photomicrographs showing NFBT between apical and middle third of dental alveolus previously occupied by root mesial of lower first molar in groups VEH (**B**), VEH-PBM (**C**), ZOL (**D**) and ZOL-PBM (**E**). Abbreviations and symbols: bt, bone tissue; ii, inflammatory infiltrate; †, statistically significant difference in relation to VEH; ‡, statistically significant difference in relation to VEH-PBM; \*, statistically significant difference in relation to ZOL. Staining: HE. Original magnification: 400×. Scale bars: 150 μm.

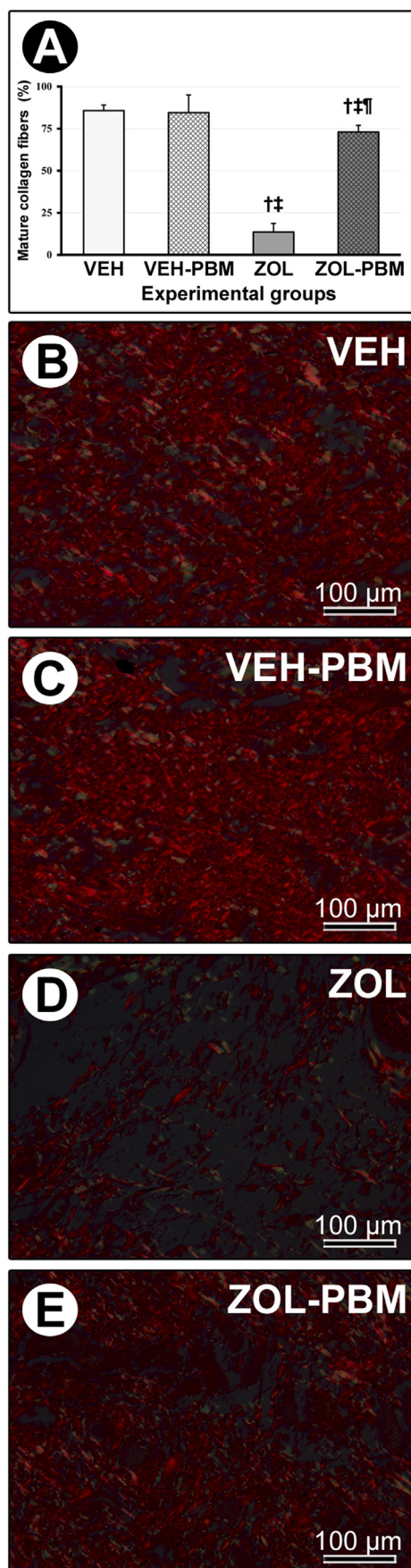
and its major risk factors were defined. The present experimental model was established based on such epidemiological studies [6,7]. We used senile female rats, since the disease mainly affects women with old age. The rats were treated with an oncologic dose of zoledronate given that this dose and this bisphosphonate were related to the majority of cases of MRONJ. Tooth extraction and the presence of periodontal disease are important local risk factors for MRONJ [6,7]. Therefore, in the present study periodontitis was induced experimentally, the tooth was extracted and the repair process or triggering of MRONJ was analyzed.

The histopathological analysis performed in this study showed that zoledronate impaired the regenerative capacity of the epithelial tissue, since no tissue recovery was observed at 28 days postoperatively. These results are in agreement with previous studies that demonstrated that BPs exert an adverse effect on keratinocytes of the oral mucosa. These studies showed that BPs are able to decrease proliferation [31–35], migration ability [31,36], intercellular adhesion [32] and maturation of keratinocytes [32], which are essential events for the regeneration process of the epithelial tissue. Furthermore, the viability of mature keratinocytes is considerably impaired by BPs since these drugs are able to induce apoptosis [36,37] and premature senescence [38] in such cells, which would affect the integrity of the epithelial tissue structure.

In this study, the histopathological and immunohistochemical analysis showed that zoledronate prevented total connective tissue repair, since a persistent inflammation remained. Intense inflammatory infiltrate, increased and prolonged expression of pro-inflammatory cytokines and the negative effect on collagen fiber maturation contributed to severe impairment of the tissue repair. Morita et al. [39] demonstrated that high local levels of pro-inflammatory cytokines play an important role in the pathogenesis of MRONJ, since TNFα-, IL-1β- and IL-6-deficient mice were significantly resistant to osteonecrosis development. These authors demonstrated that most of these cytokines are derived from osteoclast precursors whose maturation was inhibited by use of BPs. Furthermore, studies show that zoledronate exerts a stimulatory effect on apoptosis in connective tissue cells [40–44], besides inhibiting their proliferation [40,41,45,46] and impairing extracellular matrix synthesis [40,44,46], particularly collagen, corroborating with the findings at the tissue level observed in this study.

The use of PBM in rats with major risk factors for MRONJ was able to exert positive effects on the repair process of the epithelial and connective tissues of the mucosa overlying the tooth extraction site, thus avoiding the onset of MRONJ. PBM exerted an anti-inflammatory and stimulatory effect on cells that structure the oral mucosa. These findings are consistent with studies showing increased cell viability when keratinocytes and fibroblasts treated with BPs are submitted to PBM [47,48]. PBM also attenuated the inflammatory response that persisted in the tooth extraction site, demonstrated by lower intensity and extension of the inflammatory process as well as by reduction of levels of the main pro-inflammatory cytokines. In addition, PBM stimulated maturation of collagen fibers, which favored the repair process of the mucosa overlying the tooth extraction site.

The post-extraction alveolar bone response was severely compromised, inhibiting the bone regeneration process, presumably by a negative action of zoledronate on osteoblasts. In agreement with the findings of the present study, Huang et al. [49] reported that zoledronate impairs osteoblast differentiation and reduces type I collagen gene expression, the main component of the organic bone matrix,



**Fig. 5.** Maturation level of collagen fibers. A: Mature collagen fibers in connective tissue of mucous membrane overlying tooth extraction site in the different experimental groups at 28 postoperative days. B-E: Photomicrographs showing maturation pattern of collagen fibers in VEH (B), VEH-PBM (C), ZOL (D) and ZOL-PBM (E). Symbols: †, statistically significant difference in relation to VEH; ‡, statistically significant difference in relation to VEH-PBM; ¶, statistically significant difference in relation to ZOL. Staining: picrosirius red in polarized light microscopy. Original magnification: 250 $\times$ . Scale bars: 100  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

besides decreasing alkaline phosphatase and osteocalcin, which are essential for the matrix biomineralization process in mature osteoblasts. The negative effects of zoledronate on osteoblasts have also been reported by several other authors [50–54].

The negative effects of zoledronate on the bone tissue repair process in the tooth extraction site were partially reversed by PBM. The histological aspect of newly formed bone tissue and the pre-existing bone tissue adjacent to the alveolus and higher NFBT were parameters that improved compared with those presented by ZOL. Studies have shown that PBM exerted a potent biostimulatory effect on osteoblast lineages treated with zoledronate [48,55]. However, another study found that PBM showed a limited effect on osteoblasts treated with zoledronate, exerting a positive effect only on type I collagen gene expression, but no effect on cell viability and proliferation, total protein, activity and alkaline phosphatase gene expression and mineralization [56].

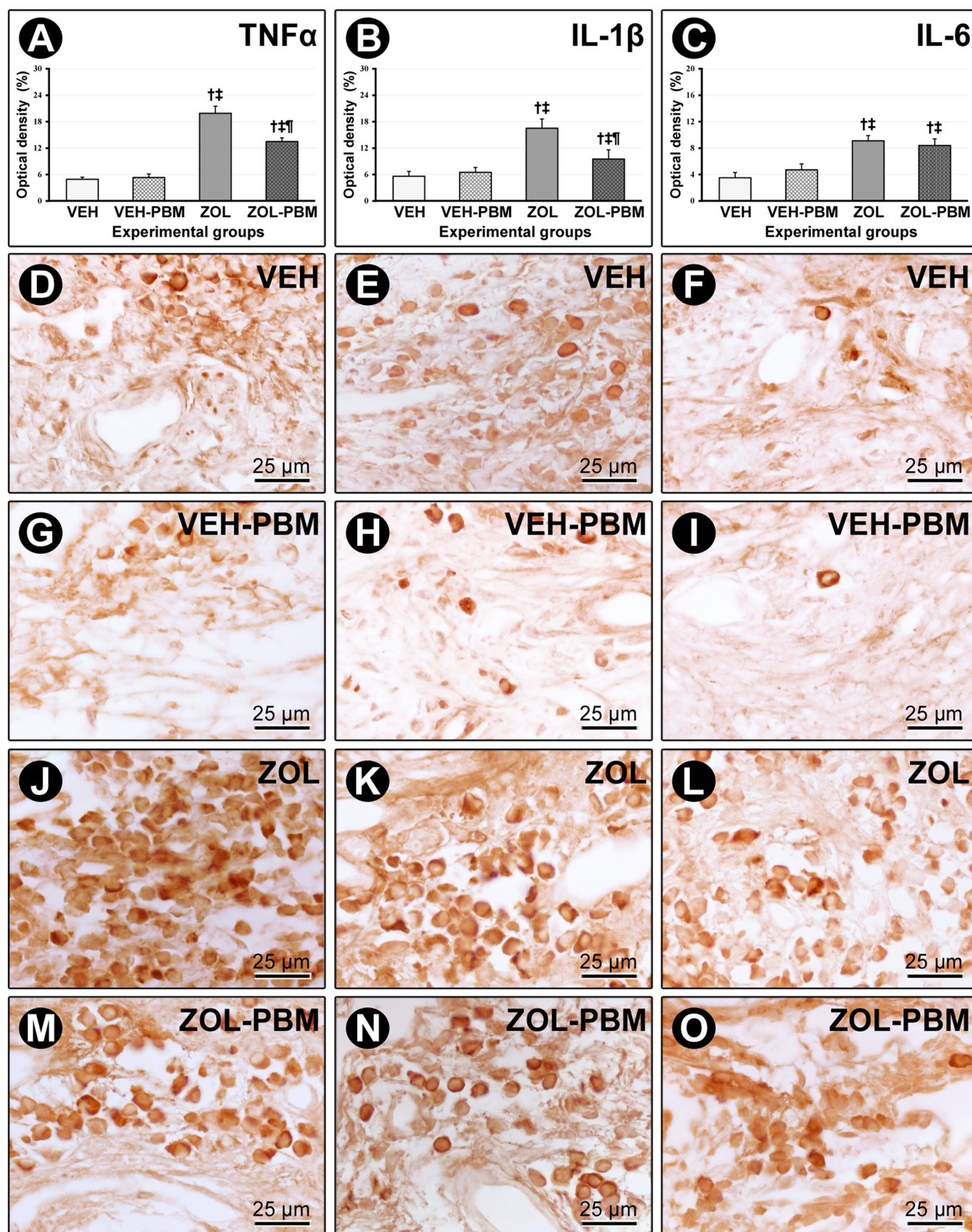
One aspect of great importance in PBM is in the laser parameters. In the present study, an InGaAlP laser device, 660 nm, 0.035 W power, energy point of 2.1 J/point, density energy of 74.2 J/cm<sup>2</sup> was used for 60 s. The irradiation was carried out at a single point in the center of the dental alveolus with the laser tip positioned parallel to the long axis and in contact with the treated area. The use of these parameters was based on previous studies from our research group with some modifications, showing PBM effectivity (InGaAlP; 660 nm; 0.035 W; 4.2 J; 120 s; 74.2 J/cm<sup>2</sup>) as an adjuvant therapy in rats with experimentally induced periodontitis submitted to 5-fluorouracil chemotherapy treatment [27]. A previous study showed positive effects of PBM using diode laser (AlGaAs; 685 nm; 0.030 W; 4 J; 134 s; 67 J/cm<sup>2</sup>) in rat MRONJ prevention [57], corroborating the results of the present study. The experimental protocols using red range laser and their effectivity in MRONJ preventive therapy are similar. There are no human clinical studies adopting PBM with low-level laser for MRONJ prevention. However, association of antibiotics (pre- and postoperatively) with multiple PBM sessions using Nd:YAG laser in defocus mode (2 mm of distance, 1.25 W, 60 s, 320  $\mu$ m), after tooth extraction in patients who had previously developed MRONJ in another site, showed to be an efficient therapy [58].

A few systematic reviews have currently shown that the combination of antibiotics and/or surgical therapy with PBM may represent a promising strategy for the treatment of osteonecrotics injury [22–24]. However, the adoption of preventive measures is ideal in the case of MRONJ, since this disease affects patient quality of life, resulting in serious sequelae, and often hinders the treatment of the underlying disease, such as certain malignancies. Similar to all experimental animal studies, our findings can be used for guiding further clinical studies. Clinical studies for the establishment of a standard preventive protocol to be followed by dentists in patients with chronic use of BPs and undergoing invasive dental interventions is something necessary in view of the increasing use of anti-resorptive drugs. The incorporation of PBM to conventional preventive treatments should be considered for enhancing the effectiveness of therapeutics.

## 5. Conclusion

Within the limits of this study, it can be concluded that zoledronate significantly impairs the post-extraction repair process by decreasing





(caption on next page)



**Fig. 6.** Immunolabeling pattern for TNF $\alpha$ , IL-1 $\beta$  and IL-6. A-C: Immunolabeling optical density for TNF $\alpha$  (A), IL-1 $\beta$  (B) and IL-6 (C) in connective tissue of mucous membrane overlying tooth extraction site in different experimental groups at 28 postoperative days. D-O: Photomicrographs showing immunolabeling pattern for TNF $\alpha$  (D, G, J, M), IL-1 $\beta$  (E, H, K, N) and IL-6 (F, I, L, O) in VEH (D-F), VEH-PBM (G-I), ZOL (J-L), ZOL-PBM (M-O). Symbols: †, statistically significant difference in relation to VEH; ‡, statistically significant difference in relation to VEH-PBM; ¶, statistically significant difference in relation to ZOL. Original magnification: 1000 $\times$ . Scale bars: 25  $\mu$ m.

the repair capacity of both soft and hard tissues, which would be among the etiopathogenic factors of MRONJ. According to the parameters used in this study, PBM is able to stimulate the post-extraction tissue repair process by minimizing the negative consequences of zoledronate. Thus, PBM is a promising preventive therapy strategy to avoid the onset of MRONJ.

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## References

- [1] R.G. Russell, Bisphosphonates: the first 40 years, *Bone* 49 (2011) 2–19.
- [2] M.J. Rogers, J.C. Crockett, F.P. Coxon, J. Monkken, Biochemical and molecular mechanisms of action of bisphosphonates, *Bone* 49 (2011) 34–41.
- [3] S.L. Ruggiero, T.B. Dodson, J. Fantasia, R. Goodday, T. Aghaloo, B. Mehrotra, F. O'Ryan, American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaws - 2014 update, *J. Oral Maxillofac. Surg.* 72 (2014) 2381–2382.
- [4] A.A. Khan, A. Morrison, D.L. Kendler, R. Rizzoli, D.A. Hanley, D. Felsenberg, L.K. McCauley, F. O'Ryan, I.R. Reid, S.L. Ruggiero, A. Taguchi, S. Tetradis, N.B. Watts, M.L. Brandi, E. Peters, T. Guise, R. Eastell, A.M. Cheung, S.N. Morin, B. Masri, C. Cooper, S.L. Morgan, B. Obermayer-Pietsch, B.L. Langdahl, R.A. Dabagh, K.S. Davison, G.K. Sándor, R.G. Josse, M. Bhandari, M. El Rabbany, D.D. Pierroz, R. Sulimani, D.P. Saunders, J.P. Brown, J. Compston, International task force on osteonecrosis of the jaw. Case-based review of osteonecrosis of the jaw (ONJ) and application of the international recommendations for management from the international task force on ONJ, *J. Clin. Densitom.* 20 (2017) 8–24.
- [5] R. Gomez Font, G.M.L. Martinez, M.J.M. Olmos, Osteochemonecrosis of the jaws due to bisphosphonate treatments. Update, *Med. Oral Patol. Oral Cir. Bucal.* 13 (2008) E318–E324.
- [6] K. McGowan, T. McGowan, S. Ivanovski, Risk Factors for Medication-Related Osteonecrosis of the Jaws: A Systematic Review, *Oral Dis.* (2017) (Epub ahead of print).
- [7] S. Otto, C. Schreyer, S. Hafner, G. Mast, M. Ehrenfeld, S. Stürzenbaum, C. Pautke, Bisphosphonate-related osteonecrosis of the jaws - characteristics, risk factors, clinical features, localization and impact on oncological treatment, *J. Craniomaxillofac. Surg.* 40 (2012) 303–309.
- [8] V. Thumbigere-Math, B.S. Michalowicz, J.S. Hodges, M.L. Tsai, K.K. Swenson, L. Rockwell, R. Gopalakrishnan, Periodontal disease as a risk factor for bisphosphonate-related osteonecrosis of the jaw, *J. Periodontol.* 85 (2014) 226–233.
- [9] M. Song, A. Alshaikh, T. Kim, S. Kim, M. Dang, S. Mehrzarin, K.H. Shin, M. Kang, N.H. Park, R.H. Kim, Preexisting periapical inflammatory condition exacerbates tooth extraction-induced bisphosphonate-related osteonecrosis of the jaw lesions in mice, *J. Endod.* 42 (2016) 1641–1646.
- [10] T.L. Aghaloo, B. Kang, E.C. Sung, M. Shoff, M. Ronconi, J.E. Gotcher, O. Bezouglaia, S.M. Dry, S. Tetradis, Periodontal disease and bisphosphonates induce osteonecrosis of the jaws in the rat, *J. Bone Miner. Res.* 26 (2011) 1871–1882.
- [11] R.E. Marx, Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic, *J. Oral Maxillofac. Surg.* 61 (2003) 1115–1117.
- [12] T. Badel, I.S. Pavicin, A.J. Carek, K. Rosin-Grget, D. Grbesa, Pathophysiology of osteonecrosis of the jaw in patients treated with bisphosphonate, *Coll. Antropol.* 37 (2013) 645–651.
- [13] C.A. Migliorati, J.B. Epstein, E. Abt, J.R. Berenson, Osteonecrosis of the jaw and bisphosphonates in cancer: a narrative review, *Nat. Rev. Endocrinol.* 7 (2011) 34–42.
- [14] M.R. Allen, D.B. Burr, The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data, *J. Oral Maxillofac. Surg.* 67 (2009) 61–70.
- [15] M. El-Rabbany, A. Sgro, D.K. Lam, P.S. Shah, A. Azarpazhooh, Effectiveness of treatments for medication-related osteonecrosis of the jaw: a systematic review and meta-analysis, *J. Am. Dent. Assoc.* S0002-8177 (2017) 30300–30308.
- [16] L.F. de Freitas, M.R. Hamblin, Proposed mechanisms of Photobiomodulation or low-level light therapy, *IEEE J. Sel. Top. Quantum. Electron.* 22 (2016) 7000417.
- [17] M. Noda, A. Aoki, K. Mizutani, T. Lin, M. Komaki, S. Shibata, Y. Izumi, High-frequency pulsed low-level diode laser therapy accelerates wound healing of tooth extraction socket: an in vivo study, *Lasers Surg. Med.* 48 (2016) 955–964.
- [18] N.S. Korany, S.S. Mehanni, H.M. Hakam, E.M. El-Maghraby, Evaluation of socket healing in irradiated rats after diode laser exposure (histological and morphometric studies), *Arch. Oral Biol.* 57 (2012) 884–891.
- [19] Y. Takeda, Irradiation effect of low-energy laser on alveolar bone after tooth extraction. Experimental study in rats, *Int. J. Oral Maxillofac. Surg.* 17 (1988) 388–391.
- [20] C.N. Eroglu, S. Keskin Tunc, Effectiveness of single session of low-level laser therapy with a 940 nm wavelength diode laser on pain, swelling, and trismus after impacted third molar surgery, *Photomed. Laser Surg.* 34 (2016) 406–410.
- [21] M. Ferrante, M. Petrini, P. Trentini, G. Perfetti, G. Spoto, Effect of low-level laser therapy after extraction of impacted lower third molars, *Lasers Med. Sci.* 28 (2013) 845–849.
- [22] J. Robijns, S. Censabella, P. Bulens, A. Maes, J. Mebis, The use of low-level light therapy in supportive care for patients with breast cancer: review of the literature, *Lasers Med. Sci.* 32 (2017) 229–242.
- [23] S. Latifyan, M.T. Genot, J. Klastersky, Bisphosphonate-related osteonecrosis of the jaw: a review of the potential efficacy of low-level laser therapy, *Support Care Cancer* 24 (2016) 3687–3693.
- [24] J.B. Weber, R.S. Camilotti, M.E. Ponte, Efficacy of laser therapy in the management of bisphosphonate-related osteonecrosis of the jaw (BRONJ): a systematic review, *Lasers Med. Sci.* 31 (2016) 1261–1272.
- [25] Food and Drug Administration, Dose Calculator: Conversion Chemotherapy of Human to Animals, <http://www.accessdata.fda.gov>, Accessed date: February 2014.
- [26] P.G. Silva, A.E. Ferreira-Júnior, C.R. Teófilo, M.C. Barbosa, R.C. Lima-Júnior, F.B. Sousa, M.R. Mota, R.A. Ribeiro, A.P. Alves, Effect of different doses of zoledronic acid in establishing of bisphosphonate related osteonecrosis, *Arch. Oral Biol.* 60 (2015) 1237–1245.
- [27] L.H. Theodoro, M. Longo, E. Ervolino, C. Duque, M.L. Ferro-Alves, N.Z. Assem, L.M. Louzada, V.G. Garcia, Effect of low-level laser therapy as an adjuvant in the treatment of periodontitis induced in rats subjected to 5-fluorouracil chemotherapy, *J. Periodontol. Res.* 51 (2016) 669–680.
- [28] V.G. Garcia, L.R. Knoll, M. Longo, V.C. Novaes, N.Z. Assem, E. Ervolino, B.E. de Toledo, L.H. Theodoro, Effect of the probiotic *Saccharomyces cerevisiae* on ligature-induced periodontitis in rats, *J. Periodontol. Res.* 51 (2016) 26–37.
- [29] K. Ganganna, P. Shetty, S.E. Shroff, Collagen in histologic stages of oral submucous fibrosis: a polarizing microscopic study, *J. Oral Maxillofac. Pathol.* 16 (2012) 162–166.
- [30] T.W. King, E.M. Brey, A.A. Youssef, C. Johnston, C.W. Patrick Jr., Quantification of vascular density using a semiautomated technique for immunostained specimens, *Anal. Quant. Cytol. Histol.* 24 (2002) 39–48.
- [31] T. Saito, K. Izumi, A. Shiomi, A. Uenoyama, H. Ohnuki, H. Kato, Zoledronic acid impairs re-epithelialization through down-regulation of integrin  $\alpha$ v $\beta$ 6 and transforming growth factor  $\beta$  signalling in a three-dimensional in vitro wound healing model, *Int. J. Oral Maxillofac. Surg.* 43 (2014) 373–380.
- [32] E. Donetti, A. Gualerzi, A. Sardella, G. Lodi, A. Carrassi, C. Sforza, Alendronate impairs epithelial adhesion, differentiation and proliferation in human oral mucosa, *Oral Dis.* 20 (2014) 466–472.
- [33] H. Ohnuki, K. Izumi, M. Terada, T. Saito, H. Kato, A. Suzuki, Y. Kawano, K. Nozawa-Inoue, R. Takagi, T. Maeda, Zoledronic acid induces S-phase arrest via a DNA damage response in normal human oral keratinocytes, *Arch. Oral Biol.* 57 (2012) 906–917.
- [34] S. Saracino, R.A. Canuto, M. Maggiora, M. Oraldi, M. Scoletta, L. Ciuffreda, Exposing human epithelial cells to zoledronic acid can mediate osteonecrosis of jaw: an in vitro model, *J. Oral Pathol. Med.* 41 (2012) 788–792.
- [35] E.L. Scheller, C.M. Baldwin, S. Kuo, N.J. D'silva, S.E. Feinberg, P.H. Krebsbach, P.C. Edwards, Bisphosphonates inhibit expression of p63 by oral keratinocytes, *J. Dent. Res.* 90 (2011) 894–899.
- [36] A.M. Pabst, T. Ziebart, F.P. Koch, K.Y. Taylor, B. Al-Nawas, C. Walter, The influence of bisphosphonates on viability, migration, and apoptosis of human oral keratinocytes—in vitro study, *Clin Oral Invest* 16 (2012) 87–93.
- [37] M.A. Scheper, A. Badros, R. Chaisuparat, K.J. Cullen, T.F. Meiller, Effect of zoledronic acid on oral fibroblasts and epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis, *Br. J. Haematol.* 144 (2009) 667–676.
- [38] R.H. Kim, R.S. Lee, D. Williams, S. Bae, J. Woo, M. Lieberman, J.E. Oh, Q. Dong, K.H. Shin, M.K. Kang, N.H. Park, Bisphosphonates induce senescence in normal human oral keratinocytes, *J. Dent. Res.* 90 (2011) 810–816.
- [39] M. Morita, R. Iwasaki, Y. Sato, T. Kobayashi, R. Watanabe, T. Oike, S. Nakamura, Y. Kenko, K. Miyamoto, K. Ishihara, Y. Iwakura, K. Ishii, M. Matsumoto, M. Nakamura, H. Kawana, T. Nakagawa, T. Miyamoto, Elevation of pro-inflammatory cytokine levels following anti-resorptive drug treatment is required for osteonecrosis development in infectious osteomyelitis, *Sci. Rep.* 7 (2017) 46322.
- [40] M.J. Ravosa, J. Ning, Y. Liu, M.S. Stack, Bisphosphonate effects on the behaviour of oral epithelial cells and oral fibroblasts, *Arch. Oral Biol.* 56 (2011) 491–498.
- [41] M.A. Scheper, A. Badros, R. Chaisuparat, K.J. Cullen, T.F. Meiller, Effect of zoledronic acid on oral fibroblasts and epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis, *Br. J. Haematol.* 144 (2009) 667–676.



- [42] C. Walter, A. Pabst, T. Ziebart, M. Klein, B. Al-Nawas, Bisphosphonates affect migration ability and cell viability of HUVEC, fibroblasts and osteoblasts in vitro, *Oral Dis.* 17 (2011) 194–199.
- [43] M. Scheper, R. Chaisuparat, K. Cullen, T. Meiller, A novel soft-tissue in vitro model for bisphosphonate-associated osteonecrosis, *Fibrogenesis Tissue Repair* 3 (2010) 6.
- [44] M.J. Simon, P. Niehoff, B. Kimmig, J. Wiltfang, Y. Acil, Expression profile and synthesis of different collagen types I, II, III and V of human gingival fibroblasts, osteoblasts, and SaOS-2 cells after bisphosphonate treatment, *Clin Oral Investig* 14 (2010) 51–58.
- [45] T.N. Pansani, F.G. Basso, A.P. Turirioni, C. Kurachi, J. Hebling, C.A. de Souza Costa, Effects of low-level laser therapy on the proliferation and apoptosis of gingival fibroblasts treated with zoledronic acid, *Int. J. Oral Maxillofac. Surg.* 43 (2014) 1030–1034.
- [46] Y. Acil, B. Moller, P. Niehoff, K. Rachko, V. Gassling, J. Wiltfang, The cytotoxic effects of three different bisphosphonates in-vitro on human gingival fibroblasts, osteoblasts and osteogenic sarcoma cells, *J. Craniomaxillofac. Surg.* 40 (2012) 229–235.
- [47] J.Y. Lee, I.R. Kim, B.S. Park, Y.D. Kim, I.K. Chung, J.M. Song, S.H. Shin, Effect of low-level laser therapy on oral keratinocytes exposed to bisphosphonate, *Lasers Med. Sci.* 30 (2015) 635–643.
- [48] C. Walter, A.M. Pabst, T. Ziebart, Effects of a low-level diode laser on oral keratinocytes, oral fibroblasts, endothelial cells and osteoblasts incubated with bisphosphonates: an in vitro study, *Biomed. Rep.* 3 (2015) 14–18.
- [49] X. Huang, S. Huang, F. Guo, F. Xu, P. Cheng, Y. Ye, Y. Dong, W. Xiang, A. Chen, Dose-dependent inhibitory effects of zoledronic acid on osteoblast viability and function in vitro, *Mol. Med. Rep.* 13 (2016) 613–622.
- [50] F.G. Basso, A.P. Silveira-Turirioni, J. Hebling, C.A. de Souza Costa, Zoledronic acid inhibits human osteoblast activities, *Gerontology* 59 (2013) 534–541.
- [51] I.R. Orriss, M.L. Key, K.W. Colston, T.R. Arnett, Inhibition of osteoblast function in vitro by aminobisphosphonates, *J. Cell. Biochem.* 106 (2009) 109–118.
- [52] S. Pozzi, S. Vallet, S. Mukherjee, D. Cirstea, N. Vaghela, L. Santo, E. Rosen, H. Ikeda, Y. Okawa, T. Kiziltepe, J. Schoonmaker, W. Xie, T. Hideshima, E. Weller, M.L. Bouxsein, N.C. Munshi, K.C. Anderson, N. Raje, High-dose zoledronic acid impacts bone remodeling with effects on osteoblastic lineage and bone mechanical properties, *Clin. Cancer Res.* 15 (2009) 5829–5839.
- [53] A. Naidu, P.C. Dechow, R. Spears, J.M. Wright, H.P. Kessler, L.A. Opperman, The effects of bisphosphonates on osteoblasts in vitro, *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 106 (2008) 5–13.
- [54] K. Anavi-lev, Y. Anavi, G. Chaushu, D.M. Alon, G. Gal, I. Kaplan, Bisphosphonate related osteonecrosis of the jaws: clinico-pathological investigation and histomorphometric analysis, *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 115 (2013) 660–666.
- [55] H. Bayram, H. Kenar, F. Taşar, V. Hasirci, Effect of low level laser therapy and zoledronate on the viability and ALP activity of Saos-2 cells, *Int. J. Oral Maxillofac. Surg.* 42 (2013) 140–146.
- [56] F.G. Basso, A.P. Turirioni, D.G. Soares, V.S. Bagnato, J. Hebling, C.A. de Souza Costa, Low-level laser therapy for osteonecrotic lesions: effects on osteoblasts treated with zoledronic acid, *Support Care Cancer* 22 (2014) 2741–2748.
- [57] J.B.B. Weber, R.S. Camilotti, J. Jasper, L.C.O. Casagrande, F.L.D.M. Maito, Effect of low-level laser therapy on tissue repair after dental extraction in rats administered zoledronic acid and dexamethasone, *J. Biomed. Opt.* 22 (2017) 58001.
- [58] P. Vescovi, I. Giovannacci, E. Merigo, M. Meleti, M. Manfredi, C. Fornaini, S. Nammour, Tooth extractions in high-risk patients under bisphosphonate therapy and previously affected with osteonecrosis of the jaws: surgical protocol supported by low-level laser therapy, *J. Craniofac. Surg.* 26 (2015) 696–699.