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Effects of low dose bisphosphonates treatment on the mandibular bone in rats with and without mini-implants application: an experimental model

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Abstract. Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ) is a pathological condition observed in patients underwent oral surgical procedures during bisphosphonates treatment. Although several studies have focused on the BRONJ, the exact pathophysiological pathways remain still unclear. In this study we aimed to observe the effects of the low-dose bisphosphonates administration on mandibular bone of rats with and without mini-implants application. For this study we used 28 male Wistar rats, divided in in two groups: 1) control group, treated with saline solution (n = 14 rats); 2) ZA group, treated with low dose zoledronic acid (n=14 rats). After 6 weeks of treatment, the half of each group underwent mini-implant application. All rats were sacrificed and their mandibles were analyzed by light microscopy and scanning electron microscopy (SEM). Our data have shown a healthy bone tissue of control group both with and without mini-implant application. In the treated group empty osteocyte lacunae have been observed and they slightly increase in rats with mini-implant application. Although that, no bone exposition has been observed. By that, during low dose zoledronic acid treatment we can observe the presence of empty osteocytes lacunae, also called "primary lesion", that not seem to be sufficient alone to determine osteonecrosis of the jaw spontaneously or even after mini-implant application.

Keywords: mandibular bone, osteonecrosis, bisphosphonate, mini-implant, osteocytes lacunae.

1. INTRODUCTION

Bisphosphonates (BPs) is a category of drugs that are widely used in various pathological conditions such as hypercalcemia associated with malignancy, Paget's disease, Gorham-Stout syndrome, lytic bone metastasis, breast cancer and for treating osteoporosis [1-9]. This category of drugs plays a role

in regulating bone cells behavior acting directly on bone remodeling. On the basis of their chemical properties are divided into two groups: no nitrogen-containing and nitrogen-containing BPs [10-13]. The non-nitrogen group, such as etidronate and clodronate, is mainly used for metabolic disorders. The nitrogen-containing BPs, such as zoledronate (ZA) and pamidronate, are the gold standard for neoplastic bone diseases [14].

Although the use of BPs determines positive effects in various pathological conditions, their use could be associated to high risk of jaw or maxillary bone necrosis; more often it could involve in osteonecrosis of the jaw (ONJ). The ONJ in a very low percentage of cases could occur spontaneously while in the most cases it is triggered by certain factors such as the use of zoledronic acid [15], the cumulative dose of bisphosphonates, dentoalveolar surgery [16-19] or trauma [20]. According to Advisory Task Force on Bisphosphonate-Related Osteonecrosis of the Jaws (BRONJ), osteonecrosis is commonly described as exposed jaw bone condition without heal within 8 weeks after identification in patients undergoing treatment with BPs and without irradiation therapy to the craniofacial region [21].

Despite the BRONJ is a severe condition, especially in cancer patients, the exact pathophysiological mechanisms are still unknown; for these reasons, it is necessary to understand what the mechanisms are underlying the BRONJ to choose the best preventions and /or curation therapy.

The most discussed hypothesis about the pathophysiological mechanisms of the BRONJ during BPs treatment considers severe the suppression of bone turnover that in turn lead to necrotic bone, depending on the drug type, dosage and other trigger events as trauma application [22-28]. Our previous studies performed on animal model have shown that in rats treated with low dose bisphosphonates (zoledronic acid), the first signs that could lead to bone death, such as empty osteocytes lacunae, appear only after 30 days of treatment and we did not observe spontaneous bone exposure [28,29]. For this reason, we have hypothesized that the presence of empty lacunae may be a predisposing condition to the death of the bone just in case of a trauma application. On this basis, we have repeated the experiment on animal model to observe the low-dose bisphosphonates effects on bone in which a mini-implant has been inserted, comparing it to rats treated with BPs without dental implants, to understand if the presence of empty osteocytes lacune during low dose zoledronate treatment could represent an accelerator of necrotic lesion in case of trauma application.

2. MATERIALS AND METHODS

2.1. Animals and inoculation

In our experiment we used 28 male Wistar rats (each weighing 250g. And aged 7 weeks) coming from Janvier Labs (Saint Berhevin, France). All the rats were placed in individual cages, where they had food and water ad libitum for the duration of the experimental phase. The protocol required the rats to be kept at a constant temperature (22 °), under a 12-h light /dark cycle. The protocol complied with the guidelines of the European Parliament and of the Council on the protection of animals used for scientific purposes, ARRIVE (Animal Research: Reporting of In Vivo Experiments) [30] and Directive 2010/63 / EU. The protocols have been authorized by the Ethics Committee of the University of Messina. Two groups of rats have been tested: 1) control group, treated with saline solution injection, (n = 14); 2) ZA group treated with zoledronic acid (ZA), (n = 14). The ZA group was treated with 0.1 mg / kg zoledronate via intraperitoneal injection, three times a week for 6 weeks, while the control group have been treated with saline solution. The methods and timing of the experimental phase followed the designs of previous studies [29]. After a week from the end of BP and saline solution treatment, 7 rats for each group underwent to mini-implants application in two sides of mandible by a surgical protocol that will be described below while the other 7 rats for each group didn't undergo to mini-implant application. After 4 weeks from the surgical positioning procedure, all rats (with and without mini-implant) were euthanized under general anesthesia and were perfused with 4% paraformaldehyde (Sigma-Aldrich) and each mandible has been divided into two parts, one part direct for histological analyses and the second part for SEM [31-34].

2.2. Mini-implant Positioning Technique

The placement of the mini-implants was performed on the rat under intraperitoneal general anesthesia (Ketamine 100 mg/kg, Atropine 0.4 mg/kg, Diazepam 8 mg/kg) simulating the surgical procedures applied for human. A mucosa anesthesia was performed in the posterior mandibular region using as local anesthetics articaine with adrenaline diluted at 2%. The inoculums were performed in the periosteal region using an insulin syringe with a 30gauge needle. A mucotomy was performed using a blade 11, the bone was mixed at a speed of 1100 rpm using a 1.2 mm diameter drill bit, titanium mini-implant (1.5-mm diameter and 3-mm length) was positioned manually using a dedicated screwdriver.

2.3. Histological preparation

Bone samples were post-fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 4 hours and then rinsed in 13 M phosphate buffer (pH 7.3); subsequently, they have been decalcified in ethylenediaminetetraacetic acid (pH 7.2; Hach Company, Loveland, CO, USA) for 30 days, dehydrated in ethanol (from 50% to 100% alcohol) and embedded in paraffin. Paraffin block were cut in 8 um sections with the Leica RM2255 microtome (Leica Microsystems GmbH). After coating the sections into lysinated slides, hematoxylin and eosin staining has been performed at room temperature (H&E; Abbey Color, Philadelphia, PA, USA) [35-38].

2.4. SEM analysis

Bone samples were post-fixed with 2% glutaraldehyde (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in 0.1 M phosphate buffer (Sigma-Aldrich) and then they were dehydrated with ethanol and amyl acetate. After critical point dryer process (Leica Microsystems GmbH, Wetzlar, Germany), the fractured surfaces were coated with a Plasma Sciences CrC-100 Turbo-Pumped sputtering system and observed using a Phenom G2 Pro scanning electron microscope (Phenom-World B.V., Eindhoven, The Netherlands).

2.5. Statistical analysis

We have observed 100 lacunae for ZA group and 100 for ZA group with mini-implant. We observed 10 lacunae taken from 10 random microscopic fields at 20x magnification and we counted how many empty lacunae there were using ImageJ software. After that, we calculated the mean of empty osteocytes lacunae for ZA-group and ZA- group with mini-implant application. The means were compared by T-student test to evaluated the possible statistically significant differences. The means were also turned in a percentage shown in a pie-chart.

3. RESULTS

3.1. H&E staining

The mandibular bones of control group (saline solution injections) showed the characteristics of the healthy bone. This compact bone was characterized by the presence of full osteocytes lacunae with osteocytes that occupy the entire space within the lacunae and full haversian and Volkman's canals (Fig. 1A). The mandibular bone of control group (saline solution injection) after mini-implant applications shows a healthy bone tissue with full lacunae and canals with the same characteristics of the bone of control group without mini-

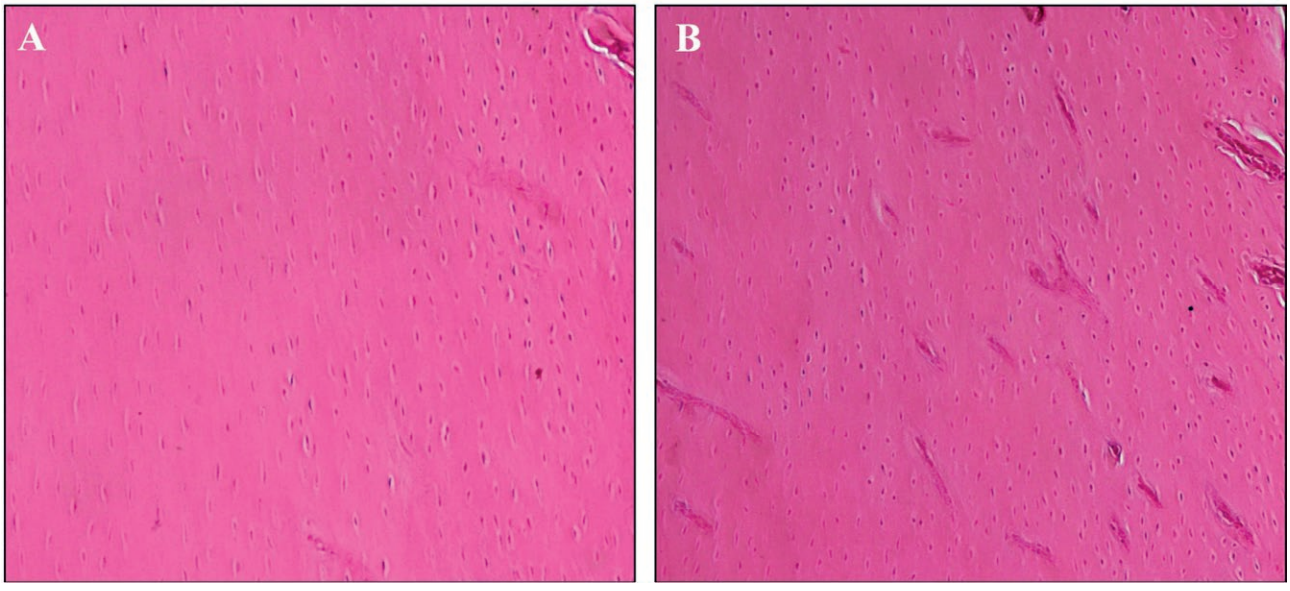

Figure 1. Hematoxylin-eosin staining of bone tissue from rats treated with saline solution (control group) without (A) and with mini-implant application(B). In both cases it is possible to observe a healthy tissue with full osteocytes lacunae and osteocytes that occupy most of the space within the lacunae. No empty osteocyte lacunae and no signs of inflammation have been observed.

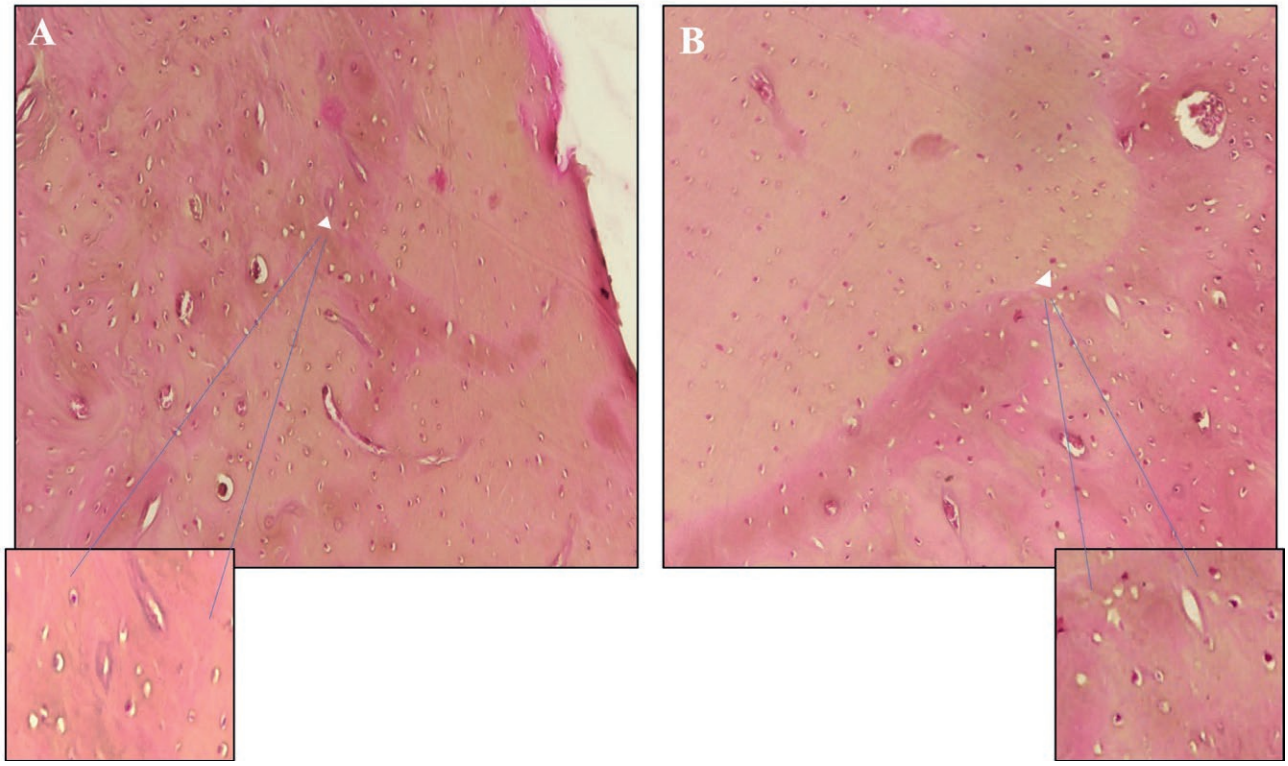

Figure 2. Hematoxylin-eosin staining of bone tissues from rats treated for 6 weeks with zoledronate (0,1 mg/Kg) (ZA group) without (A) and with (B) mini-implant application. The bone of rats without mini-implant application shows the presence of some empty osteocytes lacunae (A, head arrow); in several region of the bone tissue the osteocytes seem to be small and they don't occupy the entire space within the lacunae (A). The bone of rats with mini-implant application shows an increased number of empty osteocytes lacune (B, head arrow). Zoom of empty osteocytes lacunae in the corners of A and B pictures.

implant application; no evidence of inflammatory processes has been observed indicating a total recovery of structural features after 4 weeks of trauma application (Fig. 1B).

In the mandibular bones of rats at 6 weeks of treatment (ZA group), some empty osteocytes lacunae were observed among full osteocytes lacunae where the osteocytes don't occupy the entire space within the lacunae (Fig. 2A).

The ZA group with mini-implants, inserted into the mandibular bone cortex, exhibited a slight increase of empty osteocytes lacunae among full osteocytes lacunae where the osteocytes don't occupy the entire space within the lacunae (Fig. 2B).

3.2. SEM Images

In biopsies of the mandibular bones of control group, the observation of the fracture surface showed the physiological presence of osteocytes lacunae, Haversian systems and Volkmann canals (Fig. 3A).

The bone of control group after mini-implant application showed a full recovered tissue with full lacunae and canals and no evidence of inflammatory processes (Fig. 3B).

In rats group treated with BPs for 6 weeks (ZA group), the fracture surface of biopsy bone fragments frequently showed areas with irregularly spherical or elongated morphology. Moreover, empty osteocytes lacunae were detected (Fig. 4A).

In ZA group with implants placement, an alteration of the morphological structure is visible and empty osteocytes lacunae increased, compared to the mandibular bone of ZA group without mini-implants insertion (Fig. 4B).

3.3. Statistical analysis

Results shows that for every 10 osteocytes lacunae observed we find a mean of 2 and 2,6 of empty osteocytes lacunae, for ZA group and ZA group with mini-implant respectively. The T-student test showed a no statistically significant differences between the ZA without

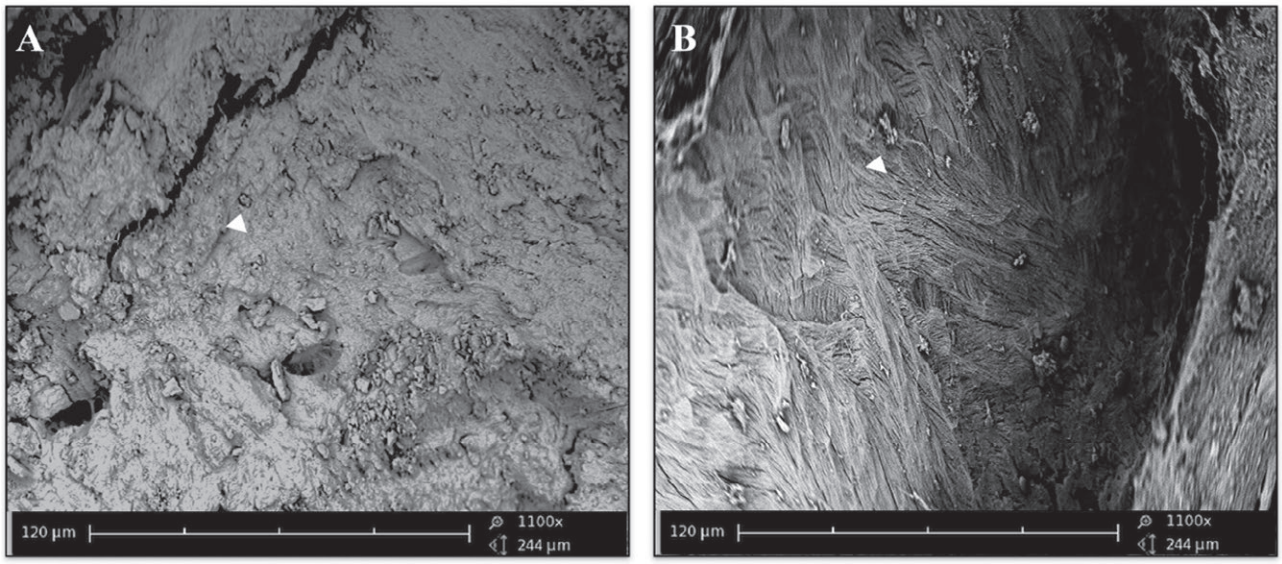

Figure 3. Scanning electron microscopy pictures of bone tissue from rats treated with saline solution (control group) without (A) and with mini-implant application(B). In both cases it is possible to observe a healthy tissue with full osteocytes lacunae and osteocytes that occupy most of the space within the lacunae. No empty osteocyte lacunae and no signs of inflammation have been observed.

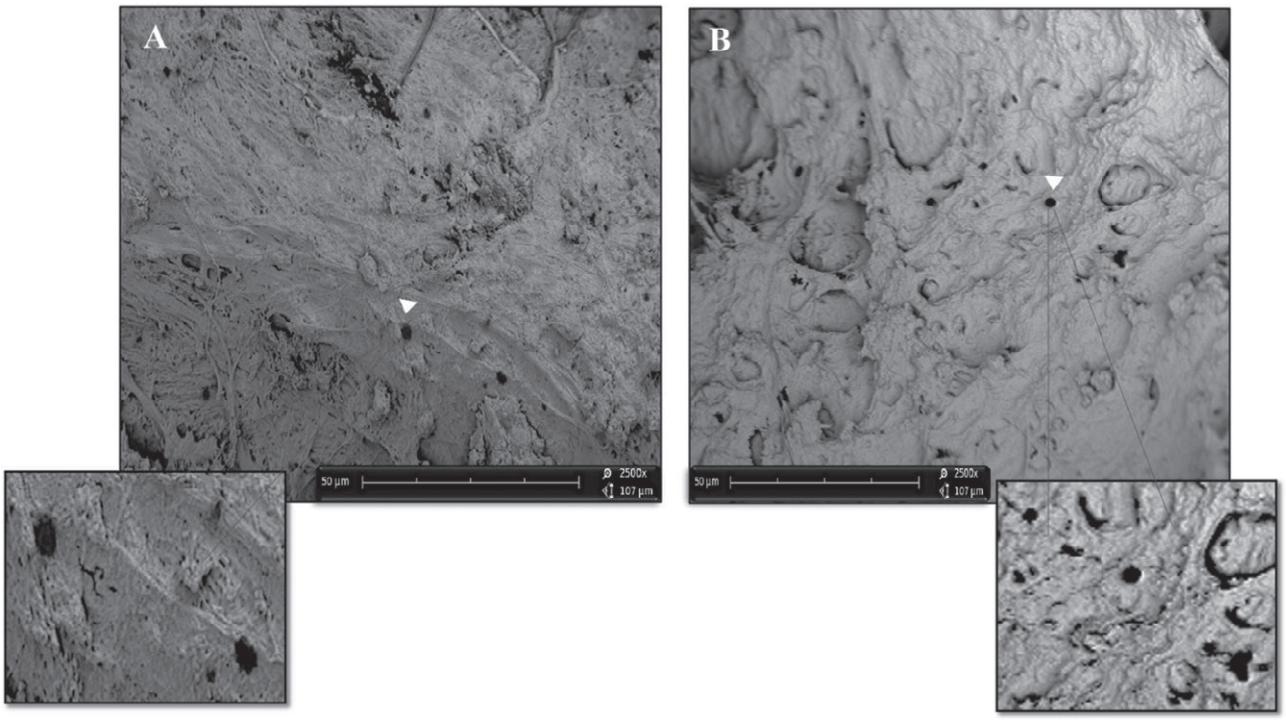

Figure 4. Scanning electron microscopy pictures of bone tissues from rats treated with zoledronate (0,1 mg/Kg) (ZA group) without (A) and with (B) mini-implant application. The bone of rats without mini-implant application shows the presence of some empty osteocytes lacunae (head arrow) (A). The bone of rats with mini-implant application shows an increased number of empty osteocytes lacune as evidenced by head arrow (B). Zoom of empty osteocytes lacunae in the corners of A and B pictures.

ZA-Group	M	DS
ZA-Group without mini implant.	2	1,333333
ZA-Implant-Group	2,6	0,966092

Figure 5. Table that shows the means of empty osteocytes lacunae that we found in 10 microscopic fields both for ZA group without mini-implant application and ZA group with mini-implant application. M= mean; DS= standard deviation. P>0,05.

and ZA with mini-implant application groups. We have turned the means in a percentage shown in a pie-chart (Fig. 5).

4. DISCUSSION

BRONJ is a severe condition that could occur in patients treated with bisphosphonates spontaneously or after trigger events; in fact, tooth extractions and dentoalveolar surgical procedures, such as positioning of dental implants, are represented as precipitating or trigger events for the manifestation of BRONJ in subjects receiving BPs and other antiresorptive drugs [39]. Although several studies have been performed on this severe condition, the etiopathology of BRONJ still remains undefined. BPs are known to have an inhibitory action on bone turnover after surgical procedures [40]. In literature, it was observed that the incidence of BRONJ depends on several factors (the method of administration, the BPs, dose, etc.). Other factors as periodontal diseases and local infection are closely linked to BRONJ [41]. Several authors have observed that the cases of BRONJ increase in patients whose route of administration is intravenous and oral. [42-43]. A study by Henry et al. [44] has shown how the duration of treatment with BPs can lead to an adverse event. Despite that, it is difficult to detect early structural alteration that could lead to necrosis in human. For these reasons, the use of an experimental model seems to be necessary to better understand the structural and molecular mechanism involved in BRONJ. Our previous studies, conducted on bone and gingival tissue samples taken surgically in the perilesional area, showed an alteration of the structural architecture, with the presence of empty lacunae, absence of matrix and disorganization of the fibrillar component. Our results confirm the presence of empty osteocyte lacunae in samples treated with bisphosphonates without mini-implant, and show that in the samples with mini-implant, the number of empty osteocyte lacunae increases slightly [27,28]. The present study has been performed on animal model to evaluate the modification that occur during zoledronic acid treatment with and without the application of trauma such as mini-implant insertion. For this reason, we studied two groups of rats: control group, treated with saline solution and ZA group, treated with zoledronic acid; the half of each group underwent mini-implant application. Our results obtained by Histological and SEM examinations showed that the saline solution injection doesn't causes structural alteration of bone tissue both with and without mini-implant applications. In ZA group, bone tissue structural alterations characterized by the presence of empty osteocyte lacunae have been observed. Moreover, we haven't observed a statistically significant difference in empty osteocytes lacunae between ZA group without and ZA group with mini-implant. No cases of bone exposure have been observed. These results are in accordance with our previous study performed on rats

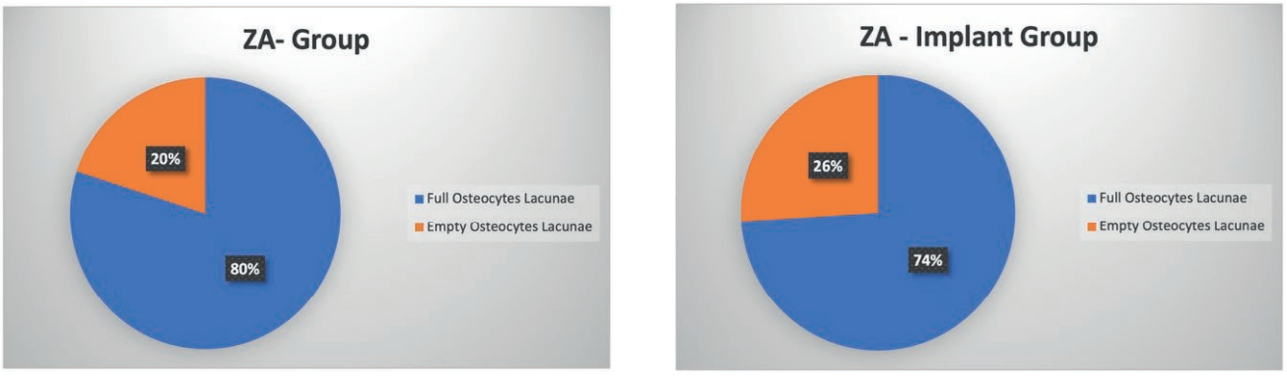

Figure 6. Pie chart showing the percentage of empty and full osteocytes lacunae in bone tissue from rats treated with BPs (ZA group) without (A) and with (B) mini-implant application.

treated with Zoledronate for 45 days without trauma application [29] demonstrating that the mandibular bone at 7 and 15 days of treatment exhibited similar features to those of healthy bone while after 30 and 45 days of treatment a structural alteration in large surface areas of the bone with empty osteocytes lacunae and a marked reduction in the number of osteoblasts and osteoclasts were observed with no cases of bone exposition. Moreover, the present data are also in accordance with Huja et al. [45] that administered a ZA dose of 0.1 mg/kg intraperitoneally without performing extractions and obtained 0% of osteonecrotic lesions and Kobayashi et al. [46] which showed no signs of osteonecrosis (0%) in the jaw bones of experimental animals treated with a ZA dose of 0.250 mg/kg for subcutaneous administration and with extractions. Conversely, Senel et al. [47] detected 20% of ONJ lesions with the same dosage of ZA but in combination with dental extractions and Biasotto et al. [48] showed 100% of ONJ lesions at a ZA dose of 0.04 mg/Kg for intravenous administration; Maahs et al [49] found 80% of osteonecrotic lesions at a ZA dose of 0.6 mg/kg for intraperitoneal administration. The present work shows at morphological level the early bone structural modification, such as empty osteocytes lacunae, that could be involved in BRONJ. Although that, our data support that the existence of these histological features during low dose zoledronic acid treatment is not sufficient to determine osteonecrosis of the jaw spontaneously or even after mini-implant application. By that, the presence of empty osteocytes lacunae could represent the first morphological events, also called "primary lesion" from which, together with other trigger events and/or individual variables, osteonecrosis can occur. It will be interesting to perform future studies to understand how the "primary lesion" can evolve during high dose zoledronic acid treatment.

5. CONCLUSION

Our experimental data obtained by low dose zoledronic acid treatment on rat show "primary lesions" that not lead to osteonecrosis spontaneously or after mini-implant application. Hence, BRONJ should not only be related to a unique factor, but the combination of more factors and more variables would make patients more susceptible to BRONJ. In fact, the bone necrosis and exposure could also depend on long term BP treatment, individual variables or on concomitant oral cavity pathology. That seem to support the possibility of oral surgical intervention in those patients treated with low doses of zoledronic acid.

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