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Original Article:

Stereological Measurement of Histopathological Changes in Subcomponents of Mandibular Bone Trabeculae after Mechanical Trauma in Zoledronate-induced Rats

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Abstract

Introduction: Stereological methods to measure structural changes play an important role for diagnosing and evaluating the healing process of diseases in organs. Zoledronate, used to treat bone diseases, causes osteonecrosis of the jaw following mechanical injuries and is a challenge in clinical settings. This experiment aimed to investigate histopathological changes in subcomponents of mandibular bone trabeculae after mechanical trauma in zoledronate-induced rats by using stereological methods.

Materials and Methods: Fifty female rats (220-270g) were randomly assigned to experimental and control groups. 25 rats in the experimental group were injected 0.06 mg/kg zoledronic acid (ZA) intraperitoneally, and 25 animals in the control group were injected saline. Injection was performed once a week for 8 weeks, and then dental extraction was done for each animal. Four weeks after extraction, the rats were euthanized and the right hemimandibles were removed, decalcified, cut into serial sections at 5 microns, stained with hematoxylin-eosin, and subjected to stereological analyses. Data were analyzed by using SPSS version 26.

Results: The volume of bone matrix and empty lacunae was significantly higher in experimental rats than that in controls four weeks after extraction ($P < 0.05$). Reduction of the volume of osteocytes, osteoblasts, osteoclasts, and Howship's lacunae in experimental group was observed when compared with the controls ($P < 0.05$).

Conclusion: The stereological methods in the present study provided a standardized and reproducible methodology for quantitative analysis of histopathological changes and can be used as a basis for future studies investigating preventive or therapeutic strategies for Medication-Related Osteonecrosis of the Jaw (MRONJ) that are evidence-based and associated with accurate and valid data.

Keywords: Bisphosphonates, Osteonecrosis, Mandible, Rats

1. Introduction

Zoledronic acid is a drug used for treatment of osteoporosis and malignant bone metastases. Despite their therapeutic benefits, a potentially severe complication of large-dose, long-term bisphosphonate (BPs) treatment is osteonecrosis of the jaw in patients consuming them [1, 2].

Bisphosphonate-related osteonecrosis of the jaws (BRONJ) was first reported in 2003 by Marx, and the reports of cases of this disease are increasing [3].

Most cases have occurred following invasive dental interventions, for example tooth extractions, and a few

have occurred spontaneously, largely after zoledronic acid administration [2, 4].

Further studies have shown that other drugs, such as denosumab and various anti-angiogenic drugs, may cause this condition. Therefore, a broader name was assigned to that: medication-related osteonecrosis of the jaw (MRONJ) [5].

The diagnosis of this disease is based on the history of bisphosphonate consumption and the occurrence of symptoms such as necrosis of uncovered bone in the mouth for eight weeks, without any radiation to the craniofacial area [6, 7].

Although more than two decades have passed since the first report of MRONJ, its exact pathology is still unknown, and treatment of it in clinical situations remains a challenging issue [8].

Additionally, as our population ages, reliance on antiresorptive medications and concern about their side effects appear to increase.

Given that the low prevalence of MRONJ prohibits design for clinical trials, the application of animal models for preclinical studies is needed to investigate the pathology and potential treatment or prevention of MRONJ [9].

Some clinical and semiquantitative histopathological studies on MRONJ have been previously performed in rats, but there are no stereological studies [10-12].

Stereology refers to the quantitative analysis of three-dimensional objects based on their two-dimensional appearance on histological, MRI, CT or ultrasound section images. This knowledge has a special place in experimental and clinical pathology and histology and has opened a new window in the diagnosis and treatment of diseases.

Determining volume is an important criteria in understanding the microstructures of the organs, and this criterion has been considered in many diseases.

Determining the volume of organs has been done with various methods. Nowadays, various stereological methods have been introduced to determine the volume of the desired organ structures, among which the Cavalier method has been introduced as the golden standard method. The reason is that the stereological error in this method is less and there is no presupposition about the shape, size and position of the investigated organ [13-15].

Even though it is feasible to conduct volumetric evaluations based on DXA BMD measurement, FRAX adjusted with TBS, 3D CT and MRI, it should be noted that these techniques are currently limited to static assessments only. In contrast, appropriately prepared histological sections can be used for either static or dynamic assessments [16].

Objectives

Therefore, the present study was done to measure the volume of bone matrix, osteocytic lacunae, osteocytes, osteoblasts, osteoclasts and howship's lacunae in the mandibular bone trabeculae after mechanical trauma in zoledronate induced rats using stereological approaches.

2. Materials and Methods

Experimental animals

For this study, 50 female Sprague-Dawley rats (220-270 g) were provided and were maintained under standard laboratory conditions, including a 12-hour light/dark cycle with controlled temperature (22◦C ± 2◦C). Laboratory food and tap water were freely available.

Experimental design

The zoledronic acid regimen was designed based on the recommendations of Zandi et al. (2016) to induce MRONJ in rats [17]. 10 days after acclimation to the laboratory conditions, the rats were weighed, numbered, and randomly separated into experimental (ZA-treated) and control groups. The experimental group (n=25) was intraperitoneally injected with 0.06 mg/kg ZA once a week. The control group (n=25) was intraperitoneally administered with equivalent 0.9% normal saline once a week. Body weight was recorded before the administration of the solution to regulate the dose for each animal. Eight weeks after administration, dental extraction was done for each animal under general anesthesia. The rats continued to receive treatment after extraction. Four weeks after surgery, the animals were euthanized for removal of the right hemimandible of each animal Figure 1.

Surgical procedure

Anesthesia was administered by intraperitoneal injections of ketamine (75 mg/kg), and midazolam (7.5 mg/kg). Then animals were placed in a hand-made unit, especially for dental extraction. Adequate access and visualization of the surgical field were achieved using the dental unit of experimental rat. surgery was done according to the technique suggested by Moghadam et al. [18]. Immediately after the surgery, the tooth extraction sockets were sutured.

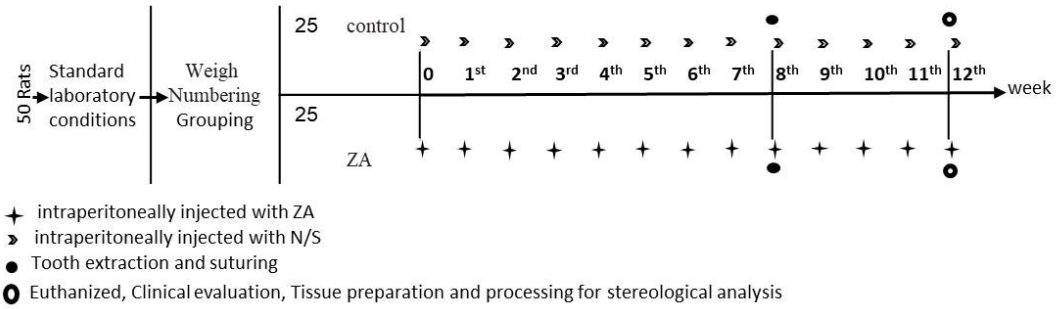

Figure. 1. Outline of applications, surgical procedure, and euthanasia, related to time

Clinical evaluation

Four weeks after surgery, clinical evaluation in each animal was done for unhealed gingiva, exposed necrotic bone, and intraoral or extraoral fistulae.

Tissue preparation and processing for stereological analysis

The right hemimandible of each animal was dissected, cleaned and weighed. The samples obtained in the present study were fixed in formalin 10% for two days and decalcified with EDTA (10%) for two months. EDTA was changed twice a week and demineralization was confirmed by physical tests. After tissue processing, demineralized samples were embedded in a paraffin block. Sections of 5-µm (for estimation of volume) in thickness were cut buccolingually by microtome. One section of each 25 sections was mounted on the microscope slides by systematic random sampling technique. Choosing the first section was done randomly [14, 19, 20]. After staining sections with hematoxylin and eosin, photographs were taken under the light microscope. Stereological analyses were performed on these micrographs.

Estimation of volume of subcomponents of mandibular bone trabeculae

A point counting grid consisting of crosses with an area (1·61 cm2) was randomly placed on the photographs of the tissue sections. The number of crossings hitting total section of the mandible was calculated. Estimation of total volume of mandible was done with this formula: Eq. (1): $V = T.a/p.\sum P$ (1)

Where "T" was the cross-sectional thickness of successive transects, "a/p" was the area per point, and "∑P" was the sum of all points hitting the transects [14].

The volume fraction of the sub-components of mandibular bone trabeculae was used to express the proportion of a sub-component in the whole structure (mandible) using the following formula Eq. (2):

$$Vv\,(X,Y) = (Volume\ of\ X\ phase\ in\ Y\ reference\ space)/(Volume\ of\ Y\ reference\ space) \quad (2)$$

Where "Vv (X, Y)" represents the volume fraction of phase "X" in the reference volume "Y".

Based on the above information, the same points counted to estimate the volume of the mandible are used to calculate the volume fraction of subcomponents in the mandibular tissue using the following formula Eq. (3):

$$Vv\,(Structure, total\ mandible) = (\sum P\ Structure)/(\sum P\ total\ mandible) \quad (3)$$

where "∑P structure" was the number of points placed over on the profiles of the bony trabeculae, bone matrix, osteocytic lacunae, osteocytes, osteoblasts, osteoclasts and howship's lacunae and "∑P total mandible" was the number of points located on the complete mandible.

The total volume of the subcomponents was achieved by multiplying the volume fraction by the whole volume of the mandible Eq. (4) [21].

$$V(structure) = Vv\,(structure).V\,(total\ mandible) \quad (4)$$

Calculation of Coefficient of error (CE)

Gundersen and Jensen formula was applied to calculate coefficient of error [13].

Statistical analyses

Statistical analyses were done using SPSS version 26. To assess normal distribution, Kolmogorov–Smirnov test was performed. For those that followed a normal distribution, the difference between two groups was analyzed using the independent t-test. For those that did not pass the test, Mann–Whitney U test was done for assessment of difference between two groups. Levene's test was used to evaluate the homogeneity of variance.

Data were presented as mean ± standard deviation. P<0.05 was statistically significant.

3. Results

Clinical findings

As shown in Figure 2, four weeks after extraction, intraoral examination of the animals in control group exhibited complete mucosal covering at extraction site with no inflammation and discoloration (Figure 2B). In contrast, the experimental animals presented unhealed mucosa and necrotic bone was exposed in extraction sites (Figure 2C). In some experimental rats, an extraoral fistula was found at the submental region (Figure 2E).

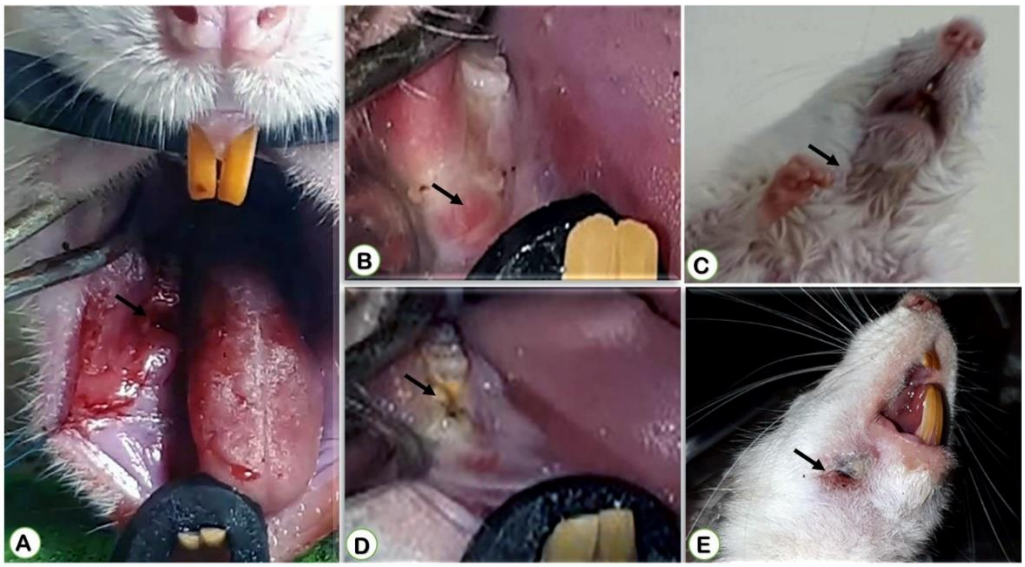

Figure 2. Intraoral and extraoral photographs extraction sites in control and experimental rats: Image A shows the dental socket in the representative rat immediately after surgery, indicated by the black arrow. Image B presents complete soft tissue healing of the extraction site in a representative rat from the control group, versus exposure of necrotic bone with no soft tissue healing in zoledronic acid–treated (experimental) group (Image C), four weeks after surgery showed by the black arrows. Image D exhibits a representative rat from the control group with healthy submental region versus osteonecrosis extending to the submental region through an extraoral fistula in the ZA-treated group (Image E), four weeks after surgery indicated by the black arrows.

Histopathological findings

Four weeks after tooth extraction, microscopic evaluation of tissue sections of mandibles stained with H&E in the control group showed no bone necrosis, typical osteocytes within lacunae, and vascularity in marrow cavities. Osteoclasts were seen as cells containing several nuclei located on bone surfaces and osteoclastic activity generating large resorption lacunae (Howship's lacunae). Furthermore, most of osteoblast-related cells were located on bone surfaces.

In contrast, microscopic presentation of the right mandibles in the experimental group was characterized by necrotic bony trabeculae demonstrating empty osteocytic lacunae or lacunae containing apoptotic osteocytes and absence of vascularity in bone interstices. The necrotic bone showed abnormal shallow peripheral resorption lacunae. The absence of osteoblasts or apoptotic osteoblasts were found in bone surfaces Figure. 3.

Stereological findings

As shown in Figure. 4 and Figure. 5, the mean volume (mm^3±SD) of osteoblasts, osteocytes, osteoclasts and Howship's lacunae in ZA-treated animals was significantly decreased (0.17±0.05, 0.98±0.12, 0.06±0.03, and 0.03±0.01, respectively) in comparison with the control animals (0.30±0.06, 2.9±0.35, 0.14±0.04, and 0.08±0.03, respectively) ($p < 0.001$).

In addition, a reduction in the average volume (mm^3± standard deviation) of bony trabeculae of hemimandible, osteocytic lacunae and bone matrix were observed in animals treated with ZA (101±8.9, 3.9±0.4, and 96±8.9, respectively) as compared to control groups (88±5.1, 0.8±0.05, and 83±5.1, respectively) ($p<0.001$). The mean of CE (coefficient of error) was 2.45%.

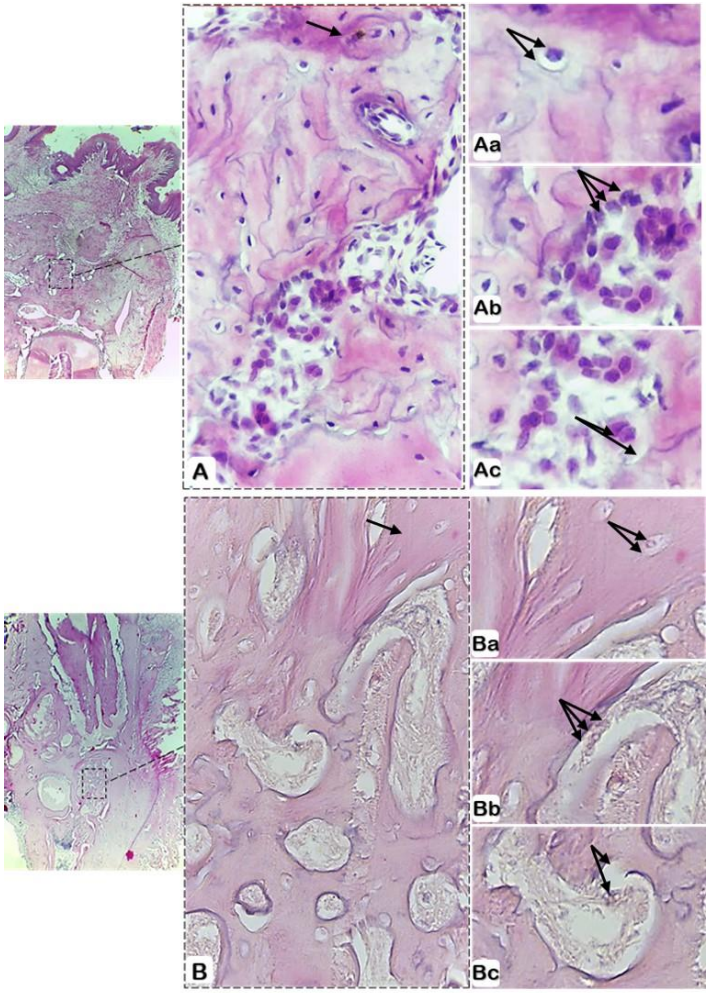

Figure 3. Histopathology of the right hemimandible of control vs. ZA-induced animals, four weeks after dental extraction (×40). A. and B. were magnified (×100) figures of the dashed black boxes. Image A indicates normal healing and vascularity in a control animal. Image B. exhibits that the architecture of the bone was impaired. Necrotic trabeculae with many empty osteocytic lacunae were observed. In addition, lack of vascularity in marrow cavities was seen, in ZA-treated animals. Aa, Ab and Ac were highly magnified (×400) figures of A. the black arrow in Aa meant cellular lacuna and normal osteocyte within lacuna, Ab meant normal osteoblasts and Ac meant multinuclear osteoclast with large resorption lacunae that occurred in control rats.
Ba, Bb and Bc were highly magnified (×400) figures of B. the black arrow in Ba meant osteocytic lacunae with apoptotic osteocyte, Bb meant apoptotic osteoblasts and Bc meant apoptotic osteoclast with irregular shallow resorption lacunae that occurred in rats treated with ZA.

4. Discussion

This study investigated the histopathological changes of the subcomponents of mandibular bone trabeculae after mechanical trauma of the jaws in zoledronic acid treated rats using stereological approaches. The important parameters were estimation of volume of osteoblasts, osteocytes, osteoclasts, osteocytic lacunae, Howship's lacunae and bone matrix.

In this study, zoledronic acid was administered intraperitoneally, based on the protocol developed by Zandi et al. [17]. The dose of ZA was consistent with that used to manage malignancies in humans (4 mg ZA monthly) [22]. Tooth extraction was done as mechanical trauma [23;24].

Although healing of extraction sockets in humans usually lasts 2–3 months, it occurs much more quickly in rats. Therefore, samples from experimental and control rats were collected four weeks post tooth extraction for stereological analyses in our study [17].

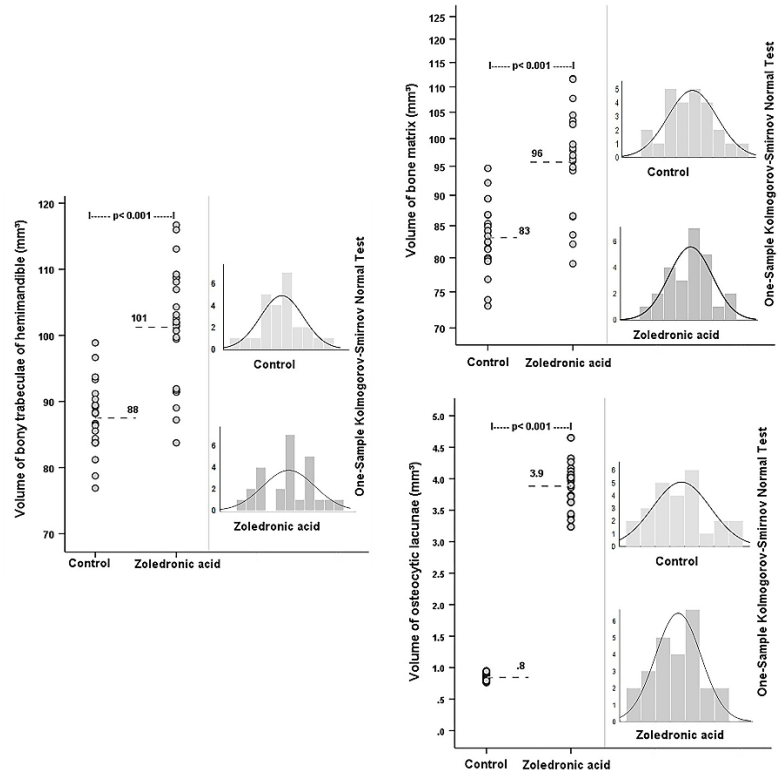

Figure 4. Stereological analyses of the volume of bony trabeculae of the hemimandible, bone matrix, and osteocytic lacunae (mm^3) in control and zoledronate-induced animals, four weeks after surgery

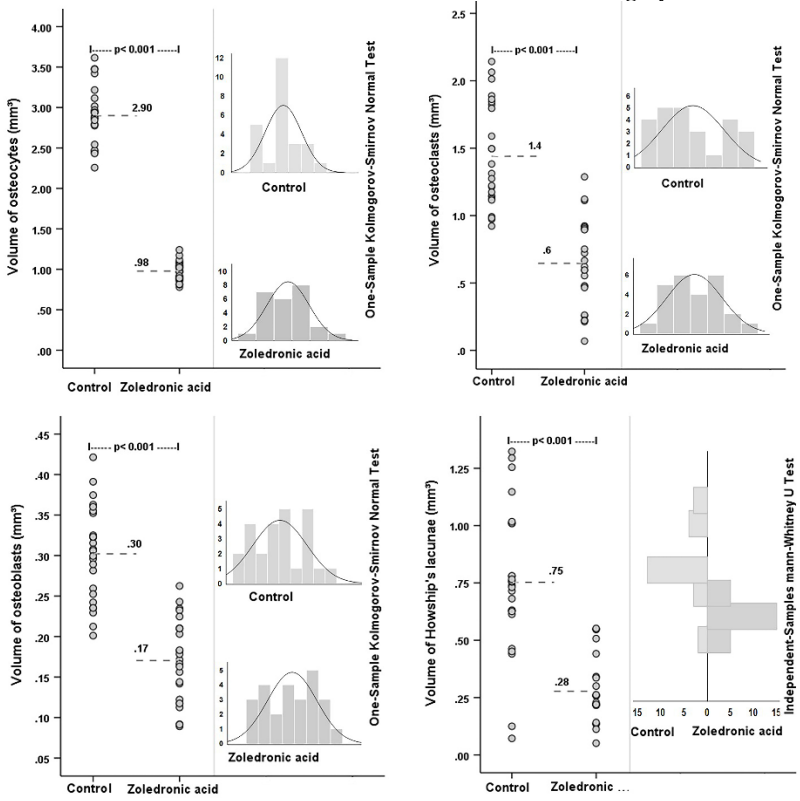

Figure 5. Stereological analyses of the volume of osteocytes, osteoblasts, osteoclasts, and Howship's lacunae (mm^3) in control and zoledronate-induced animals, four weeks after surgery

Four weeks after tooth extraction, a MRONJ-like disease was established in rats and major clinical manifestations of the human disease, including exposed necrotic bone and unhealed gingiva in the extraction site, were observed. In some ZA-treated animals, an extraoral fistula was found in the maxillofacial region. In contrast, in control rats, it was observed that extraction sockets were covered with epithelium completely. Our observations agree with clinical observations in studies [25] [26] [27].

Stereological results in this research established osteonecrosis in zoledronate induced rats.

Our stereological study showed reduction in the volume of osteoblasts in experimental rats compared to the control rats. This result agrees with the research performed by Kavanagh et al., Zara et al., and Manzano-Moreno et al., which reported that ZA inhibits the activity and function of osteoblasts and induces osteoblast apoptosis [28] [29, 30].

Comparing the volume of osteocytes between the ZA-treated groups versus the saline-treated groups demonstrated statistically significant results. A decrease in osteocyte volume was observed in the ZA groups compared to saline groups. Xin Chen et al. and Schwartze et al. showed that tooth extraction is associated with multiple mechanical and thermal injuries leading to osteocyte apoptosis and death [31] [32].

Four weeks post-surgery, the volume of empty osteocytic lacunae in ZA-treated animals was statistically increased in ZA-treated animals compared to controls. This is due to the decrease in the volume of osteocytes that leads to the increase in the volume of spaces without these cells, which are called lacunae. Dodson et al. suggested that BPs target osteocyte lacunae and gain access to osteocytes through the channel system and induce cell death [33].

Four weeks after extraction, ZA-treated rats exhibited decrease in the volume of osteoclasts when compared with control rats. This result agrees with other studies [34, 35] [29] [2] [36, 37].

Kavanagh et al. discovered that BPs exert a strong inhibition on osteoclasts after jaw injury during extraction. BPs directly affect osteoclasts, inhibit osteoclast osteolysis, and induce osteoclast apoptosis by affecting the activity of lymphocyte progenitors. Therefore, the bone turnover of the jaw is inhibited excessively and causes osteonecrosis of the jaw [28].

The volume of Hawship's lacunae decreased in the group treated with ZA compared to controls. This finding agrees with the results of Okawa et al., 2022, who discovered osteoclasts at the surface of alveolar bone forming abnormal shallow bone resorption lacunae in ZA-treated animals. In contrast, in the control groups, osteoclastic activity caused large resorption lacunae (Howship's lacunae) on the bone surface [38].

Four weeks after extraction, the ZA group showed significantly increase in volume of bone matrix compared to the control group. This result is in accordance with researches that reported high doses of ZA increase the thickness of trabecular bone [39] [40].

In this work, the average CE was 2.45%, which points to the acceptable sectioning and density of points in the grid [13, 41-43].

Stereological analyses of the present study were limited to assessment of volumetric amounts of osteoblasts, osteocytes, osteoclasts, osteocytic lacunae, howship's lacunae and bone matrix. We suggest that the number of osteoblasts, osteocytes, osteoclasts, the length of blood vessels and resorption bone surfaces were estimated.

5. Conclusion

It seems that histopathological changes after mechanical trauma in zoledronate-induced rats are linked with volumetric changes. The unbiased stereological method in the present study provides a standardized and reproducible methodology for volumetric analysis of histopathological changes of subcomponents of mandibular bone trabeculae in a rat model. Furthermore, these results can be used as an experimental basis for the future studies to develop preventive or therapeutic strategies for MRONJ.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Qom University of Medical Sciences (IR.MUQ.REC.1400.110).

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Author's contributions

All authors equally contributed to this research.

Conflict of interest

The authors have no conflicts of interest.

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