Research article

Extent of surgical trauma may not be a key factor in Medication-related osteonecrosis of the jaw – a pilot study

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Abstract

The pathogenesis of Medication-Related Osteonecrosis of the Jaw (MRONJ) is not fully understood, however, surgical trauma is thought to play a role. Therefore, the aim of the current pilot study was to compare the incidence and characteristics of MRONJ following single or multiple molar tooth extractions in a rat model. To this aim, twenty male Lewis rats were treated with subcutaneous injection of zolendronic acid (ZA), an established bone anti-resorption agent, (7.5 μg/kg) and dexamethasone (Dex), (1 mg/kg), or saline, once a week, for 11 weeks. At three weeks, the first or both first and second maxillary molar teeth were extracted. Eight weeks following extraction, rats were sacrificed and extraction sites were evaluated. Clinical macroscopic examination showed MRONJ-like lesions in all single extraction ZA/Dex-treated rats, showing exposed bone. In the control and multiple extraction ZA/Dex-treated groups, none of the rats showed visible signs of MRONJ. Histological characteristics of MRONJ were found in all ZA/Dex-treated rats (both single and multiple extractions), whereas rats treated with saline showed almost no empty lacunae and necrotic bone. In conclusion, the extent of the surgical field may not be the key factor in MRONJ development since only rats with single tooth extraction displayed exposed bone. However, histological characteristics were identified in both models. Therefore, preclinical studies that aim to evaluate histological features of MRONJ may use both models, whereas when a clinically exposed bone is required, the single tooth extraction model appears to be preferable. Further large scale studies are warranted to corroborate the present findings.

Keywords

Osteonecrosis; Histopathology; Pathogenesis; Wound healing

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1. Introduction

Bisphosphonates (BSP) are used clinically to manage cancer-related conditions including hypercalcemia of malignancy, skeletal-related events associated with bone metastases in the context of solid tumors including breast cancer, prostate cancer and lung cancers as well as for the management of multiple myeloma [1]. Furthermore, BSP are widely used for the treatment of osteoporosis and other metabolic disorders by increasing bone mineral density, decreasing fracture risk, and inhibiting bone resorption [2].

BSP-related osteonecrosis of the jaw (BRONJ) which was first described by Marx in 2003, is a serious adverse effect of BSP therapy [3]. Most BRONJ cancer patients were treated with concurrent I.V. medication of nitrogen containing BSP (such as zoledronate and pamidronate) and steroids (dexamethasone) [4]. Recently, with the discovery of osteonecrosis of the jaw in patients taking Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL) antibody and Vascular endothelial growth factor (VEGF) antagonists, the definition of BRONJ has been modified to Medication-Related Osteonecrosis of the Jaw (MRONJ) [1]. Although rare, MRONJ is a debilitating disorder that is usually associated with pain, bone sequestration, tooth loss, intraoral and extraoral fistulae, and jaw fracture [3, 5].

The prevalence of MRONJ ranges from 0.7-18.6% among cancer patients [1, 6], 0.017-0.04% in patients with I.V. BSP therapy [7] and 0.00038-0.21% in patients receiving long-term oral BSP therapy [8]. In recent years, the incidence of MRONJ has decreased owing to early screening and initiation of appropriate dental care [1]. The American Society for Bone and Mineral Research task force considers that systemic risk factors associated with chemotherapy affect the occurrence and aggravation of MRONJ, and concurrent use of chemotherapeutic regimens and steroids have a synergistic effect on MRONJ [1].

Although the first MRONJ case was reported over a decade ago, the pathophysiology of the disease has not been fully elucidated. However, several known risk factors were reported such as age, periodontal disease, smoking, diabetes, steroid therapy and immunosuppression [1]. Several hypotheses were proposed in an attempt to explain the confined localization of MRONJ exclusively to the jaws. These include altered bone remodeling or over-suppression of bone resorption [9–11], inhibition of angiogenesis [12], constant

micro-trauma; suppression of innate or acquired immunity; vitamin D deficiency [13]; soft tissue BSP toxicity [14]; dental disease or bacterial infection alone [15, 16] or in combination with fungal and viral infections [17, 18]. Dentoalveolar surgery, especially tooth extraction, is considered a major risk factor for developing MRONJ. Several studies report that among patients with MRONJ, tooth extraction is a common predisposing event (52-61% of patients with ONJ underwent tooth extraction) [19].Owing to the development of MRONJ in patients with multiple confounding factors, it is very difficult to identify the underlying pathogenesis determinants of the disease. Therefore, it is imperative to develop animal models with a high incidence of MRONJ with minimal environmental and genetic variance.

In a recent study, Jang et al., found that the combination of zolendronic (ZA) acid and dexamethasone (Dex) increased the occurrence of MRONJ in a rat model, and that a surgical stimulus, such as extraction, plays an important role as a trigger factor, increasing the incidence of MRONJ [4].Thus, we hypothesized that by increasing the surgical stimulus (i.e. extraction of two adjacent molar teeth compared with a single tooth extraction) would increase the prevalence of MRONJ. The aim of the current pilot study was to compare the incidence of MRONJ and characteristics following single or multiple tooth extractions in a rat model. The results of this study could enable further investigations in the field of MRONJ.

2. Materials and Methods

The study was performed in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures, or with the European Communities Council Directive of 24 November 1986 (86/609/ EEC), and in accordance with the ARRIVE guidelines and with local laws and regulations. The study protocol was approved by the Committee for the Supervision of Animal Experiments at the Faculty of Medicine, Technion, I.I.T. (approval # IL0580514).

2.1. Establishment of a MRONJ model in Lewis rats

In order to increase the prevalence of MRONJ, combined treatment with both ZA and Dex in addition to molar extraction was used to induce MRONJ [4]. Male Lewis inbred rats ($n = 20$, 13 weeks, ~300 g) were used in the experiment. Rats were treated with subcutaneous (s.c.) injection of ZA (Hospira, Almere, Holand) 7.5 μg/kg and 1 mg/kg Dex (Kern pharma, Barcelona, Spain) once a week, for 11 weeks ($n = 10$); control rats were treated by s.c. injection of saline in the same volume and duration ($n = 10$) [20]. At the third week (in addition to s.c. injection), rats were anaesthetized by intramuscular injection of 100 mg/kg bw Ketamin (Ketaset, Fort Dodge, Iowa, USA) and 5 mg/kg bw Xylazin (Eurovet, Cuijk, Holland) and all animals underwent unilateral tooth extraction: In the single tooth extraction group (ZA/Dex = 5; Saline = 5), the first maxillary molar was extracted while in the multiple tooth extraction group (ZA/Dex = 5; Saline = 5), the first and second maxillary molars were extracted**.** Three days post tooth extraction, rats were treated with 0.3 mg/kg bw Buprenorphine (Vetamarket, Shoham, Israel) and 50 mg/kg bw Cephalexin (Norbrook laboratories, Newry, Ireland) that were injected s.c. Rats were fed water-soaked rat chow and water ad libitum. ZA/Dex administration protocol was maintained until the animals were sacrificed. All rats were sacrificed by CO_2 asphyxiation 8 weeks after teeth extraction.

Evaluation of MRONJ occurrence was performed by clinical and histological analyses.

2.2. Clinical measurements

Eight weeks after extraction, the presence of exposed bone was identified, measured and recorded (clinical photos taken with a 105 mm lens digital camera).

2.3. Histology and histomorphometry

The part of the maxilla surrounding the extraction socket was sawed out and specimens were fixed immediately in 4% paraformaldehyde for 2 days. Fixed specimens were decalcified in 10% EDTA (Sigma-Aldrich, MS, USA), for 4 weeks, embedded in paraffin and sectioned (5μm). For determination of soft tissue and bone morphology: sections were stained with hematoxylin and eosin (H&E). Two stained sections (~20 μm apart) from each specimen were captured by a digital camera (Olympus DP70, Olympus, Tokyo, Japan) with a calibration scale and analyzed morphometrically using imageJ software (NIH, Bethesda, MD, USA). The area of the extraction site was identified adjacent to the second molar in the single extraction model and adjacent to the third molar in the multiple extraction model. Histomorphometric measurements were performed at these sites. Epithelial thickness (microns) was measured at three points in the extraction site, and an average of these measurements was calculated for each specimen. In MRONJ cases, epithelial discontinuation was measured in the most coronal mesio-distal dimension (Fig. 1).

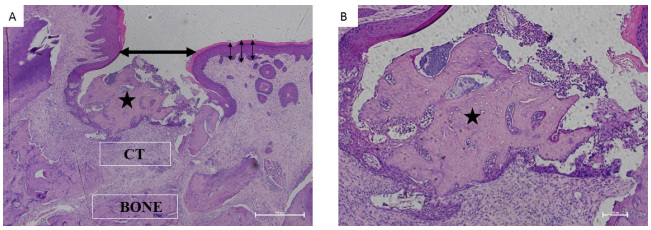

Fig. 1. Region of interest (ROI) for histomorphometric analysis. (A) Representative histological image of single extraction ZA/Dex-treated group. Area of extraction site was identified and histomorphometric measurements were taken for determination of epithelial thickness (vertical arrows), epithelial ulceration and discontinuation (horizontal arrows), and bone necrosis (black asterisk). Blood vessel density was measured in the connective tissue (CT) and in the basal bone (bone). (B) Higher magnification of the area of bone necrosis (black asterisk), demonstrate the number of empty lacunae with extensive inflammatory infiltrate.

Inflammatory infiltrate in the extraction site was detected in the connective tissue adjacent to the necrotic bone. Inflammatory infiltrate (including polymorphonuclear and mononuclear cells) was graded semi-quantitatively:

Low (0) - no inflammation < 10 cells; Mild (1) < 100 cells;
Medium (2) 100-200 cells; High (3) > 200 cells.

In order to quantify the area of osteonecrosis, areas with empty lacunae were identified and the number of empty lacunae was counted manually. Bone necrosis was defined as three or more empty lacunae per 1000 μm^2 [21]. In order to calculate the blood vessel density, 10 microscopic fields (at ×40 magnification) in the connective tissue as well as in the basal bone, were randomly selected and blood vessels were manually counted (Fig. 1).

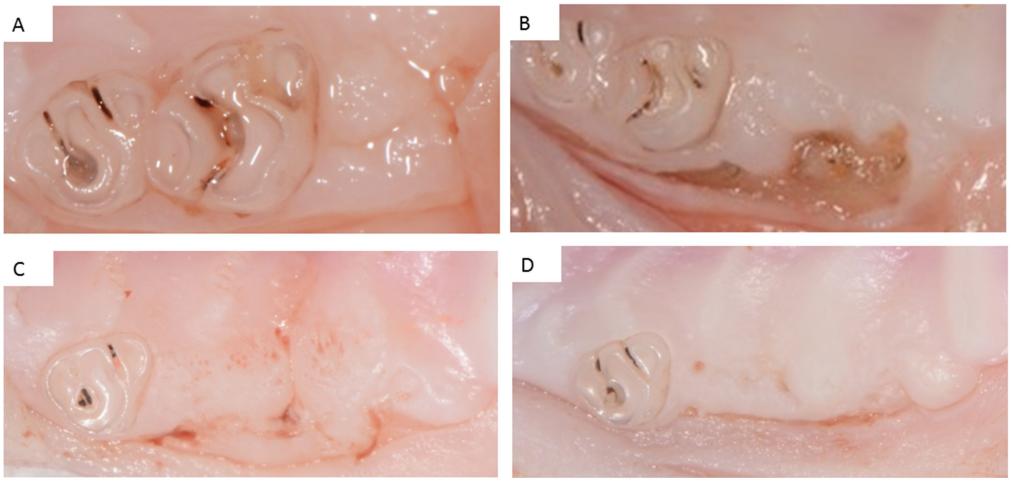

Fig. 2. Clinical healing of the extraction sites in ZA/Dex and saline groups. (A-B) Clinical images of the first molar extraction sites. (A) Saline (control) group demonstrating normal mucosa. (B) The ZA/Dex group revealing the exposed bone. (C-D) Clinical images of the multiple extraction group demonstrating normal healing. (C) saline (control). (D) ZA/Dex-treated group.

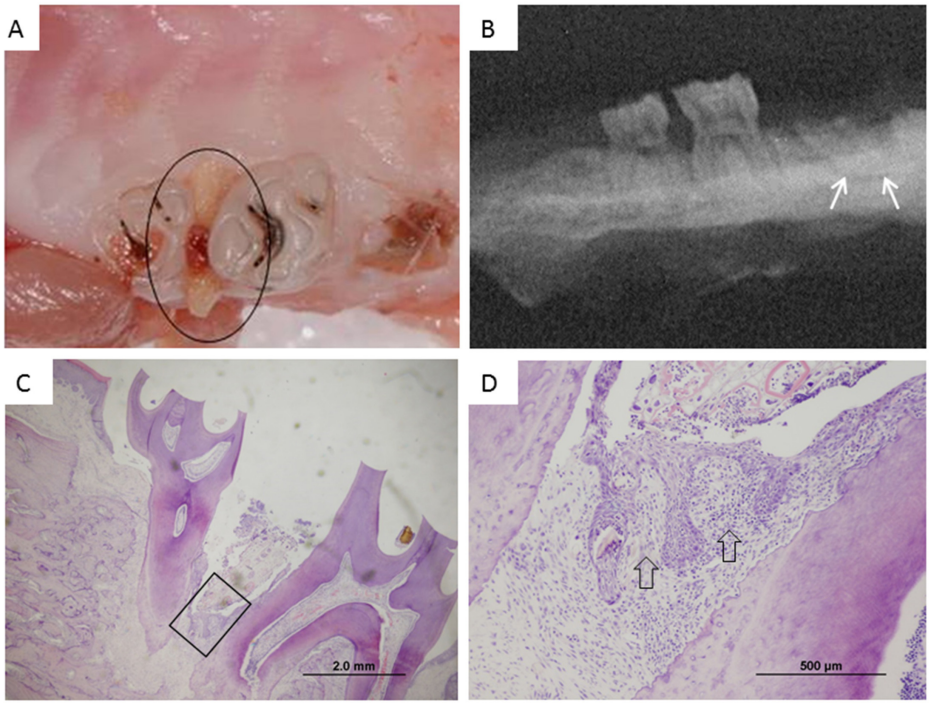

Fig. 3. Open contact between the 2nd and 3rd molars associates with food impaction, inflammation and bone loss in the single tooth extraction group. (A) Clinical macroscopic view showing open contact and food impaction. (B) A representative radiograph of single extraction ZA/Dex group demonstrating open contact point between M1 and M2 with significant bone loss. At the extraction socket area, a sequestrum was observed (arrow). (C) Histological section (H&E) of open contact area with food impaction, ulcerated epithelium and bone loss. (D) Higher magnification of the rectangular region demonstrating extensive inflammation (arrows).

Immunohistochemistry-CD31 antibody which recognizes endothelial cells served as a marker for blood vessel counting. Briefly: antigen retrieval of the samples was performed, followed by blocking non-specific binding sites (Background bluster, Innovex, Bioscience). After washes with PBS, the sections were incubated for 1 hour with a primary antibody against CD31 (Novus Biologicals, Colorado, USA), diluted 1:100. After extensive washing, samples were incubated with horseradish peroxidase (Zytomed system, Berlin, Germany). 3,3'-Dia- minobenzidine (DAB) (SuperPicture™, Thermo Fisher Scientific, Massachusetts, USA) was applied for 15 minutes and gently washed. Finally, sample dehydration and mounting were performed. Slides were visualized with an Olympus CX31 microscope (Olympus CX31, Olympus optical CO, LTD Philippines) equipped with an Olympus DP12.

Table 1. Histomorphometric analysis of total ZA/Dex-treated rats compared to all saline treated rats.

Mean ± SD	Area of necrosis (μm^2)	Empty lacunae ($1/mm^2$)	BV Bone/area ($1/mm^2$)	BV CT/area ($1/mm^2$)
Saline ($n = 8$)	0	3.13 ± 8.84	7.23 ± 1.62	6.52 ± 2.26
ZA/Dex ($n = 9$)	154.88 ± 194.06	22.44 ± 9.79	2.86 ± 1.43	3.44 ± 1.63
P-value (Saline vs ZA/Dex)	0.04	0.0007	< 0.0001	0.0054

Blood vessel density in the connective tissue (BV and CT, respectively) and blood vessel density in the bone (BV bone).

2.4. Estimation of sample size and power

According to the literature, MRONJ-like lesions occur in 50% of the animals after administration of bisphosphonates and tooth extraction. In contrary, we except to find no MRONJ occurrence in rats that were treated with saline and underwent tooth extraction. Therefore, 5 animals in each group were considered sufficient.

2.5. Statistical analysis

StatPlus® (AnalystSoft, Vancouver, BC, Canada) and JMP 10.0 (SAS Institute, Cary, NC, USA) statistical packages were used. Descriptive statistics which included means and medians, ranges and standard deviation (SD) were initially tabulated. Comparisons between control (saline) and test (ZA/Dex) groups and between one or two extractions were performed using unpaired t-test and two way anova analysis. Significance level was set at 5%.

3. Results

3.1. Clinical evaluation (Macroscopic examination)

Three rats died during anesthesia, therefore were not included in the study. Surviving animals demonstrated good hemostasis, and gained body weight; overall, 10 rats in the single tooth extraction group (ZA/ Dex = 5; Saline = 5), and 7 rats in the multiple tooth extraction group (ZA/Dex = 4; Saline = 3) were included in the analysis. MRONJ-like lesions (i.e. exposed bone) were evident clinically in all the single extraction ZA/Dex-treated rats (Fig. 2B). In contrast, all saline-treated rats (single and multiple extraction groups) failed to show clinical signs of MRONJ (Fig. 2A, 2C). In the ZA/Dex multiple extraction group, minimal evidence of incomplete healing was observed only following magnification (Fig. 2D). In addition, in all single tooth extraction cases (both saline and ZA/Dex), we found open contacts between the 2nd and 3rd molars that were associated with food impaction (Fig. 3).

3.2. Histological and histomorphometric analyses

Tissue sections obtained from the saline groups (single and multiple tooth extraction models) showed normal soft and hard tissue healing. Oral mucosa presented continuous epithelium with developed rete ridges and wide non-inflamed underlying connective tissue. Underneath, the basal bone exhibited features of mature bone with normal bone remodeling and cellular lacunae (Fig. 4A, 4B). The tissue sections obtained from the ZA/Dex-treated groups that demonstrated exposed bone clinically (single tooth extraction group), showed discontinuity of the epithelium with exposed fragments of necrotic bone and sequestrum surrounded by extensive inflammatory infiltrate that consisted of mononuclear cells (Fig. 4C, 4D).

Histomorphometric analysis was performed for epithelial thickness, epithelial discontinuation, inflammatory infiltrate, blood vessel density (BVD), empty lacunae and area of necrotic bone. When comparing total ZA/Dex to total saline treated rats, epithelial thickness was not significantly different between the groups and ranged between 157.5 μm-325.1 μm. Epithelial discontinuation (ulceration) was evident solely in ZA/Dex rats. Inflammatory infiltrate grade was higher in the ZA/Dex group (2.22 ± 0.833) compared with the saline group (1.125 ± 1.356). The number of empty lacunae and the area of necrotic bone were higher in the ZA/Dex treated group ($p = 0.0007$, $p = 0.04$ respectively), while blood vessel density in the connective tissue and bone were decreased in the ZA/Dex-treated group ($p = 0.0054$ and $p < 0.0001$, respectively) (Table 1).

When comparing single versus multiple tooth extraction groups (Fig. 5), clinically exposed bone was evident in the ZA/Dex-treated single extraction rats only, however, histological and histomorphometric analyses revealed evidence for necrotic bone and empty lacu-

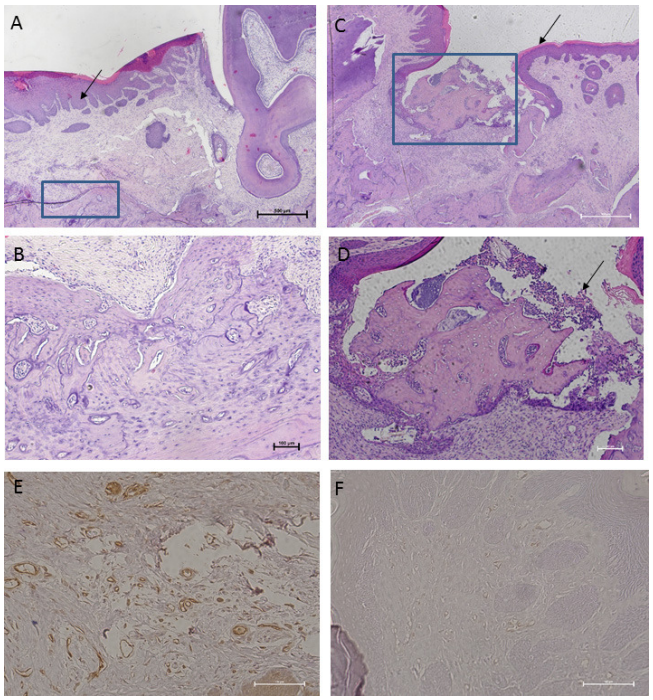

Fig. 4. Histological characteristics of MRONJ. (A-B) H&E staining sections of the saline (control) treated rats, and (C-D) ZA/Dex-treated rats. (A) Continuous epithelium, developed rete ridges (black arrows) with underlying wide connective tissue and bone in the tooth extraction area. (B) Higher magnification of the basal bone, demonstrates normal bone remodeling, bone formation with cellular lacunae. (C) Discontinuity of the epithelium and sequestrum formation (arrow). (D) Magnified area of the exposed necrotic bone, displaying lack of vascularity, empty lacunae and inflammatory cell infiltration (arrow). (E-F) blood vessels stained with anti-CD31 in control (E) and ZA/Dex (F).

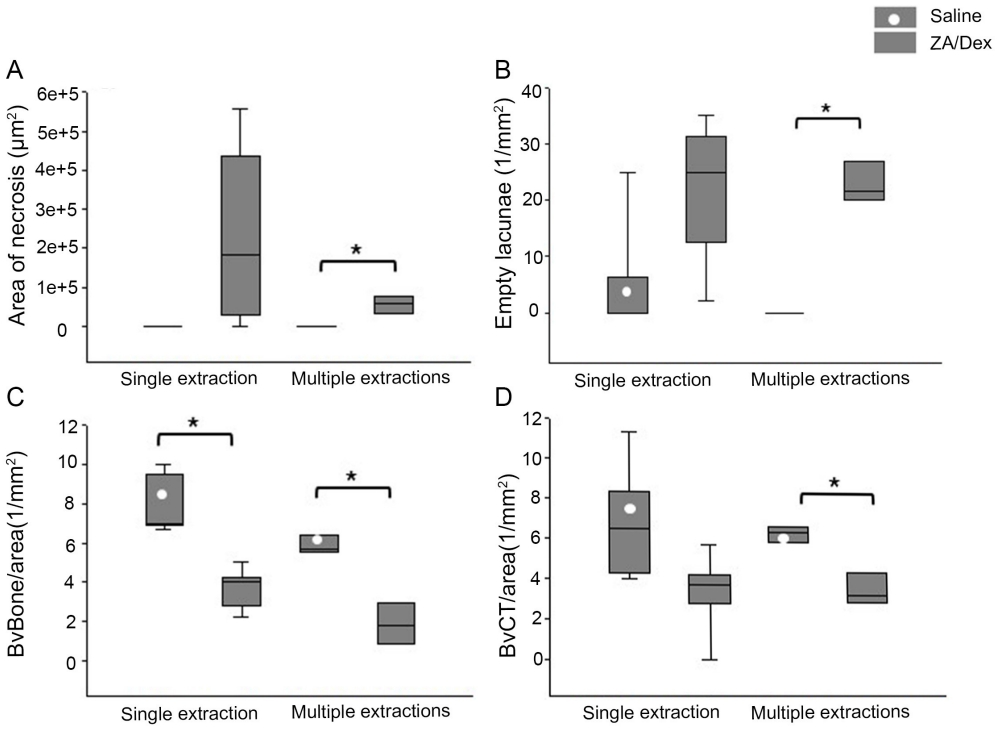

Fig. 5. Histomorphometric analysis of ZA/Dex and saline groups. (A-D) Comparison between ZA/Dex- and saline-treated rats, in single and multiple tooth extraction models $*p < 0.05$. Blood vessel density in the connective tissue (BV and CT, respectively) and blood vessels density in the bone (BV bone).

nae in both the single and multiple extraction rats that were treated with ZA/Dex. In the multiple extraction cases, epithelial ulceration (epithelial discontinuation) was too small to be detected clinically. Accordingly, several histomorphometric parameters (empty lacunae; area of necrotic bone and BVD in the CT) showed significant differences between ZA/Dex and saline groups in the multiple extraction models that were not significant in the single extraction models (Fig. 5A, 5B, 5C). However, BVD in bone was lower in the ZA/Dex versus saline groups in the single ($p = 0.0008$) and multiple ($p = 0.0039$) extraction models (Fig. 5C).

4. Discussion

MRONJ was described for the first time by Marx on 2003, as exposed bone in the oral cavity in patients taking BSP to treat osteoporosis [3]. Most of the literature in this field is limited and is based on human clinical case reports that show that MRONJ emerged after tooth extraction or surgery. Nevertheless, several case reports have presented "spontaneous development of MRONJ" (without prior surgical intervention) that could be attributed to the presence of chronic infection around teeth or dental implants [22]. In order to gain insight into the pathogenesis of the disease we aimed to establish a MRONJ model in the rat. We hypothesized that similar to humans, by increasing the extent of the surgical trauma, we would elevate the incidence of MRONJ, in this rat model. The results of the present study showed that all the rats that underwent single tooth extraction in the ZA/Dex-treated group exhibited clinical evidence of exposed bone and characteristics of MRONJ histologically. Unexpectedly, rats that were treated with ZA/Dex and underwent multiple extractions showed minimal clinical signs of MRONJ that could not be detected with the naked eye. However, evidence for the disease was found histologically. Therefore, our findings suggest that the degree of surgical trauma may not be the main risk or trigger factor for MRONJ development.

Even though MRONJ diagnosis is based on clinical examination of the patient and identification of the exposed bone that proceeds for at least eight weeks, there are several histological characteristics that are associated with MRONJ lesions. Histologically, MRONJ is characterized by diverse tissue changes, including necrotic bony trabeculae with empty osteocyte lacunae and granulation tissue. The inter-trabecular space is infiltrated by inflammatory cells including neutrophils, lymphocytes, and plasma cells as well as decreased vascularization and number of osteoblasts [23, 24]. In the present study, none of the rats in the multiple extraction groups showed exposed bone clinically, however, histological characteristics were displayed. One explanation for this surprising result may be due to the micrometer scale ulceration in the epithelium that can only be detected microscopically. However, since MRONJ diagnosis requires the clinical appearance of exposed bone for diagnosis, this multiple extraction model is inferior to the single extraction model.

Za/Dex-treated rat groups showed decreased blood vessel density in the oral mucosa and alveolar bone, increased number of empty lacunae and higher grading of the inflammatory infiltrate compared with control rats treated with saline. In general, most of the histological parameters were similar among single tooth or multiple tooth extraction models. However, the mean area of necrotic bone in the ZA/Dex-treated rats that underwent single tooth extraction was 4-fold higher in comparison to the multiple tooth extraction treatment (0.235 mm^2 vs 0.0545 mm^2, $p < 0.05$). These histological findings are in accord with the clinical observations in which we found higher incidence of exposed bone in a single tooth extraction model.

To interpret the results of the current study, mechanisms and risk factors for MRONJ should be discussed. Interestingly, MRONJ is restricted to the jaw bone. Unlike long-bones, the jaw is covered with a thin oral mucosa that separates the underlining bone from oral flora and protects the bone from mechanical trauma caused by food impaction. Injury or ulceration to the oral mucosa exposes the underlining jaw to bacterial and fungal contamination that may contribute to MRONJ development. Furthermore, in a study by Duzan et al., gingival mechanical local damage promoted Th17 cell migration and contributed to the potentiation and exacerbation of local oral immunity which contributed to pathogenic bone loss [25]. In the current research, clinical examination of rats in the single tooth extraction group showed open contact points between the second and third molars that were associated with food impaction, soft tissue ulceration and significant bone loss between the second and third molars. Based on this observation, we hypothesize that chronic local trauma to the oral mucosa, caused by the food impaction, may play an important role in MRONJ development. Alternatively, the neighboring teeth with their bacterial load or the presence of neighboring teeth that keep the wound open and enable bacteria to enter the socket, may aggravate MRONJ.

It is unclear whether or not MRONJ is induced by tooth extraction or by the surgery itself. Alternatively, as an unproven hypothesis, MRONJ may already exist as "microlesions" in the alveolar socket prior to tooth extraction or surgery, e.g. in periodontal teeth requiring extraction, and become visible after extraction or surgery. BSP are known to bind to bone at neutral pH and are dissociate from the bone in an acidic environment. During bone resorption, the acid pH in the resorption lacunae increases the release of BSP from hydroxyapatite resulting in high local BSP concentrations and therefore MRONJ development [25]. Local acidic milieus are common in infections and wound healing after surgical procedures. Furthermore, Marx (2014) described that osteoclastic resorption of BSP-loaded bone results in osteoclast cell death in which the cell lyses, releasing the BSP drugs to reenter the local bone or bone marrow in a re-dosing effect [27]. In the single tooth extraction model, the presence of open contact points indicates that tooth migration occurred. Since tooth migration occurs due to osteoclastic resorption, this "re-dosing effect" may further explain the difference in MRONJ occurrence between the two different extraction models.

The restrictive location of MRONJ to the jaw, maybe due to the high bone turnover rate and limited vasculature of the jaw. Our findings support this hypothesis as we found a reduced vasculature in the oral mucosa and in the alveolar bone of rats that were treated with ZA/DEX in comparison to control rats that were treated with saline [1]. Previous studies noted that suppression of angiogenesis can result in the development of MRONJ, and that serum vascular endothelial growth factor (VEGF) levels might be a predictive marker of MRONJ [27].Furthermore, these findings are supported by studies about cancer patients treated with ZA who exhibited decreased circulating VEGF levels [29].

To explore preventive and future treatment strategies for MRONJ, there is a burning need to establish reliable preclinical animal models that mimic clinical and histological characteristics of MRONJ. Moreover, being able to establish MRONJ-like lesions in high incidence is a prerequisite to allow further research in this field.

Several animal models for induction of MRONJ have been described using different animal species, medications and surgical triggers [30]. Overall, the incidence of MRONJ-like lesions largely varied between 0% to a 100% among the studies. In accordance with our results, Marino el al., (2012), showed that I.V. injection of ZA 20 μg/kg and 1st mandibular molar extraction resulted in clinical MRONJ-like lesion in 60% of the rats [31]. Unlike our results, other studies that combined ZA with multiple teeth extractions found a higher percentage of exposed bone. In a miniature pig model, extraction of three molars and I.V. administration of ZA caused MRONJ appearance in 80%-100% of the pigs [32]. A possible explanation for this discrepancy is the heterogeneity between the studies, for example, large versus small animals, differences in medication dosages and modes of treatment. In large animal models (e.g. sheep or pig), the dimensions of MRONJ-like lesions are on a centimeter scale enabling detection of lesions with the naked eye. Nevertheless, the advantage of choosing small animal models in research is obvious, especially in studies that can rely on histological characteristics of MRONJ to meet study aims. However, clinical detection of necrotic bone is crucial for MRONJ diagnosis, treatment and prevention studies.

5. Conclusions

Within the limits of the current pilot study with a predominantly small sample size, the extent of the surgical field may not be the key factor in MRONJ development since only single tooth extraction showed exposed bone. However, histological characteristics were identified in both single and multiple teeth extraction models. Therefore, preclinical studies that aim to evaluate histological features of the disease may use both models, whereas when clinically exposed bone is required, the single tooth extraction model is preferred. The effects of chronic micro-trauma, food impaction and tooth migration should also be considered.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Ruggiero SL, Dodson TB, Fantasia J, *et al*. American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw – 2014 update. *J Oral Maxil Surg*, 2014; 72(10): 1938-1956.

[2] Marx RE, Sawatari Y, Fortin M, Broumand V. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. *J Oral Maxil Surg*, 2005; 63(11): 1567-1575.

[3] Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. *J Oral Maxil Surg*, 2003; 61(9): 1115-1117.

[4] Jang HW, Kim JW, Cha IH. Development of animal model for Bisphosphonates-related osteonecrosis of the jaw (BRONJ). *Maxillofacial Plastic and Reconstructive Surgery*, 2015; 37(1): 18.

[5] Otto S, Pautke C, Hafner S, *et al*. Pathologic fractures in bisphosphonate-related osteonecrosis of the jaw—review of the literature and review of our own cases. *Craniomaxillofacial Trauma and Reconstruction*, 2013; 6(03): 147-154.

[6] Walter C, Al-Nawas B, Grotz KA, *et al*. Prevalence and risk factors of bisphosphonate-associated osteonecrosis of the jaw in prostate cancer patients with advanced disease treated with zoledronate. *Eur Urol*, 2008; 54: 1066-1072.

[7] Papapoulos S, Chapurlat R, Libanati C, *et al*. Five years of denosumab exposure in women with postmenopausal osteoporosis: results from the first two years of the FREEDOM extension. *J Bone Miner Res*, 2012; 27(3): 694-701.

[8] Gabbert TI, Hoffmeister B, Felsenberg D. Risk factors influencing the duration of treatment with bisphosphonates until occurrence of an osteonecrosis of the jaw in 963 cancer patients. *J Cancer Res Clin*, 2015; 141(4): 749-758.

[9] Baron R, Ferrari S, Russell RG G. Denosumab and bisphosphonates: different mechanisms of action and effects. *Bone*, 2011; 48(4): 677-692.

[10] Lacey DL, Boyle WJ, Simonet WS, *et al*. Bench to bedside: elucidation of the OPG-RANK-RANKL pathway and the development of denosumab. *Nat Rev Drug Discov*, 2012; 11(5): 401-419.

[11] Roelofs AJ, Thompson K, Gordon S, Rogers MJ. Molecular mechanisms of action of bisphosphonates: current status. *Clin Cancer Res*, 2006; 12(20): 6222s-6230s.

[12] Yamashita J, McCauley LK. Antiresorptives and osteonecrosis of the jaw. *Journal of Evidence Based Dental Practice*, 2012; 12(3): 233-247.

[13] Hokugo A, Christensen R, Chung EM, *et al*. Increased prevalence of bisphosphonate-related osteonecrosis of the jaw with vitamin D deficiency in rats. *J Bone Miner Res*, 2010; 25(6): 1337-1349.

[14] Reid IR, Bolland MJ, Grey AB. Is bisphosphonate-associated osteonecrosis of the jaw caused by soft tissue toxicity? *Bone*, 2007; 41(3): 318-320.

[15] Dimopoulos MA, Kastritis E, Bamia C, *et al*. Reduction of osteonecrosis of the jaw (ONJ) after implementation of preventive measures in patients with multiple myeloma treated with zoledronic acid. *Ann Oncol*, 2008; 20(1): 117-120.

[16] Hoff AO, Toth BB, Altundag K, *et al*. Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. *J Bone Miner Res*, 2008; 23(6): 826-836.

[17] Sedghizadeh PP, Kumar SK, Gorur A, *et al*. Microbial biofilms in osteomyelitis of the jaw and osteonecrosis of the jaw secondary to bisphosphonate therapy. *The Journal of the American Dental Association*, 2009; 140(10): 1259-1265.

[18] Sedghizadeh PP, Yooseph S, Fadrosh D, *et al*. Metagenomic investigation of microbes and viruses in patients with jaw osteonecrosis associated with bisphosphonate therapy. *Or Surg Or Med Or Pa*, 2012; 114(6): 764-770.

[19] Vahtsevanos K, Kyrgidis A, Verrou E, *et al*. Longitudinal cohort study of risk factors in cancer patients of bisphosphonate-related osteonecrosis of the jaw. *J Clin Oncol*, 2009; 27(32): 5356-5362.

[20] Sonis ST, Watkins BA, Lyng GD, Lerman MA, Anderson KC. Bony changes in the jaws of rats treated with zoledronic acid and dexamethasone before dental extractions mimic bisphosphonate-related osteonecrosis in cancer patients. *Oral Oncol*, 45(2), 164-72.

[21] Bi Y, Gao Y, Ehirchiou D, *et al*. Bisphosphonates Cause Osteonecrosis of the Jaw-Like Disease in Mice. *The American Journal of Pathology*, 2010; 177(1), 280-290.

[22] Thumbigere-Math V, Ma'Ann CS, Gopalakrishnan R, *et al*. Bisphosphonate-related osteonecrosis of the jaw: clinical features, risk factors, management, and treatment outcomes of 26 patients. *J Oral Maxil Surg*, 2009; 67(9): 1904-1913.

[23] Bertè R, Arcari A, Bernuzzi P, *et al*. Jaw avascular bone necrosis associated with long-term use of bisphosphonates. *Tumori*, 2005; 92(4): 361-361.

[24] Hansen T, Kirkpatrick CJ, Walter C, Kunkel M. Increased numbers of osteoclasts expressing cysteine proteinase cathepsin K in patients with infected osteoradionecrosis and bisphosphonate-associated osteonecrosis—a paradoxical observation? *Virchows Archiv*, 2006; 449(4): 448-454.

[25] Dutzan N, Abusleme L, Bridgeman H, *et al*. On-going mechanical damage from mastication drives homeostatic Th17 cell responses at the oral barrier. *Immunity*, 2017; 46(1): 133-147.

[26] Rao NJ, Wang JY, Yu RQ, Leung YY, Zheng LW. Role of Periapical Diseases in Medication-Related Osteonecrosis of the Jaws. *Biomed Res Int*, 2017; 2017(2): 1-8.

[27] Marx RE. A decade of bisphosphonate bone complications: what it has taught us about bone physiology. *Oral & Craniofacial Tissue Engineering*. 2014; 29: e247-581.

[28] Vincenzi B, Napolitano A, Zoccoli A, *et al*. Serum VEGF levels as predictive marker of bisphosphonate-related osteonecrosis of the jaw. *J Hematol Oncol*, 2012; 5(1): 56.

[29] Santini D, Vincenzi B, Dicuonzo G, *et al*. Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients. *Clin Cancer Res*, 2003; 9(8): 2893-2897.

[30] Aghaloo TL, Cheong S, Bezouglaia O, *et al*. RANKL inhibitors induce osteonecrosis of the jaw in mice with periapical disease. *J Bone Miner Res*, 2014; 29(4): 843-854.

[31] Marino KL, Zakhary I, Abdelsayed RA, *et al*. Development of a rat model of bisphosphonate-related osteonecrosis of the jaw (BRONJ). *J Oral Implantol*, 2012; 38(S1): 511-518.

[32] Pautke C, Kreutzer K, Weitz J, *et al*. Bisphosphonate related osteonecrosis of the jaw: a minipig large animal model. *Bone*, 2012; 51(3): 592-599.