Bone 122 (2019) 45–51

Contents lists available at ScienceDirect

Bone

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

Full Length Article

Effects of pH alteration on the pathogenesis of medication-related osteonecrosis of the jaw

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ARTICLE INFO

Keywords:
Osteonecrosis of the jaw
Bisphosphonates
Acids
Bases
Animal model

ABSTRACT

Introduction: An acidic environment has been recognized to increase catabolic activities and inhibit osteoblastic deposition, and also exhibited in the pathogenesis of various bone diseases. The aim of the study was to investigate the role of systemic and local pH alteration in the pathogenesis of medication-related osteonecrosis of the jaw (MRONJ).

Material and methods: Initially, MRONJ was induced in 54 Sprague-Dawley rats via subcutaneous bisphosphonate injections, once a week for 8 weeks. A week prior to bisphosphonate termination, surgical intervention was performed and rats were divided into 3 groups—alkalotic, acidic and control group, wherein each received $NaHCO_3$, NH_4Cl and normal saline, respectively for 8 weeks. Upon sacrifice, blood was sent for arterial blood pH analysis and their mandibles were subjected to histomorphometric and μCT analyses. ONJ was histologically defined as necrotic bone persisting for eight weeks after surgical intervention.

Results: Each intervention exemplified its expected outcome wherein each group exhibited a borderline alkalotic (7.43 ± 0.05) and acidic state (7.27 ± 37), respectively ($P < 0.05$). Acidic group showed a higher occurrence of MRONJ (95%) compared to that of alkalotic group (60%) and control (76.9%). Histomorphometric and microstructural evaluation revealed that acidic group presented deteriorated bone architectures with significantly higher necrotic bone fraction, clusters of empty lacunae, N.Oc/B.Pm and lower B.Ar./T.Ar, BV/TV, Tb.Th ($P < 0.05$). Alkalotic group showed possible protective effects against ONJ versus acidic group, however these trends were not statistically significant.

Conclusions: An acidic milieu aggravated ONJ development in an animal model. Further investigations are needed to elucidate the exact role of acid-base balance in MRONJ pathogenesis and possible benefits of alkali supplementation for the prevention.

1. Introduction

Precise maintenance of the acid-base balance is paramount since bone cells are found to be extremely sensitive to pH changes [1,2]. To demonstrate these conditions in a cellular level, researchers subjected osteoclasts and osteoblasts in an acidic environment. These studies showed that acidosis exerts an inhibitory action on matrix mineralization, reciprocal with osteoclast activation response. Hence, osteoblastic deposition of alkaline mineral in bone is decreased, and osteoclastic resorptive activity is increased to maximize the availability of H^+ in solution to buffer protons [3–5]. Such condition decrease expression of extracellular matrix genes in osteoblasts, including collagen [2,6,7]. In addition, it favors osteoblastic production of certain factors such as prostaglandin E_2, receptor activator of nuclear factor-kappaB ligand (RANKL), macrophage colony stimulating factor (M-CSF), which further enhances osteoclastic resorption [5,8,9].

Meanwhile, administration of an alkali to counteract such imbalances was found to increase osteoblastic mineral deposition and attenuation of osteoclastic activities in a rat calvaria study [5]. Various clinical studies on alkali supplementation in menopausal adults demonstrated results of lowered calcium excretion, bone resorption markers- N- and C-terminal telopeptide and serum amino-terminal

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https://doi.org/10.1016/j.bone.2019.02.007
Received 3 December 2018; Received in revised form 9 January 2019; Accepted 6 February 2019
Available online 08 February 2019
8756-3282/

propeptide of type 1 procollagen [10–12]. These findings are of clinical significance since increases in bone turnover are associated to bone loss and fracture risks [13,14], conditions warranting pharmacologic supplementation and therapy for most cases.

In addition, the pH changes not only affect cellular mechanisms but also the pharmacokinetics and pharmacodynamics of certain medications acting on bone metabolism, such as bisphosphonates (BP). Normally, BP adsorbs to the mineral component of bone and interfere with osteoclast actions. It selectively binds to bone at neutral pH and is released into the resorption lacunae, in the presence of an acidic milieu created by the osteoclasts [15–17]. Osteoclasts, in particular, internalize these BP molecules through fluid-phase endocytosis [15]. Once engulfed, BP exerts its intracellular effects in osteoclast function and survival to inhibit bone resorption [18]. This has led to its clinical use in treating various hyper-resorptive bone disorders. However, this mechanism has been associated to one of the drug's adverse effects involving non-healing jaw lesions in cancer patients receiving high-dose intravenous BP [19]. Later termed as medication-related osteonecrosis of the jaw (MRONJ), this condition is associated with anti-resorptive and anti-angiogenic agents [20] and is still an area of ongoing investigation.

One of the proposed mechanisms of MRONJ pathogenesis is the increased local concentration of dissociated BP due to an acidic environment [15,21,22]. With increased local concentration, toxic effects on different cell types such as osteoclasts, oral keratinocytes and mesenchymal cells, are suggested in various studies [16,23,24]. On the other hand, the jaw is subjected to frequent periapical infections, inflammatory responses, trauma and dentoalveolar surgery, unlike any other bones because of the presence of teeth. These factors are reported to cause a decrease in local pH and were found to predispose BP-exposed patients to MRONJ [21,25]. Hence, this anatomic vulnerability and local acidosis as part of the risk factors for ONJ pathogenesis was proposed.

It was not the goal of this study to elucidate the exact cellular interplay of BP-related osteoclastic apoptosis and low pH-related osteoclastic activation, proven in vitro but to reflect an acidic milieu in an in vivo study. Hence, this study aimed to determine whether systemic and local acidic environment aggravate the development of MRONJ in the animal model [26]. In addition, the study investigated the role of alkali supplementation in MRONJ prevention.

2. Material and methods

2.1. MRONJ animal model

Fifty-four, 16-week male Sprague Dawley rats, weighing from 200 to 230 g were purchased from the Institute of Ewha Medical Research (Seoul, Korea) for this experiment. The animals were housed in cages (3 animals/cage), maintained in controlled environment with a temperature of 22 ± 2 °C, 50% ± 10% relative humidity, and a 12-h light/dark cycle. All animals were given a week of acclimatization period before commencement of interventions and have access to standard rodent diet and water, ad libitum. This animal study was approved by and performed in accordance with the guidelines provided by the institutional animal research ethics committee.

The present model was based on our previous protocol [26], wherein all rats were given subcutaneous BP once a week for 8 weeks (100 μg/kg, Zometa™, Novartis Pharma AG, Basel, Switzerland), followed by extraction of all lower left molars on the 7th week of BP injections (Figs. 1 and 2), under general anesthesia with tiletamine hypochloride and zolazepam hypochloride solution (15 mg/kg, Zoletil™ 50, Virbac Laboratories, Carros, France) and xylazine (10 mg/kg Rompun®, Bayer AG, Lever-kusen, Germany). Extraction sockets were allowed to heal through secondary intention. Once hemostasis was achieved after gauze packing, rats were placed in recovery positions and monitored daily. Antibiotics and analgesics were administered post-operatively. BP administration was carried out for another week before dividing them into their respective groups.

2.2. Administration of PH-altering agents

Animals were divided into alkalotic group (group 1, $n = 20$), acidic group (group 2, $n = 20$) and control ($n = 14$). All administered agents were given in an 8-week period via oral route and local mucosal injections before sacrifice.

2.2.1. Alkalotic group

This group ($n = 20$) was further divided into 2 sub-groups. The first sub-group (group 1–1, $n = 10$), were given alkaline supplementation via drinking water with 0.25 M of $NaHCO_3$, ad libitum for 8 weeks. The 2nd sub-group (group 1–2, $n = 10$) was given local injections of 4.2% of $NaHCO_3$, twice a week for 8 weeks. The local injections were administered on the buccal mucosa of the extracted teeth under inhalation anesthesia using isoflurane. Concentrations used were based on previous animal model of alkalosis (for group 1-1) and clinical dosages of managing metabolic acidosis (for group 1-2) [27].

2.2.2. Acidic group

The acidic group (group 2, $n = 10$) comprised of 2 sub-groups wherein the first sub-group (Group 2–1, $n = 10$) were given 0.28 M NH_4Cl + 0.5% sucrose in drinking water for 8 weeks ad libitum. The 2nd (group 2–2, $n = 10$) received 2% NH_4Cl local injection with the same frequency as in the previous group. Concentrations for group 2-1 were based on a previous rat animal study of metabolic acidosis induction [28], and relative safety of the dosage was based on a rat toxicity and carcinogenicity experiment for group 2-2 [29].

2.2.3. Control group

The control group ($n = 14$) was locally given 0.5 ml normal saline injections twice a week for 8 weeks.

2.3. Animal sacrifice and arterial blood gas analysis

After 8 weeks of administration of the respective agents, the animals were put under general anesthesia using the same protocol. Arterial blood was collected via cardiac puncture using 23-gauge, heparinized arterial blood collection syringes (BD Preset ™, Becton, Dickinson and Company, Plymouth, United Kingdom) and were then immediately sent for arterial blood-gas (ABG) analysis (RAPIDPoint® 500PH Blood Gas Systems, Siemens Healthcare, Erlangen Germany). Rats were then sacrificed via CO_2 chamber and specimen collection was performed. Classification of blood pH states were based on normal values for anesthetized rats [30].

2.4. Macroscopic and radiographic analysis

The hemi-mandibles were trimmed, photographed and immersed in 0.2% glutaraldehyde (Daejung Chemicals & Metals Co., LTD, Gyeonggi-do, Korea). Each specimen was sent for micro-architectural assessment using micro-computed tomography (μCT, SkyScan1173 Ver 1.6, Bruker-CT, Kartuizersweg 3B 2550 Kontich, Belgium). The acquisition settings were at a voxel size of 5.33 μm^3 with an X-ray tube voltage of 60 kVp and the intensity current of 133 μA. An entire region of the mandible (2240 slices; 8 μm/slice) was scanned for each sample. Microarchitecture 3D data registration, thresholding and segmentation within the conforming volume of interest (VOI) was performed using DataViewer and CTAn Imaging Softwares (Bruker microCT, Kartuizersweg 3B, 2550 Kontich, Belgium). VOI was defined as the area within the extraction socket from the crest of residual ridges to 5–10 mm above the superior wall of mandibular canal wall or inferior border of the mandible. Morphometric parameters defined are tissue volume (TV; mm^3), percent bone volume (BV/TV; %), trabecular

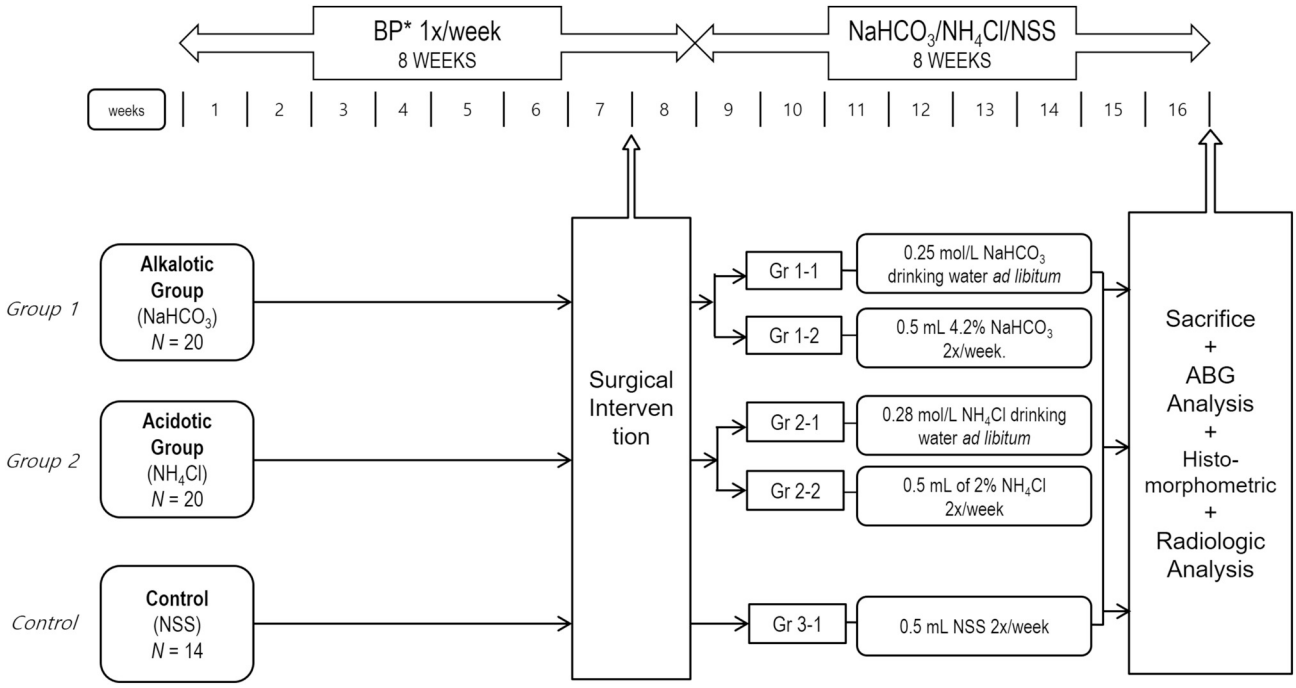

Fig. 1. Study setting.
Animals were divided into group 1 (alkalotic), group 2 (acidic) and control. Group 1 and 2 were further divided according to administration routes.

number (Tb.N; 1/mm), trabecular thickness (Tb.Th; μm), and trabecular separation (Tb.Sp; mm).

2.5. Histomorphometric assessment

Sectioning, deparaffinization, rehydration and staining with hematoxylin-eosin (HE) and Masson Trichrome (MT) were executed to determine and confirm the presence of MRONJ, based on the following histological criteria: (1) presence of ulcerative lesion with exposed and non-exposed necrotic bone as presented by clusters of > 10 empty lacunae, (2) presence of pseudoepitheliomatous-like hyperplasia of the epithelium accompanied by inflammatory cell infiltration, and (3) presence of sequestrum and/or bacterial colonies. Image capture was carried out using an Eclipse 50i light microscope (Nikon, Tokyo, Japan), equipped with CCD camera (MicroPublisher 3.3 RTV cooled, QImaging, Bethesda, MD, USA), and Image Pro Capture Kit Platform (Media Cybernetics, Bethesda, MD, USA).

Histomorphometric evaluation was performed by a single investigator in a blinded manner. Four regions of interest (ROI), located at the osteonecrotic lesion or prior extraction site were initially defined at lower magnification (50×). Images were calibrated and formatted with a uniformed ROI dimension (1000 μm × 1000 μm), which were assigned in areas within extraction sites, below the alveolar crests and approximately 5–10 mm above the inferior border of the mandible or superior wall of mandibular canal.

Further histomorphometric analysis and manual cell evaluation was done with a higher magnification (200×), using Image Pro Premier (Media Cybernetics, Bethesda, MD, USA). Measurements included tissue area (T.Ar, μm^2), bone area (B.Ar, μm^2), bone perimeter (B.Pm, μm), osteoclast number [N.Oc, #], number of empty lacunae ($\#/\mu m^2$) and necrotic bone fraction (%). Osteoclasts were restricted to bone-resorbing multinucleated cell within the bone periphery and empty lacunae were defined by a lacuna without a visible osteocyte. Necrotic bone was defined by a fraction of bone with the presence of clusters of 10 or more empty lacunae.

2.6. Statistical analysis

Statistical analysis was carried out using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Measurements of pH, histomorphometric and architectural morphometric indices were compared using analysis of variance with Bonferroni correction. Group comparison between control, acidic and alkalotic groups were performed and subsequent subgroup differences were analyzed. To analyze the MRONJ occurrence,

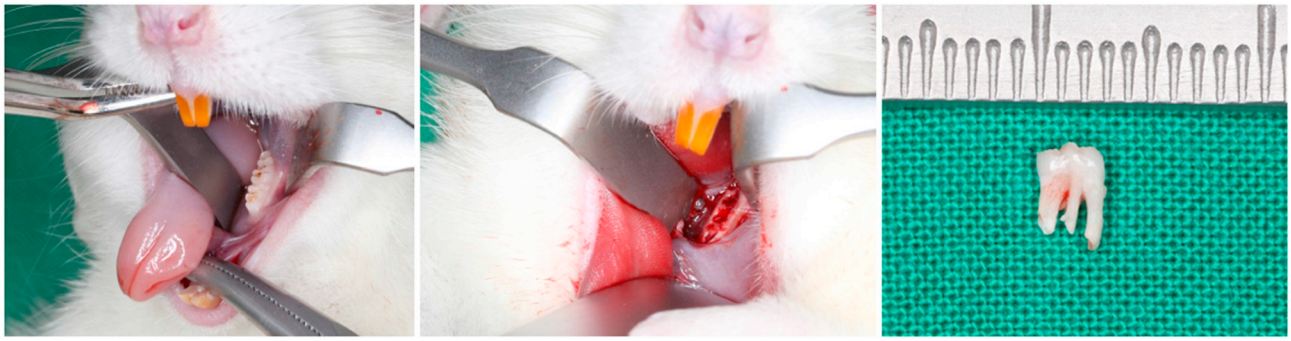

Fig. 2. Surgical intervention.
Induction of MRONJ in a rat animal model by teeth extraction under general anesthesia.

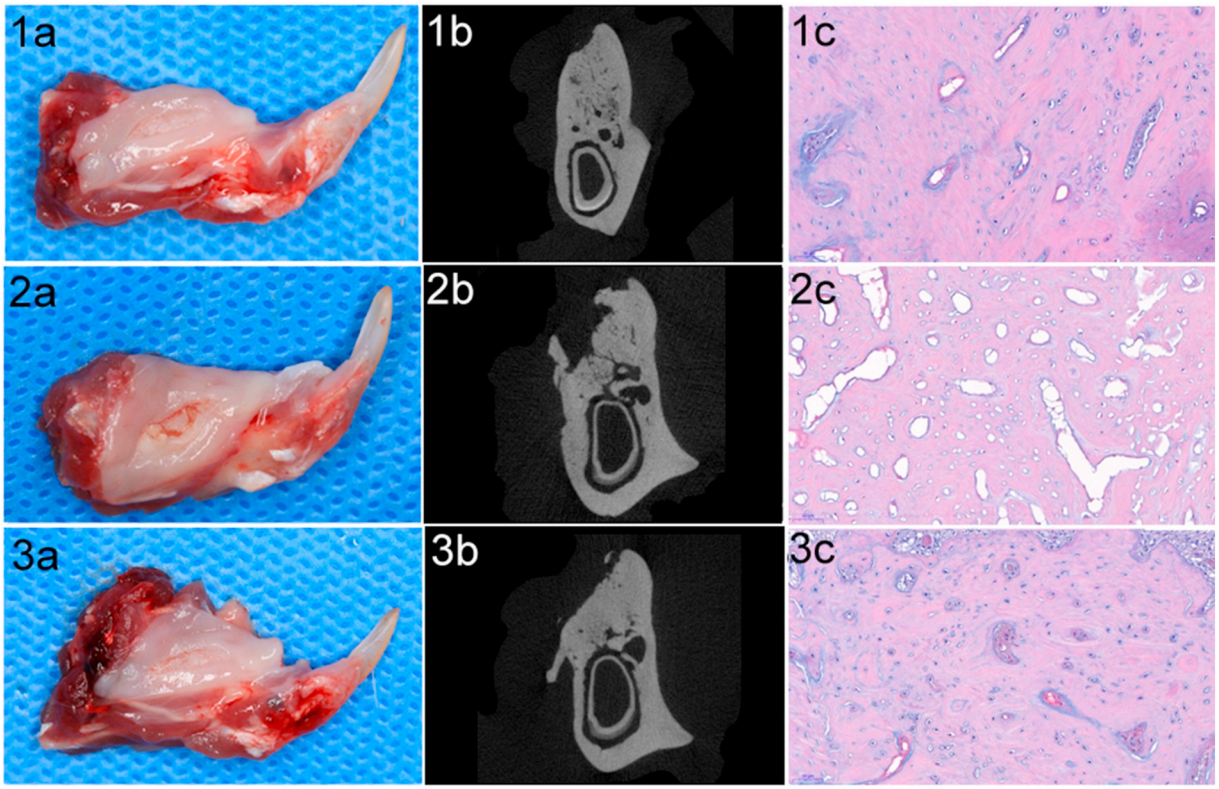

Fig. 3. Clinical, radiological and histological evaluation.
Representative images of alkalotic group (1-abc), acidic group (2-abc) and control (3-abc). Group 2 exhibits non-healing soft tissue lesion with extensive cortico-trabecular destruction and necrotic bone fractions, characterized by clusters of empty lacunae.

2 × 3 contingency table according to the three groups was made and then Chi-square test was performed. When the expected frequency was lower than 5, Fisher's exact test was performed. A P value of < 0.05 was considered statistically significant.

3. Results

Final analyses were performed omitting one of the control rats that died 2 weeks after surgical intervention. To confirm the effects of the respective agents to the systemic pH, ABG analyses were performed immediately within 15 min of blood collection. Each intervention exemplified its expected outcome wherein the pH of control was 7.38 ± 0.04 and alkalotic group exhibited a borderline alkalotic state ($P < 0.05$; 7.46 ± 0.04 for group 1-1, 7.41 ± 0.05 for group 1-2). For acidic group, group 2-1 which administrated 0.28 M NH_4Cl ad libitum exhibited a systemic pH of 7.26 ± 0.12 and group 2-2 showed a pH of 7.28 ± 0.06. ($P < 0.05$) Presence of ONJ in alkalotic group ($n = 10$ each) was both 60% as confirmed histologically, clinically and radiographically while control group exhibited 76.9% ($n = 10/13$; Fig. 3). On the other hand, most animals in the acidic group showed a higher occurrence of MRONJ, presenting a 90.0% for group 2-1 and 100% for group 2-2, which valued significantly higher among other groups ($P < 0.05$) (Fig. 4).

Histologically, necrotic bone was seen in all MRONJ animals in the acidic group along with the presence of pseudo-epitheliomatous epithelium overlying exposed and/or unexposed bone with osteolytic lesions and clusters of empty lacunae. Histomorphometric analysis revealed that acidic group ($n = 20$, 2-1 and 2-2) presented significantly lower bone area (34.30 ± 8.23, %), more necrotic bone fraction (36.33 ± 14.12, %) and clusters of empty lacunae (3.94 ± 2.27, #), and higher number of osteoclast (2.60 ± 1.33, #/μm) compared to both those of control and alkalotic group ($P < 0.05$; Table 1). In terms of administration route as represented by the sub-groups, locally administered medications resulted into more ONJ development versus those with systemic interventions, which were demonstrated by more evident ulcerative lesions with extensive destruction and disorganization of necrotic bone with cortical disruption and sequestrum. Locally administered subgroup showed higher prevalence, necrotic bone fraction and number of osteoclasts. ($P < 0.05$) Alkalotic group ($n = 20$, 1-1 and 1-2) showed possible protective effects against ONJ demonstrated by lower ONJ occurrence (60%), higher bone area (56.57 ± 14.27, %), number of osteoclasts (1.38 ± 0.63, #/μm) and necrotic bone fraction (16.98 ± 10.29, %), ($P < 0.05$ for acidic group) however these trends were not statistically significant versus control.

Three-dimensional μCT imaging demonstrated osteolytic lesions with more extensive tissue destruction and cortico-trabecular pattern disruption in the acidic group compared to the other groups. More animals in the alkalotic and control groups presented normal course of healing with new bone formation and improved architectural indices, despite signs of bone loss due to previous surgical extraction. Microarchitectural morphometric analysis showed significantly lower bone volume (BV/TV; 29.21 ± 11.98, %) and trabecular thickness (Tb.Th; 12.69 ± 5.25, μm) in acidic group. ($P < 0.05$) Locally administered acidic group (2-2) exhibited more impaired morphometric indices compared to group 2-1. Subgroup difference among alkalotic group (1-1 and 1-2) was not evident.

4. Discussion

Medication-related osteonecrosis of the jaw (MRONJ) brings about potentially severe complications in patients treated with BPs [31]. In 2014, the American Association of Oral and Maxillofacial Surgeons (AAOMS) defined MRONJ with the following characteristics in their position paper; (1) current or previous treatment with antiresorptive or antiangiogenic agents; (2) exposed bone or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region that

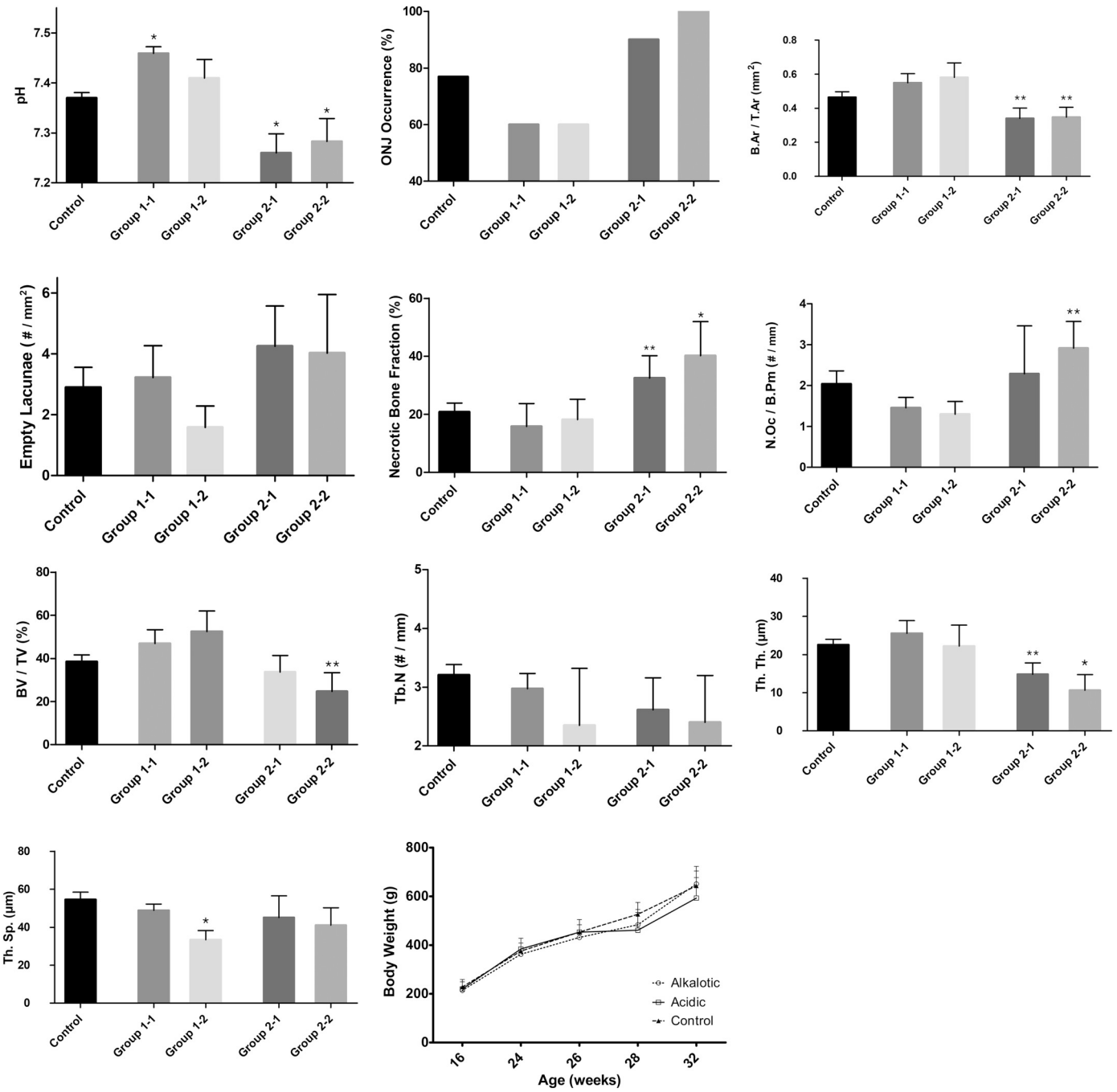

Fig. 4. Clinical, histomorphometric, bone micro-architectural morphometric evaluation among acidic, alkalotic groups and control.
Alkalotic group received 0.25 M of $NaHCO_3$ ad libitum (group 1-1, $n = 10$) and local injections of 4.2% of NaHCO3 (group 1-2, $n = 10$). Acidic group received 0.28 M NH4Cl + 0.5% sucrose ad libitum (group 2-1, $n = 10$) and local injections of 2% NH_4Cl (group 2-2, $n = 10$). Control received normal saline injection ($n = 10$).
Abbreviations; T.Ar, tissue area; B.Ar, bone area; B.Pm, bone perimeter; N.Oc, osteoclast number; BV, bone volume; TV, total volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation.
Column and bar indicates mean and standard error, respectively.
* indicates $P < 0.05$ compared to control, ** indicates non-significant versus control, but $P < 0.05$ versus alkalotic group 1-1.

has persisted for > 8 weeks; and (3) no history of radiation therapy to the jaws or obvious metastatic disease to the jaws [31]. Despite the growing number of reports and awareness linking prolonged BPs treatment with ONJ, however, the pathogenesis of this condition is still not well-defined [32,33].

To the best of our knowledge, this is the first in-vivo study inducing pH-altering conditions and determining its role on MRONJ pathogenesis. Based on the results of this study, the importance of incorporating abnormal acid-base condition in animals to be utilized for well-controlled future studies is emphasized. Also, the data presented are sufficient to favor the use of this condition to expound on the different risk factors and etiology, further leading to the discovery of effective treatment strategies for MRONJ.

Since its first report until at present, the exact pathogenesis of MRONJ remains controversial. Proposed theories include BP's effect on bone turnover suppression, inflammation, infection, angiogenesis inhibition and BP's cytotoxic effects on different cell types. Otto and colleagues proposed another perspective in which the acidic

Table 1
Clinical, histomorphometric, bone micro-architectural morphometric evaluation among acidic, alkalotic groups and control.

	Control	Group 1-1	Group 1-2	Group 2-1	Group 2-2
	Normal saline	Alkalotic, 0.25 M $NaHCO_3$ ad libitum	Alkalotic, local injections of 4.2% $NaHCO_3$	Acidotic, 0.28 M NH_4Cl ad libitum	Acidotic, local injections of 2% NH_4Cl
ONJ occurrence	76.9%	60.0%	60.0%	90.0%	100%
pH (aterial)	7.38 (0.04)	7.46 (0.04)*	7.41 (0.05)	7.26 (0.12)*	7.28 (0.06)*
B.Ar/T.Ar (%)	0.46 (0.12)	0.55 (0.17)	0.58 (0.12)	0.34 (0.09)†	0.35 (0.08)†
Necrotic bone (%)	20.84 (10.22)	15.79 (11.13)	18.17 (9.83)	32.46 (10.82)†	40.20 (16.45)*
Empty lacunae (#/mm^2)	2.45 (2.28)	2.26 (1.81)	1.42 (1.01)	4.25 (1.85)	3.62 (2.69)
N.Oc/B.Pm (#/mm)	2.04 (1.16)	1.46 (0.80)	1.30 (0.44)	2.28 (1.64)	2.92 (0.91)†
BV/TV (%)	38.59 (10.91)	46.97 (19.94)	52.42 (13.37)	33.73 (10.60)	24.69 (12.05)†
Tb. N (1/mm)	3.21 (0.65)	2.97 (0.81)	2.35 (1.36)	2.61 (0.77)	2.40 (1.11)
Tb. Th (μm)	22.51 (5.25)	25.54 (10.55)	22.19 (7.69)	14.81 (4.19)†	10.58 (5.78)*
Tb. Sp (μm)	54.51 (14.25)	48.84 (10.52)	33.41 (6.72)*	45.08 (15.87)	40.99 (12.92)

Alkalotic group received 0.25 M of $NaHCO_3$ ad libitum (group 1-1, $n = 10$) and local injections of 4.2% of $NaHCO_3$ (group 1-2, $n = 10$). Acidotic group received 0.28 M NH_4Cl + 0.5% sucrose ad libitum (group 2-1, $n = 10$) and local injections of 2% NH_4Cl (group 2-2, $n = 10$). Control received normal saline injection ($n = 10$).

Abbreviations; T.Ar, tissue area; B.Ar, bone area; B.Pm, bone perimeter; N.Oc, osteoclast number; BV, bone volume; TV, total volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation.

Results are shown as mean (SD).

* Indicates $P < 0.05$ compared to control.

† Indicates non-significant versus control but $P < 0.05$ versus alkalotic group 1-1.

environment, induced by local inflammation, infection and dentoalveolar surgery could be a predisposing factor for MRONJ progression [16,21]. When BP is released from bone due to an acidic environment, it produces cytotoxic effects on different cell types as manifested by bone and tissue destruction, and poor wound healing [15,16,24]. Bone homeostasis is dynamic and is greatly affected by many systemic and local conditions, especially by the acid-base balance and oxygen tension [1,2]. Either causative or resultant, acidosis is commonly associated to catabolic bone resorption [19,34].

Similarly, this present model exemplified non-healing extraction sockets, osteolysis, cortico-trabecular degradation and higher necrotic bone fractions in the acidic group compared to the other groups. In addition, this group exhibited a disparity in terms of clinical MRONJ and histological MRONJ diagnosis, acknowledging the subclinical, non-exposed type. This supports modification of the MRONJ classification of adding a 'stage 0' on the previously established system [20]. It is for this reason the authors defined the evidence of ONJ based on histological parameters. In terms of osteoclastic counts, acidic group exhibited less active osteoclasts, compared to both alkalotic and control, similar to the established animal models of this disease [26]. However, inactive osteoclasts were higher in this group and is consisted to a recent osteoclast profile study wherein giant, inactive osteoclasts were seen in MRONJ slides [35]. These results can support previous studies that bone in MRONJ is not necessarily devoid of osteoclasts and that inactivated osteoclasts could lead to this decrease in normal bone turnover.

The alkalotic group, which tested the potential for decreasing MRONJ manifestations by alkali supplementation, presented a 58% presence of ONJ. It is difficult to determine whether higher presence of ONJ in this group than control is attributed to the method of administration since significantly higher MRONJ occurrence were seen in rats in the local/mucosal injection sub-group than in the systemic/drinking water sub-group. Despite that the systemic sub-group's lower MRONJ occurrence may be attributed to the neutralizing ability of the sodium bicarbonate, similar to clinical trials previously mentioned [10–12]. Another study presented the same outcome wherein 100% MRONJ prevention was seen in rats by placing sodium bicarbonate-soaked collagen sponges in extraction socket [36].

In contrast to authors' previous research [26], male SD rats were used in this study. Interestingly, the incidence of ONJ in male rats was similar with ovariectomized female rats which were administered more zoledronate injection, and showed higher incidence than non-ovariectomized female rats. This might indicate the possible susceptibility of male on ONJ regardless of current absolutely higher proportion of ONJ in female patients due to osteoporosis, however, further research would be essential. One of the limitation of the study is that all of the animals had received BPs, therefore the effects of the acidosis itself couldn't be separated from the effects of acid plus BPs. Experimental intervention into acidic environment would increase the local concentration of BPs thus aggravating ONJ pathogenesis. More precise study setting to rule out the effects of BPs is necessary. In spite of all drawbacks, this design can still be utilized as in-vitro model for bone environment subjected to systemic and local pH decrease, commonly seen in the population at-risk for MRONJ. With a MRONJ incidence of 0%–0.04% in osteoporotic patients taking oral BPs [20] and approximately 1.3% in patients receiving high-dose BPs in cancer patients with bone metastases [37], the investigation for preventive and therapeutic potentials of this strategy would be necessary.

An acidic bone environment aggravated MRONJ development in this animal model as manifested by non-healing soft tissue, osteolytic lesions with clusters of empty lacuna, inactive osteoclasts and necrotic bone. Local factors such as dentoalveolar surgeries may predisposes the jaw to a decrease in pH and hence, disease development. In addition, further studies are needed to elucidate the treatment benefits of alkali supplementation in the prevention of MRONJ.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2016R1C1B2006270).

Conflict of interest

The authors declare no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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