Biomarker detection in

Bisphosphonate-related Osteonecrosis of the Jaw

Jin-Woo Kim

The Graduate School

Yonsei University

Department of Dental Science

Biomarker detection in

Bisphosphonate-related Osteonecrosis of the Jaw

Directed by Professor In-Ho Cha, D.D.S., Ph.D.

A Doctoral Dissertation
Submitted to the Department of Dental Science
and the Graduate School of Yonsei University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Jin-Woo Kim

Dec 2014

This certifies that the Doctoral Dissertation of

Jin-Woo Kim is approved.

_____

Thesis Supervisor: Professor In-Ho Cha

_____

Thesis Committee Member: **Professor Hyung-Jun Kim**

_____

Thesis Committee Member: **Professor Tae-Geon Kwon**

_____

Thesis Committee Member: **Professor Sun-Jong Kim**

_____

Thesis Committee Member: **Professor Won-Yoon Chung**

The Graduate School
Yonsei University
December 2014

Acknowledgement

Table of Contents

List of Figures and Tables

Abstract

Biomarker detection in
Bisphosphonate-related Osteonecrosis of the Jaw

Jin-Woo Kim

Department of Dental Science
The Graduate School, Yonsei University

Various bone biomarkers have been suggested for the risk assessment for osteonecrosis of the jaw, an oral complication associated with bisphosphonate (BP) use; however, no consensus has been established. The aim of this thesis was to investigate a possible biomarker for bisphosphonate-related osteonecrosis of the jaw (BRONJ) in an animal model.

Forty eight Sprague-Dawley rats were randomly divided into the two groups; bisphosphonate group (n=36) treated with weekly zoledronic acid injection and control group (n=12) treated with weekly saline injection. After 6 weeks, surgical intervention was performed, and weekly injections were continued up to 8 weeks. Rats in the bisphosphonate group were then further classified to the ONJ group and the non-ONJ group, and biomarkers, including serum CTx, Glu-OC, TRACP 5b, RANKL, and OPG, were assessed at baseline (T0), at surgical intervention (T1; 8 weeks) and at sacrifice (T2; 14 weeks). Histomorphometric analysis for quantification of osteoclasts was performed.

TRACP 5b levels and the RANKL/OPG ratio were significantly decreased over time in the ONJ group compared to the non-ONJ group ($P < 0.05$). At T2, the

area under the curve was 0.807 for TRACP 5b (sensitivity: 88.9%, specificity 66.7% at cutoff) and 0.765 for the RANKL/OPG ratio (sensitivity: 77.8%, specificity 62.9% at cutoff). TRACP 5b showed a lower least significant change (LSC; 29.6%) with lower intra-assay coefficient variability (CV; 6.32%) and inter-assay CV (11.20%) compared to those of the RANKL/OPG ratio (39.27%) and showed a higher signal-to-noise ratio (2.76) than that of the RANKL/OPG ratio (1.62). Moreover, N.Oc/T.Ar and N.Oc/B.Ar demonstrated significantly decreased number of osteoclasts in ONJ group versus non-ONJ group. ($P < 0.05$)

These results demonstrated that serum TRACP 5b and the RANKL/OPG ratio were possible biomarkers for BRONJ. These data may provide useful additional information for future ONJ research. Further studies are needed to validate these results in humans with ONJ.

Biomarker detection in
Bisphosphonate-related Osteonecrosis of the Jaw

Jin-Woo Kim

Department of Dental Science
The Graduate School, Yonsei University

(Directed by Professor In-Ho Cha)

I. Introduction

Since the first report of osteonecrosis of the jaw (ONJ) associated with bisphosphonate administration in 2003[1], there have been many attempts to establish the pathophysiological nature of this disease[2,3]. Based on the pharmacological effects of bisphosphonates on osteoclasts, ONJ has been thought to be associated mainly with the oversuppression of bone remodeling[2-4]. However, still a large part of the diseases including appropriate methods for the diagnosis, details of the mechanisms of pathogenesis, and effective treatment modalities are still not known.

Several research groups have attempted to detect biomarkers for ONJ[5,6]. Marx et al.[6] performed an uncontrolled retrospective study and found that serum C-terminal telopeptide of type I collagen (CTx) was a useful predictor of ONJ. However, contradictory results were reported in several subsequent clinical studies[5,7,8], and no conclusive evidence for other bone biomarkers has been published to date[9,10]. A recent position paper published by the American Association of Oral and Maxillofacial

surgeons stated that there was no evidence to support the use of currently proposed markers[11]. Given that the increasing global dependence on antiresorptive drugs for various bone diseases and the fact that no biomarkers have been validated for identifying patients at high risk of developing ONJ, there is an increasing need to establish biomarkers of ONJ.

Osteoclasts, which are thought to be key cells involved in the effects of bisphosphonates on ONJ, have two distinct functions: bone resorption and maintenance of bone remodeling balance. For measuring the osteoclast-dependent bone resorption, collagen-degraded molecules, such as CTx and N-terminal telopeptide of type I collagen, have significant roles in several bone diseases, such as metabolic, pathologic, and metastatic bone diseases[12]. Another function, which is independent of their resorptive activity, is regulation of the bone remodeling through coupling with osteoblasts[13-15]. This function is thought to be associated with osteoclast number and can be reflected by the levels of osteoclast-specific molecules, such as tartrate-resistant acid phosphatase isoform 5b (TRACP 5b)[16,17].

Important issue to be considered is that the osteoclast number rather than osteoclast activity may mediate the coupling between bone resorption and bone formation, which is essential for continuous bone remodeling[13,18,19]. Previously proposed ONJ markers, such as CTx, only reflect the resorptive activity[5,12], and the role of osteoclasts in bone remodeling is thought to be directly associated with bisphosphonate-mediated suppression of bone remodeling in ONJ pathogenesis[2,3], suggesting that biomarkers reflecting the degree of bone remodeling suppression may represent ONJ biomarkers. Hence, the investigator preferentially considered following osteoclast-specific markers as ONJ biomarker candidates; TRACP 5b for reflection of osteoclast number which is thought to be associated with degree of bone

remodeling[16,18,19], and circulating RANK/RANKL/OPG system markers as key molecules for regulation of osteoclasts function[20-23]. Analysis also included CTx and osteocalcin which had shown controversial clinical results.

The aim of this thesis was to identify novel biomarkers for ONJ using an animal model. Our analysis included the investigation of significant biomarkers for ONJ, the assessment of clinical performance of biomarkers, and biological interpretation through characterization of histological and radiological features of ONJ.

II. Materials and Methods

1. Establishment of Animal model and Study design

Eight-week-old female Sprague-Dawley (SD) rats (n = 48) with a mean weight of 220 g (range, 190–247 g), purchased from the Institute of Ewha Medical Research, Seoul, Korea (Orient Bio Inc., Seoul, Korea), were used in this study. Animals were housed at three rats per cage and were individually numbered using ear punches. The cages were placed in a room with filtered air at a temperature of $22 \pm 2°C$ and $50\% \pm 10\%$ relative humidity. A 12-h light/dark cycle was maintained. The animals were fed a normal rodent diet and water *ad libitum*. Animals were acclimated for 1 week prior to the beginning of the study. This study was approved by and performed in accordance with the guidelines of the institutional animal research ethics committee.

The rats were randomly divided into two groups. The bisphosphonate group (n = 36) received zoledronic acid (200 μg/kg), and the control group (n = 12) received normal saline once a week subcutaneously[24,25]. Given that the pharmacodynamics of bisphosphonates have not been determined in rodents, we determined dosing according to the following factors: (1) the oncologically relevant zoledronate doses in humans (67 μg/kg/4 weeks); (2) the relatively rapid bone metabolism of rodents; (3) the route of bisphosphonate administration; lower plasma concentrations when using the subcutaneous route compared to intravenous route; and (4) maximizing drug exposure during the relatively short experimental period.

After 6 weeks of injections, tooth extraction was performed as a surgical intervention to induce ONJ. Animals were anesthetized with an intramuscular

injection of ketamine (100 mg/kg ketamine, Yuhan Pharmacy, Seoul, Korea), xylazine

(10 mg/kg Rompun, Bayer AG, Leverkusen, Germany), and meloxicam (1.5 mg/mL

Metacam, Boehringer Ingelheim, Germany). After syndesmotomy using a sharpened

dental explorer, adequate luxation was performed until sufficient mobility of the tooth

was achieved. In this manner, the lower left first to third molars were extracted using

dental explorer in consecutive order. Gauze pressure was applied to control bleeding,

and the wounds were left open without any sutures. Weekly injections were continued

for 8 more weeks, a timeframe chosen according to the pathogenic progression of

BRONJ[11]. The animals were then sacrificed for further assessment (Fig. 1).
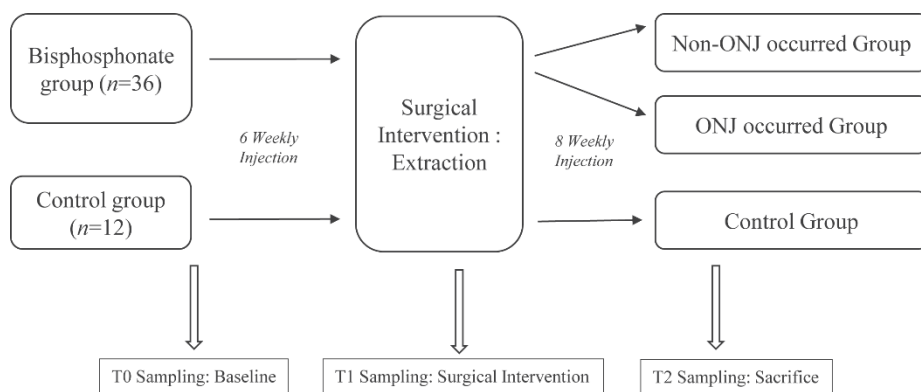

Figure 1. Study design

2. Radiological Assessment

The ONJ lesion was radiologically assessed by micro-computed tomography (μCT, Skyscan 1076, Aartselaar, Belgium). The acquisition settings were at a voxel size of 6 μm^3 with an X-ray tube voltage of 70 kVp and the intensity current of 140 μA. An entire region of the mandible (1335 slices; each slice = 8 μm) was scanned for each sample. μCT scans revealed the presence of osteolytic lesions accompanied by a wide destruction of trabecular patterns with irregular bone margins and sequestrum formation in ONJ group. In control group, the extraction socket was nearly filled with newly formed bone with normal trabecular patterns. On the other hand, non-ONJ group showed insufficient bone fill with osteosclerotic changes of trabecular patterns. (Fig. 2)

3. Histological Assessment

Samples were fixed with 4% phosphate-buffered saline formalin for 2 weeks and decalcified in 10% EDTA (pH 7.2) at 4°C for 28 days. Samples were dehydrated with a graded series of ethanol, cleaned with xylene, and immersed in paraffin. The samples were then embedded in paraffin and cut into 4-μm-thick sections. The sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H-E). We determined the presence of ONJ based on histological analysis according to the following criteria: (1) presence of an ulcerative lesion with exposed and necrotic bone and/or osteolysis, (2) presence of pseudoepitheliomatous-like hyperplasia of the epithelium accompanied by inflammatory cell infiltration, and (3) presence of sequestrum and bacterial colonies.

(a)

(b)
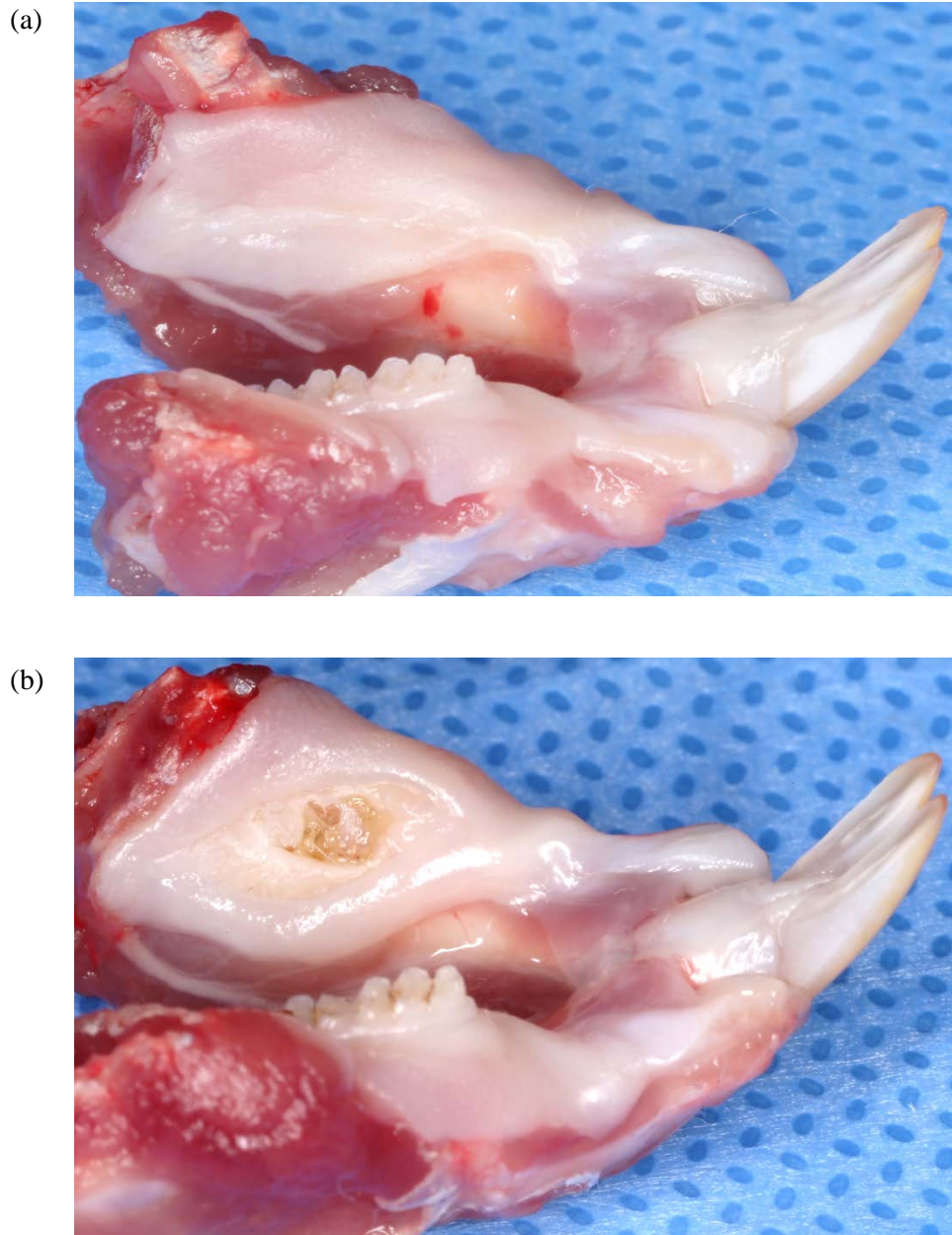
Figure 2. Representative clinical and radiological images.

(a) Non-ONJ group showing normal mucosal healing 8 weeks after surgical

intervention (b) ONJ occurred group.

(c)

(d)

(e)
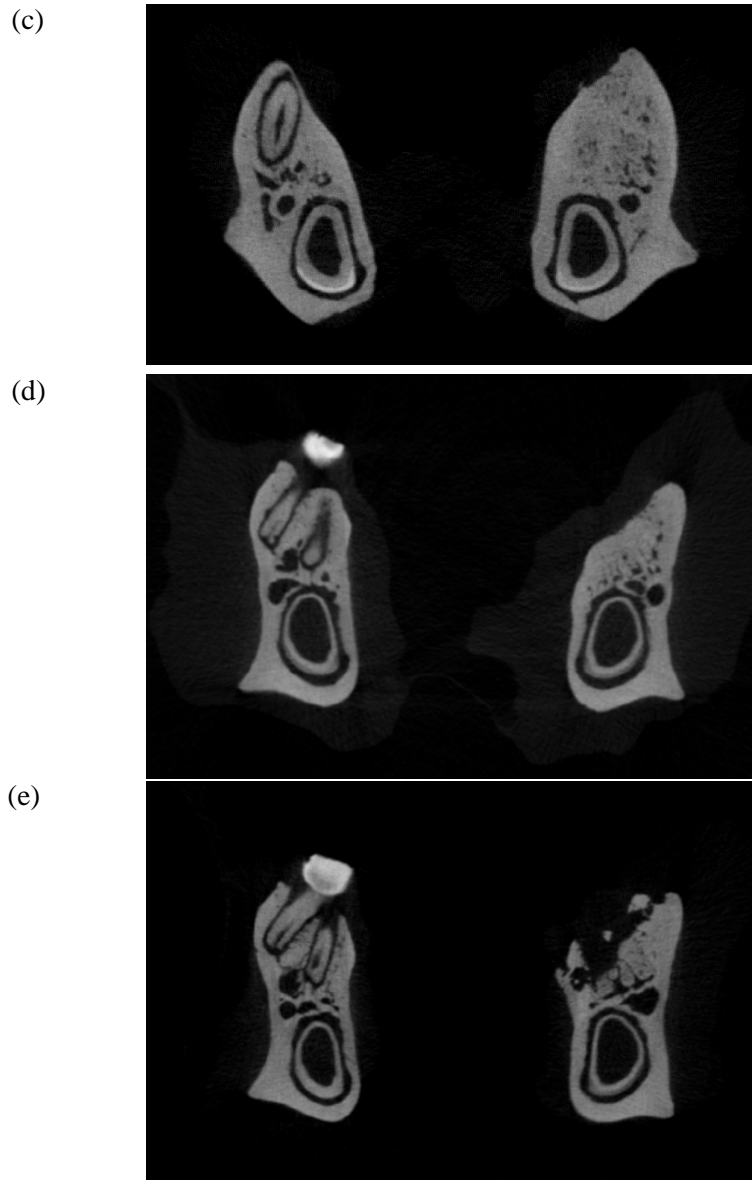
(**Fig 2. Continued**) μCT assessment revealed evident bony healing in control group (c) and non-ONJ group (d). Particularly, non-ONJ group showed osteosclerotic changes of trabecular patterns with dense woven bone and insufficient bone fill compared to control. Typical radiological features of ONJ – osteolytic lesions accompanied by a wide destruction of trabecular patterns with irregular bone margins and sequestrum – were observed in (e).

4. Histomorphometric analysis

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). In a blind-coded fashion, histomorphometric evaluation was performed in the 4 to 6 regions of interest (ROI) located at the osteonecrosis lesion or prior extraction site with Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA). Measurements included tissue area, (T.Ar, mm^2), bone area (B.Ar, mm^2) and number of osteoclast (N.Oc, #). To normalize indices, B.Ar/T.Ar (%), N.Oc/T.Ar (#/mm^2) and N.Oc/B.Ar (#/mm^2) were calculated for further analysis[26].

5. Biomarkers Investigation and Statistical Analysis

For the biomarker investigation, blood samples were obtained after 12 h of fasting at time T0 (baseline), T1 (6 weeks; surgical intervention), and T2 (14 weeks; sacrifice). Blood samples were collected through the jugular vein (T0, T1) and by cardiac puncture (T2) and were then allowed to coagulate for 20 min. Collected serum samples were separated by centrifugation (1600 × g for 15 min at 4°C), aliquoted, and stored at -70°C until analysis. The concentrations of serum CTx (RatLaps EIA, IDS, Boldon, Tyne & Wear, UK), serum undercarboxylated osteocalcin (Glu-OC; Rat Glu-Osteocalcin high sensitive EIA, Takara Bio, Shiga, Japan), serum TRACP 5b (RatTRAP, IDS, Boldon, Tyne & Wear), serum receptor activator of nuclear factor-κβ ligand (RANKL; Rat sRANKL ELISA, Cusabio, Wuhan, Hubei, China) and serum osteoprotegerin (OPG; Rat sOPG ELISA, Cusabio) were determined in accordance

with the manufacturer's instructions. All marker measurements were performed blinded and in duplicates.

According to the results of histological assessment, rats in the bisphosphonate group (n = 36) were further classified into the non-ONJ and ONJ groups. The normal distribution and homogeneity of variance for the biomarkers studied (CTx, Glu-OC, TRACP 5b, and the RANKL/OPG ratio) were checked before further statistical analyses. Overall group differences in biomarkers over time were compared with repeated measures analysis of variance (RM-ANOVA). Independent t-tests were used to test the significance of differences between the non-ONJ group and the ONJ group and between the bisphosphonate group and the control group. Receiver operating characteristic (ROC) curve analysis at T1 and T2 was used to assess the clinical performance of the biomarkers. The area under curve (AUC) and cutoff values at which the sum of the biomarker sensitivity and specificity was highest (Youden's J statistic) were calculated. The intra-assay coefficient of variability (CVa; analytical variability) of each marker was determined as the mean value of duplicated measurements. The inter-assay coefficient of variability (CVi; biological variability) was determined as the mean change observed at T2 compared with T0 in the control group. The least significant change (LSC) at $P < 0.05$ was determined based on the equation: $LSC = 2.33 \times \sqrt{(CVa^2 + CVi^2)}$[27]. The signal to noise ratio was determined by dividing the change of each marker in the bisphosphonate group at T2 (signal) with the marker's variability (noise; $\sqrt{CVa^2 + CVi^2}$). The normalized number of osteoclasts were compared using analysis of variance with Bonferroni correction. A P value of less than 0.05 was considered statistically significant. Statistical analysis was carried out using SPSS 20.0 (IBM Corp., Armonk, NY, USA) and MedCalc 12.7.0 (Medcalc software, Ostend, Belgium).

III. Results

1. ONJ determination and histological features

Of the 36 rats in the bisphosphonate group, 27 rats were histologically classified into the ONJ group, and nine rats were classified into the non-ONJ group. Representative histologic sections are presented in figure 3. Control group showed normal course of healing of extraction socket with newly filled bone and complete mucosal coverage. On the other hand, ONJ group showed extensive ulcerative lesion accompanied by exposed and necrotic bone with sequestrum and bacterial colonies. Also pseudoepitheliomatous-like hyperplasia of epithelium accompanied with inflammatory cell infiltration to the connective tissue was observed. Non-ONJ group showed mucosal coverage over extraction socket, however the presence of inflammatory process and microbial colonies were observed. Also marked decrease of bone fill was found compared to control group.
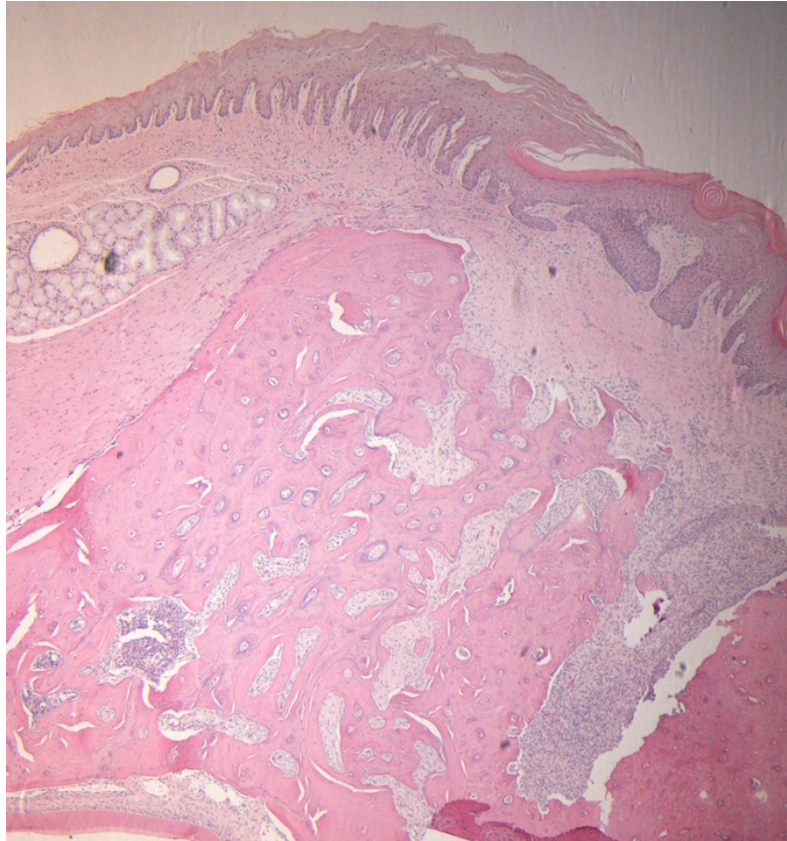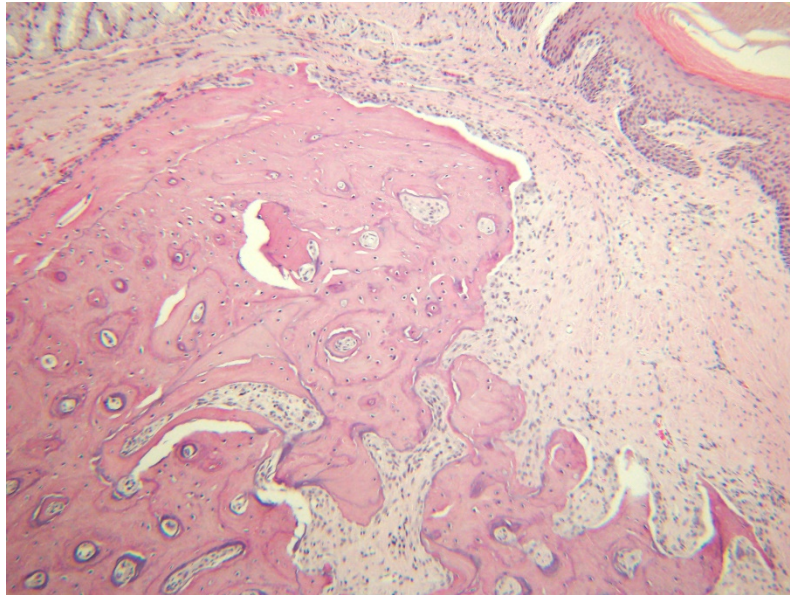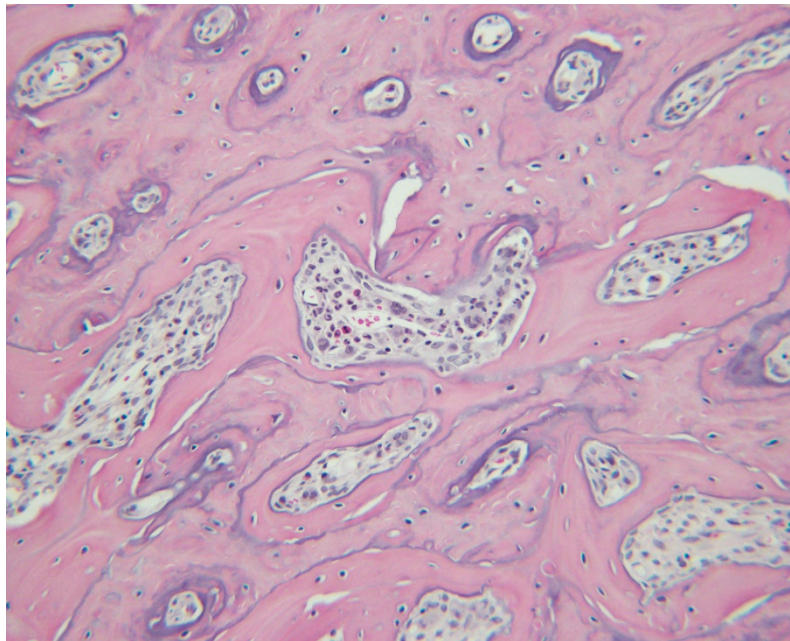
(a)

Figure 3. Representative histological features of each group

(a) Control rat showing normal healing course after post-extraction with complete
mucosal healing and sufficient bone fill. (HE, X40)

(b)

(c)

(**Fig 3. Continued**) (b, c) Normal trabecular patterns and bone remodeling are observed. Note the cellular lacunae, vascularity, and reversal line. (HE, X100, 200)

(d)
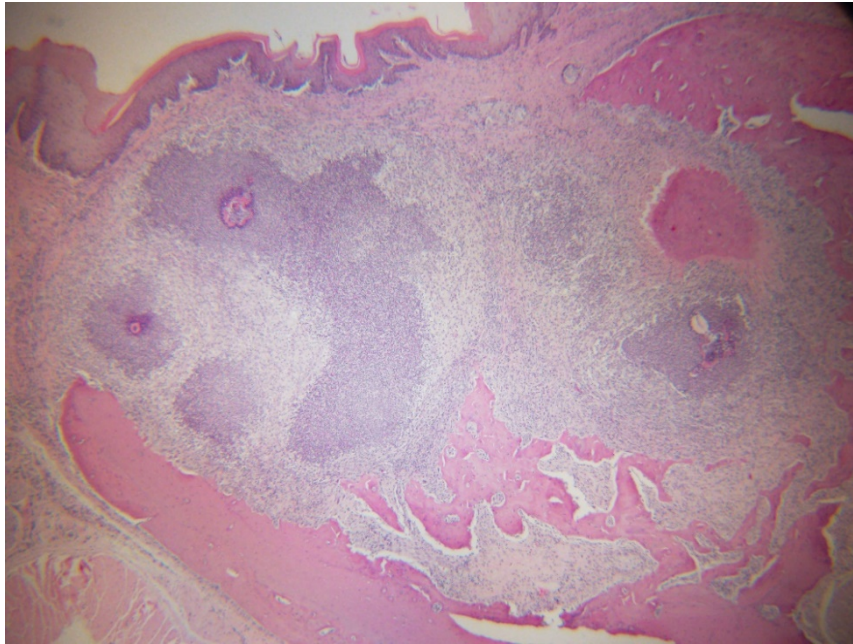
(e)
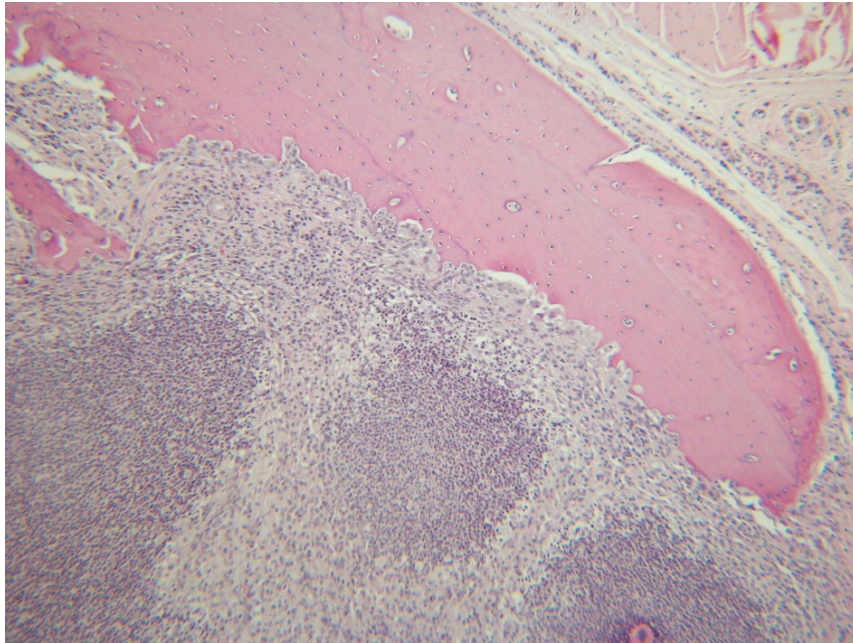
(**Fig 3. Continued**) (d) Non-ONJ rat showing normal mucosal healing, but minimal bone fill is observed with the presence of sequestrum and inflammatory cell infiltration within connective tissue. (HE, X40), (e) Non-ONJ rat showing abnormal bone remodeling patterns showing minimal bone fill from the reversal line. Note vital osteocytes and reduced vascularity. (HE, X100)

(f)

(g)
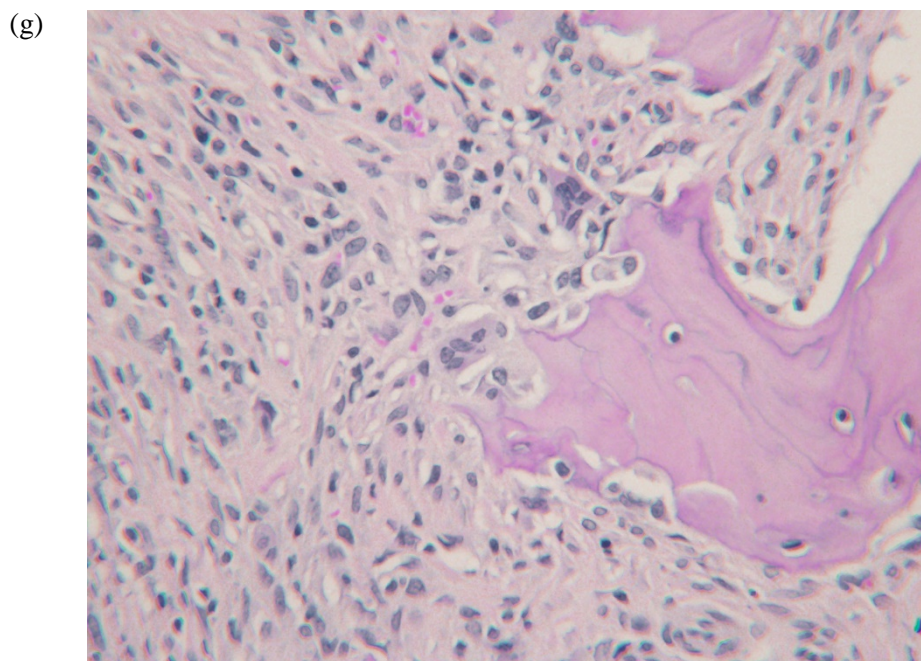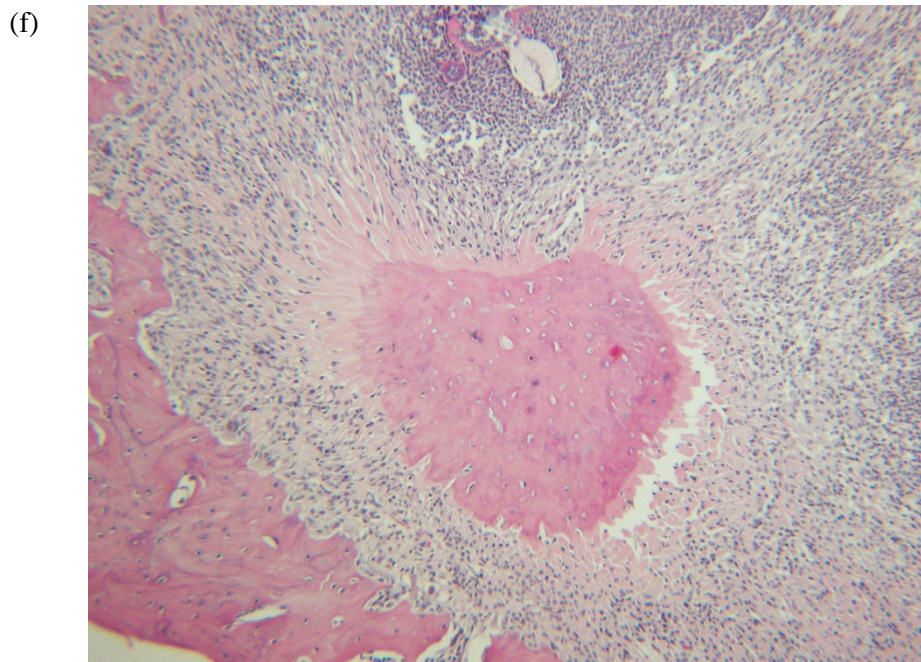
(**Fig 3. Continued**) (f) Sequestrum and infiltration of inflammatory cells (HE, X100), (g) Detached, hypernucleated, and not-resorbing inactive osteoclasts (HE, X400)

(h)
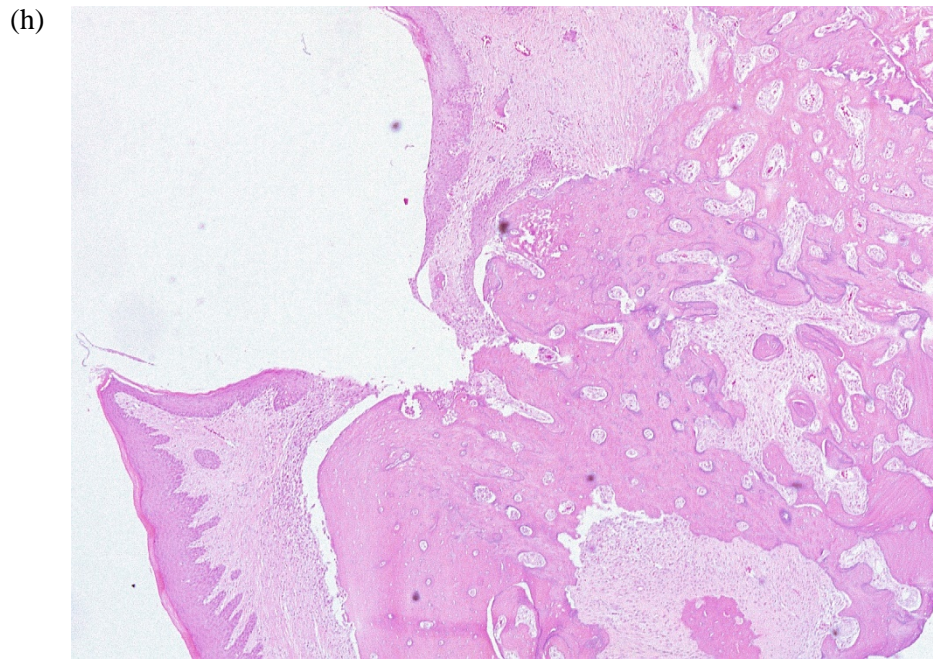
(**Fig 3. Continued**) (h) Representative ONJ section showing ulcerative mucosa
with exposed and necrotic bone with osteolysis (HE, X40)

(i)
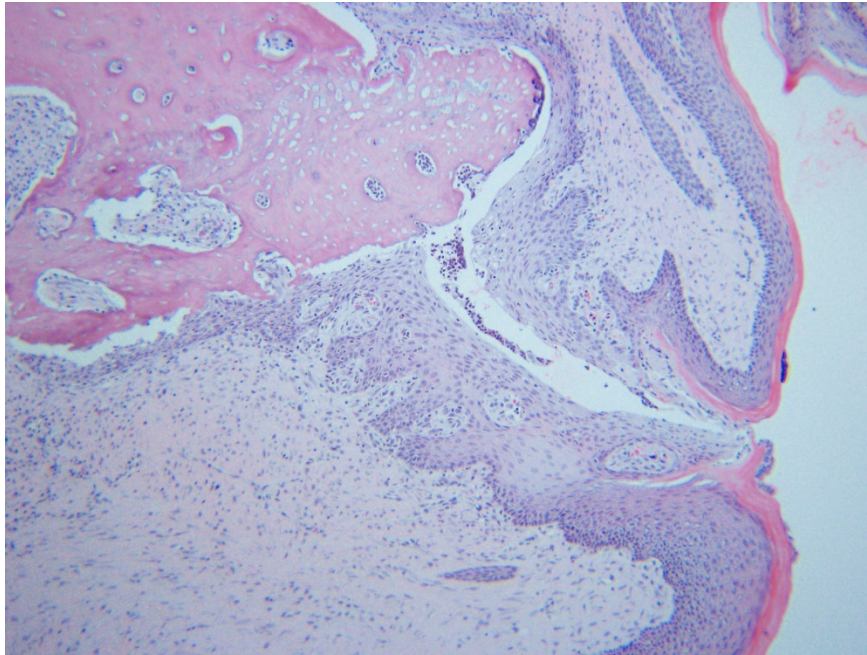
(j)
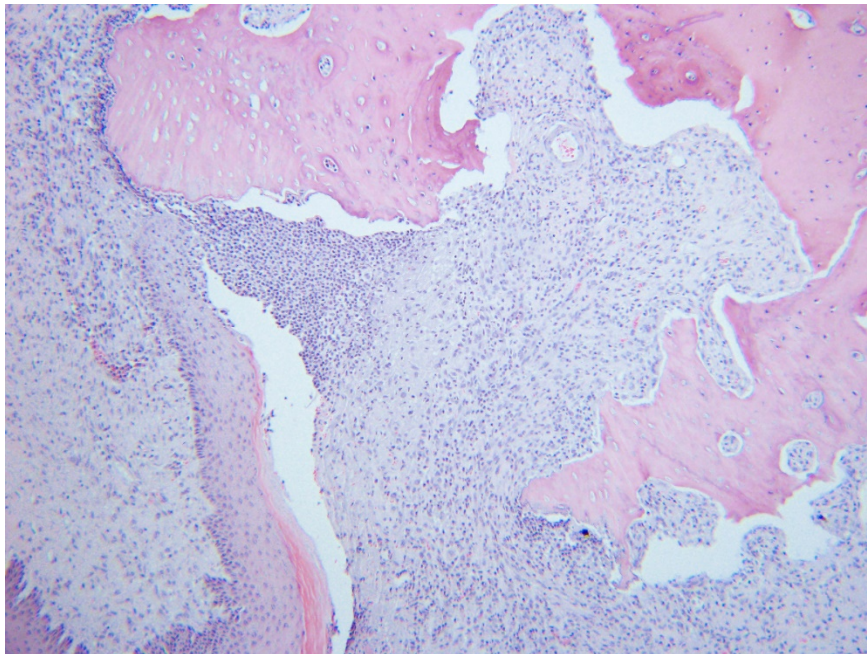
(**Fig 3. Continued**) (i,j) Histologically determined ONJ section showing necrotic bone associated with pseudoepitheliomatous hyperplasia and infiltrates of inflammatory cells. Note empty osteocytic lacunae and extensive scalloping of bone edges suggestive of interrupted Howship lacunae (HE, X100)

(k)
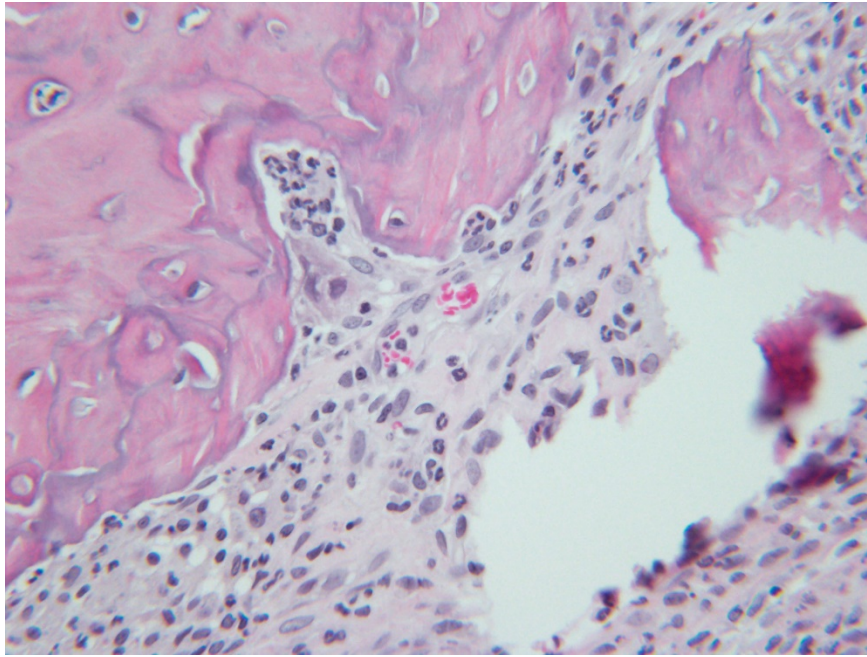
(l)
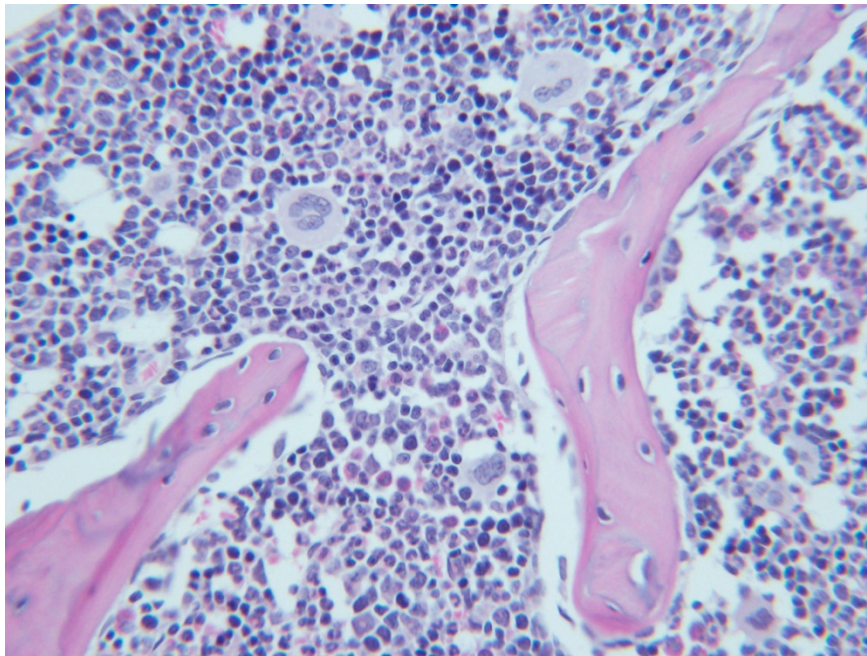
(**Fig 3. Continued**) (k) Osteoclasts attempting to resorb zoledronate loaded bone

showing apoptotic and pyknotic nuclei. (HE, X400), (l) Abnormally osteoclast-poor

hematopoietic tissue besides bone. (HE, X400)

2. Investigation of biomarkers

The values of all the studied markers are presented in Table 1. CTx, TRACP 5b, and RANKL were significantly decreased in the bisphosphonate group compared to control group at both T1 and T2.

For analysis of potential biomarkers of ONJ, we analyzed the biomarker concentrations over time between the non-ONJ group and the ONJ group. Our data demonstrated that the markers which showed significant group difference were TRACP 5b and the RANKL/OPG ratio (Figure 4). RM-ANOVA revealed the significant lower value of TRACP 5b and RANKL/OPG ratio in ONJ group over time compared to non-ONJ group. ($P<0.05$) These results were consistent at T1 for the RANKL/OPG ratio and at T2 for TRACP 5b and the RANKL/OPG ratio ($P < 0.05$).

Table 1. Values of Biomarkers between two groups

		T0	T1	T2
CTx (pg/mL)	Control	22.86 (10.81)	20.41 (11.27)	22.91 (10.15)
	Non-ONJ	21.61 (8.35)	10.50 (3.38)[*]	11.61 (7.48)[*]
	ONJ	20.70 (7.90)	10.33 (3.20)[*]	10.62 (3.54)[*]
Glu-OC (µg/L)	Control	39.59 (10.96)	42.72 (15.74)	41.28 (10.71)
	Non-ONJ	41.85 (10.66)	30.46 (11.81)	27.53 (13.69)
	ONJ	45.22 (14.38)	31.74 (12.74)	30.26 (13.52)
TRACP 5b	Control	5.07 (1.08)	4.68 (1.26)	5.25 (1.55)
	Non-ONJ	4.62 (1.12)	2.34 (1.38)[*]	3.21 (0.71)[*]
	ONJ	4.33 (1.21)	2.25 (1.14)[*]	2.40 (0.76)[*†]
RANKL (pg/mL)	Control	783.64 (141.41)	905.50 (57.46)	853.52 (92.44)
	Non-ONJ	824.55 (102.84)	693.41 (121.71)[*]	682.21 (100.60)[*]
	ONJ	798.69 (107.99)	613.21 (151.66)[*]	580.92 (103.91)[*]
OPG (pg/mL)	Control	611.99 (157.26)	657.71 (99.85)	677.79 (134.96)
	Non-ONJ	705.76 (129.69)	669.19 (169.62)	669.54 (177.29)
	ONJ	626.92 (137.34)	749.60 (184.08)	720.88 (145.53)
RANKL/OPG Ratio	Control	1.36 (0.46)	1.41 (0.27)	1.33 (0.40)
	Non-ONJ	1.19 (0.15)	1.07 (0.19)[*]	1.08 (0.32)[*]
	ONJ	1.33 (0.31)	0.85 (0.24)[*†]	0.83 (0.20)[*†]

Abbreviations; CTx, C-terminal crosslinked telopeptide of type I collagen; Glu-OC,
Undercarboxylated osteocalcin; TRACP 5b, Tartrate-resistant acid phosphatase isoform 5b;
RANKL, Receptor activator of nuclear factor-κβ ligand; OPG, Osteoprotegerin
T0; Baseline, T1; At surgical intervention (6 weeks), T2; At sacrifice (14 weeks)

Results are shown as mean (SD).
* indicates significant group difference ($P < 0.05$) compared to control group at the same time point
† indicates significant group difference ($P < 0.05$) between Non-ONJ group and ONJ group at the same time point

(a)

(b)
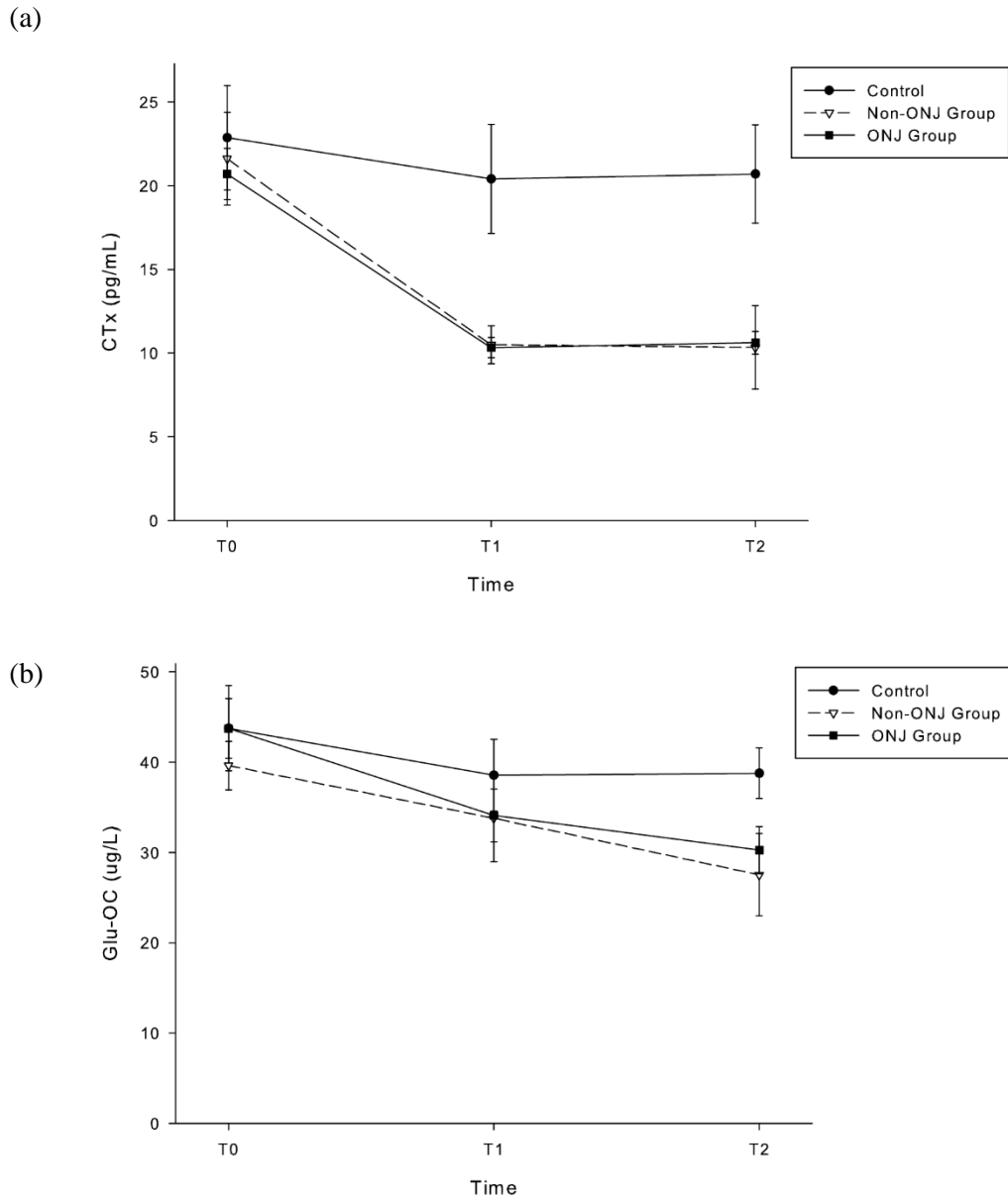
Figure 4. Trends of biomarkers over time. (a) C-terminal crosslinked telopeptide of type I collagen (CTx), (b) Undercarboxylated osteocalcin (Glu-OC)

T0; Baseline, T1; At surgical intervention (6 weeks), T2; At sacrifice (14 weeks)

The results are shown as the mean (SE).

* indicates $P < 0.05$ between Non-ONJ group versus ONJ-group.

(c)

(d)
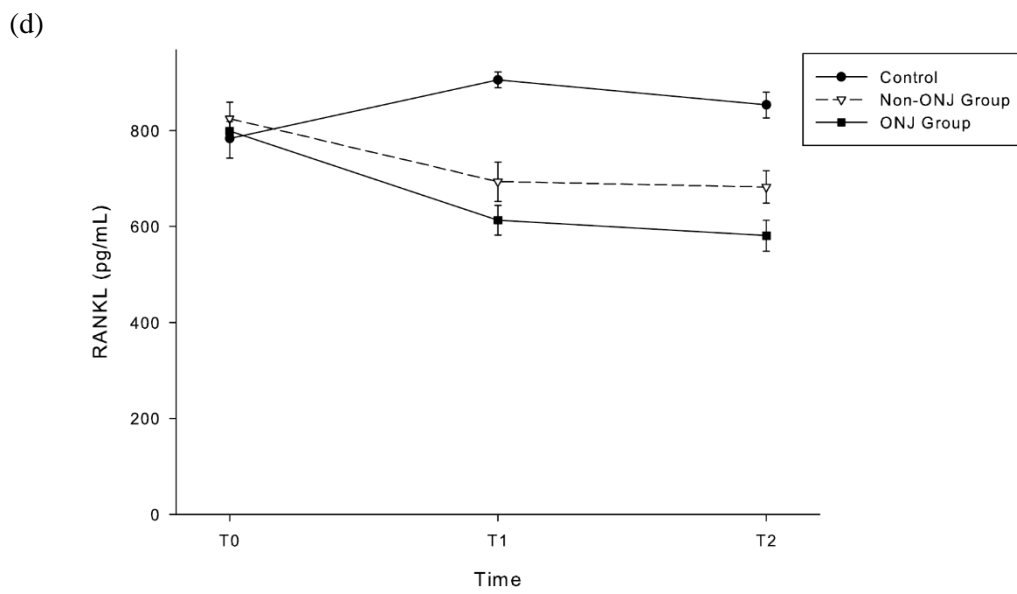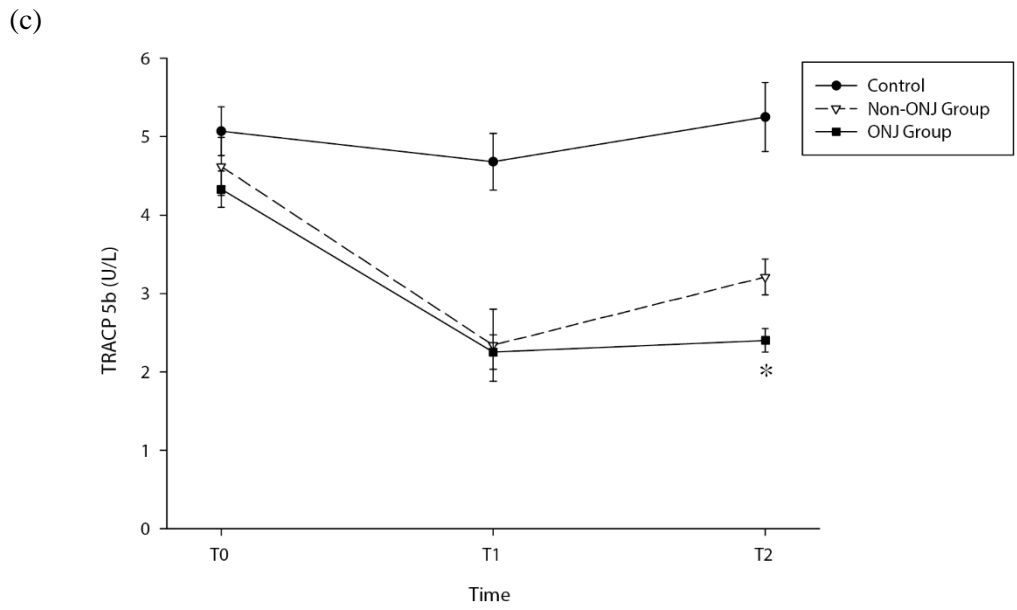
(**Fig 4. Continued**) (c) Tartrate-resistant acid phosphatase isoform 5b (TRACP 5b),
(d) Receptor activator of nuclear factor-κβ ligand (RANKL)

(e)

(f)
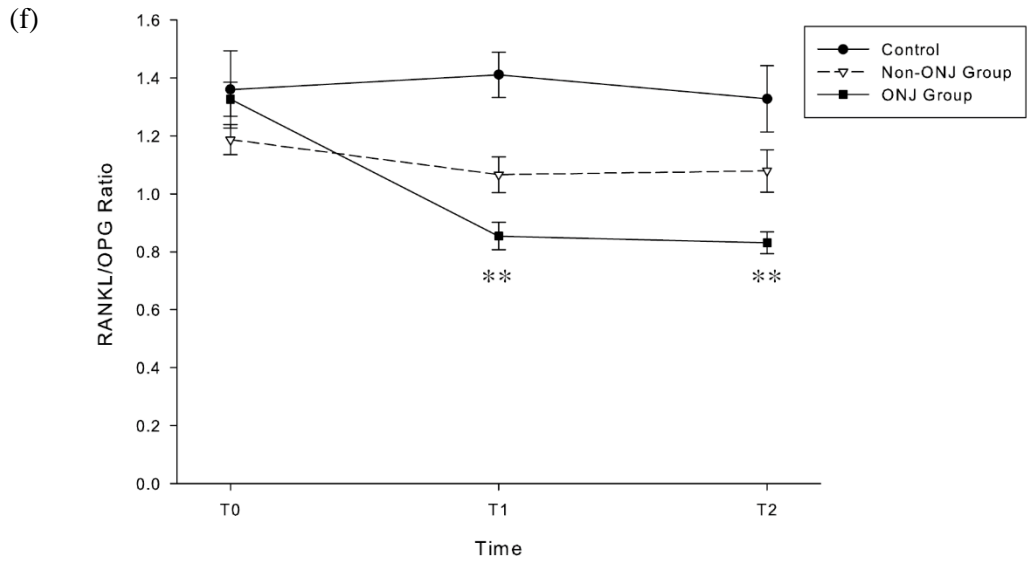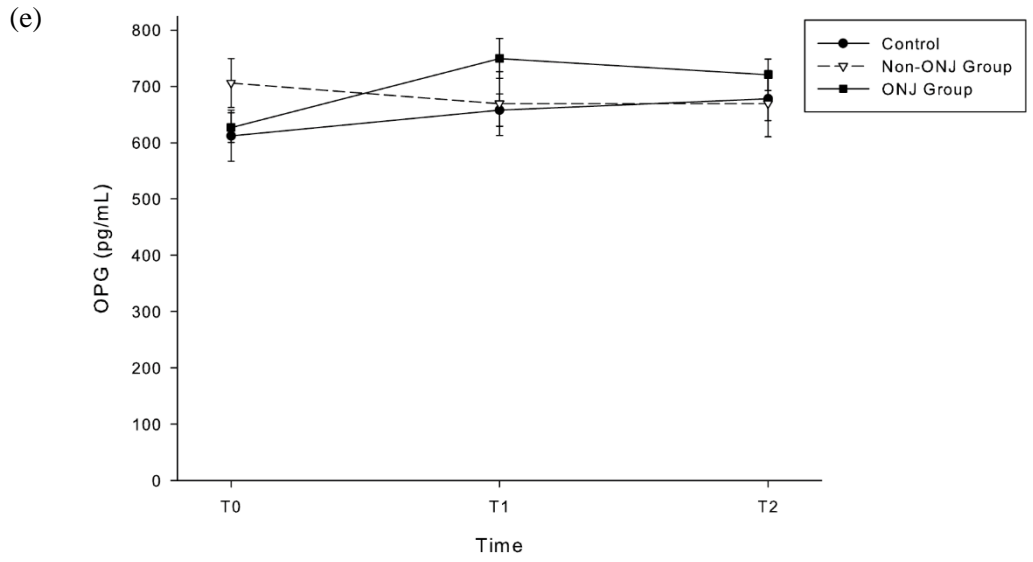
(**Fig 4. Continued**) (e) Osteoprotegerin (OPG), (f) RANKL/OPG Ratio

3. Clinical performance of biomarkers

To assess the clinical performance of TRACP 5b and the RANKL/OPG ratio as a biomarker for ONJ, we constructed ROC curves at T1 and T2 (Figure 5). For TRACP 5b at T2, the AUC was 0.807 (95% confidence interval [CI], 0.641–0.919; P < 0.001), and the cutoff value with both maximal sensitivity and specificity was 3.09 U/L or less. At this cutoff, the sensitivity and specificity of TRACP 5b for the diagnosis of ONJ were 88.9% and 66.7%, respectively. For the RANKL/OPG ratio at T1, the AUC was 0.776 (95% CI, 0.606–897; P < 0.001). The AUC at T2 was 0.765 (95% CI, 0.595–0.890; P = 0.002), and the cutoff value was 0.88 or less (sensitivity, 77.8%; specificity, 62.9%).
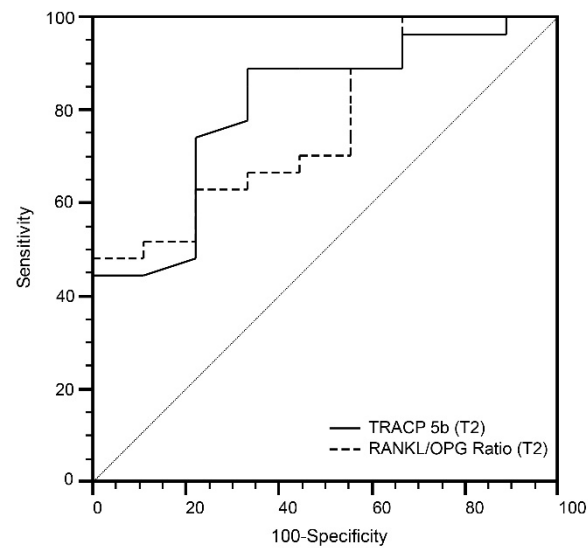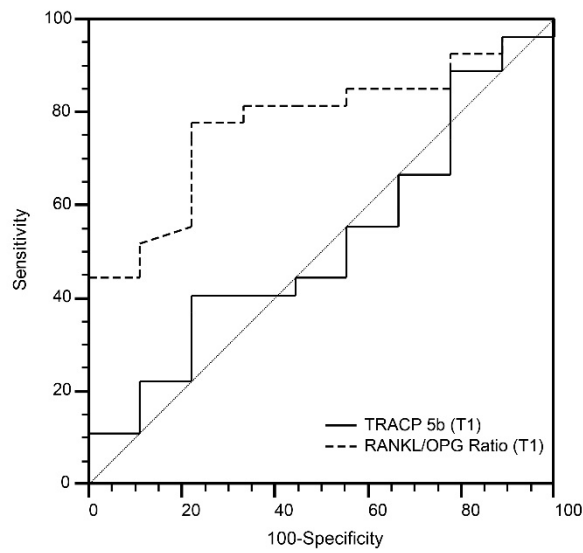
Figure 5. Receiver operating characteristic (ROC) curves of serum TRACP 5b and RANKL/OPG ratio

T1: TRACP 5b, AUC (95% CI) = 0.519 (0.346-0.688), $P > 0.05$; RANKL/OPG Ratio, AUC (95% CI) = 0.776 (0.606-0.897), $P < 0.001$

T2: TRACP 5b, AUC (95% CI) = 0.807 (0.641-0.919), $P < 0.001$; RANKL/OPG Ratio, AUC (95% CI) = 0.765 (0.595-0.890), $P = 0.002$

Individual percent changes (T2 – T0) of TRACP 5b and the RANKL/OPG ratio are shown in Figure 6. TRACP 5b had a lower LSC (29.6%) with lower intra-assay CV (6.32%) and inter-assay CV (11.20%) compared to those of the RANKL/OPG ratio (intra-assay CV, 8.14%; inter-assay CV, 14.76%; LSC, 39.27%). TRACP 5b had a higher signal-to-noise ratio (2.76) than the RANKL/OPG ratio (1.62).
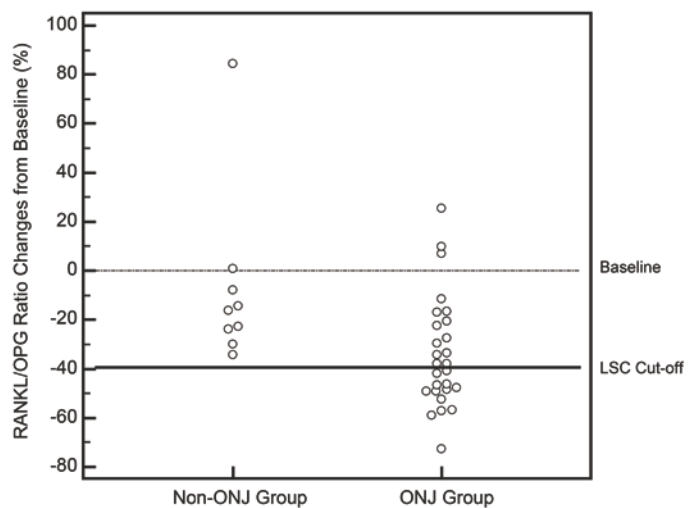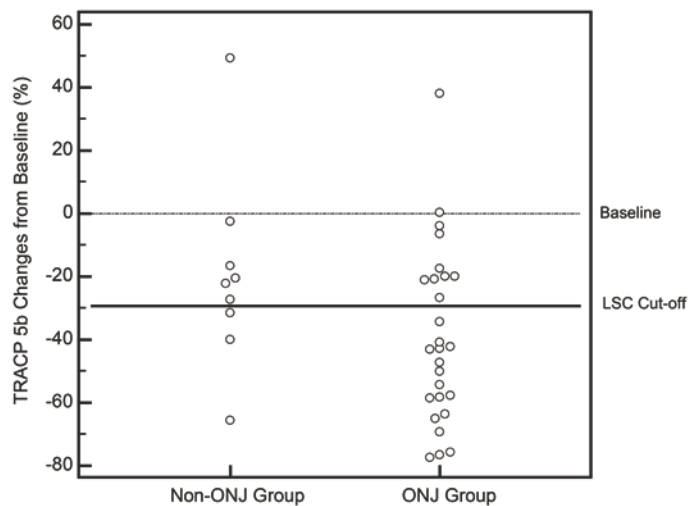
Figure 6. Individual percent changes of TRACP 5b and RANKL/OPG ratio

TRACP Intra-assay CV: 6.32% / Inter-assay CV: 11.20% / LSC: 29.96%

RANKL/OPG Ratio Intra-assay CV: 8.14% / Inter-assay CV: 14.76% / LSC: 39.27%

4. Histomorphometric analysis

In histomorphometric analysis, there was no group difference in T.Ar, B.Ar and B.Ar/T.Ar. It was evident that N.Oc of ONJ group (4.96 ± 1.83) were significantly lower than that of control (11.00 ± 2.52) and non-ONJ group (7.78 ± 1.30). ($P < 0.05$) Normalized indices, N.Oc/T.Ar and N.Oc/B.Ar demonstrated significantly lower number of osteoclasts in ONJ group than non-ONJ group. ($P < 0.05$; Fig. 7)
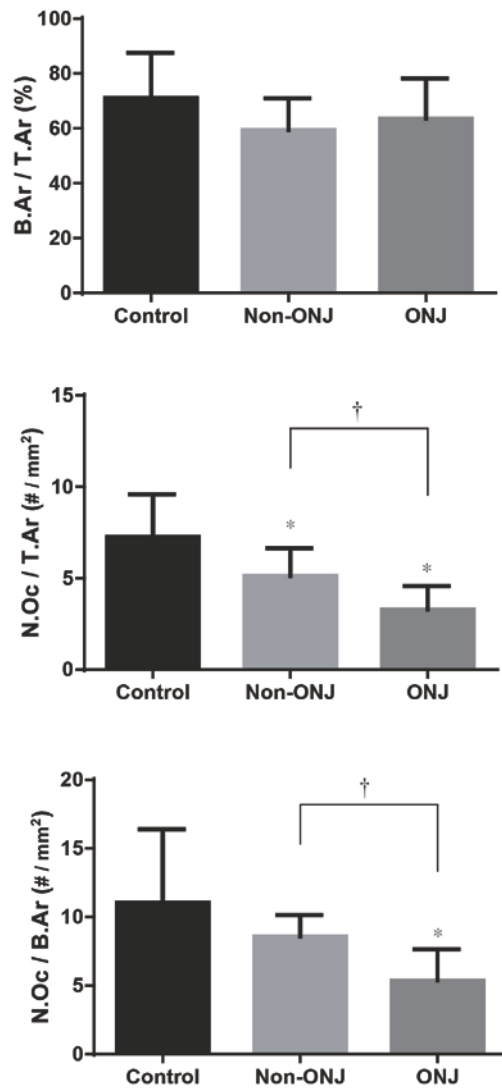
Figure 7. Histomorphometric analysis showing significantly lower number of osteoclasts in ONJ-group

* indicates $P < 0.05$ versus control, † indicates $P < 0.05$ between ONJ and Non-ONJ group

Abbreviations; T.Ar, tissue area; B.Ar, bone area; N.Oc, number of osteoclasts

IV. Discussion

This study was conducted to identify novel potential biomarkers of ONJ using several bone markers, including serum CTx, Glu-OC, TRACP 5b, RANKL, and OPG in an animal model. The results showed that serum TRACP 5b and the RANKL/OPG ratio were possible biomarkers for ONJ. Because no consensus has been reached on with the accuracy of currently proposed candidate biomarkers, this thesis was important in that it investigated possible biomarkers based on the pathogenesis of ONJ. Additionally, biomarkers may reflect dynamic conditions in the bone milieu that cannot be observed using conventional clinical or radiologic modalities; therefore, these biomarkers will function as useful tools for the diagnosis, prognosis, and risk prediction of ONJ. Furthermore, elucidation of the molecular roles of these biomarkers may provide insights into the direction of future ONJ research.

Rats in this study were designed to approximate the cumulative treatment regimen of cancer patients who received a single IV administration of zoledronic acid every 3 to 4 weeks (12 to 16 doses per year) during relatively short 14 weeks experimental period[24]. With consideration of rapid healing of rodents, which takes one-third of the time as compared to human[28], dosing frequency was determined as once a week. Although definitive pharmacokinetics of zoledronate in rodents are not fully understood, crucial features - uptake clearance of BPs by bone, renal clearance, and plasma protein binding characteristics - of rodents have been reported to be similar or slightly higher than those of human[29,30]. Moreover, a recent study reported that single bolus injection develop more ONJ lesion compared to repeated fractional dosing in spite of the equivalent cumulative dose[31]. Given the lower C_{max} (maximum serum concentration that a drug achieves in body after the drug has been administered)

and AUC (total drug exposure over time) of subcutaneous injection[30], the rats in this study received a subcutaneous zoledronate dose 3 times higher as compared to the standard IV human regimen.

As a result, 75% of zoledronate-injected rats developed ONJ lesion, which is much higher compared to the incidence of 0.8-12% of IV bisphosphonate-treated human patients[32]. More frequent dosing cycle and higher single dose are thought to bring higher total drug exposure to calcified tissue, followed by dose-dependent increase of ONJ development[33,34]. Regarding the unique pharmacologic characteristics of bisphosphonates – very low absorption rate of <1%, rapid initial uptake clearance, and very low drug plasma concentrations, direct influence of bisphosphonates concentration on serum biomarkers seems minimal[30]. However, it cannot be excluded that highly deposited bisphosphonates possibly amplified the changes of biomarker over time. Variability between species should be considered as well for interpreting results of this study. Properly designed clinical trials are urgently needed to validate the utility of biomarkers for BRONJ.

Serum TRACP 5b has been a promising marker in the fields of bone disease, especially for its exclusive specificity from osteoclast and unique characteristics as a biomarker; low diurnal variability, stable storage, being not affected by feeding and the existence of renal or hepatic failure[27,35]. Clinically, the diagnostic and prognostic efficacies of TRACP 5b have progressively been validated in osteoporosis, bone metastatic cancers, chronic renal failure, and other metabolic and pathologic bone diseases[17,36]. Because TRACP 5b reflects the osteoclast number[16,17], and osteoclast number rather than osteoclastic activity is associated with bone remodeling[13,14,18,19], the use of TRACP 5b for the assessment of oversuppressed bone remodeling seems biologically feasible. In this study, ONJ group exhibited significantly lower levels of

serum TRACP 5b than non-ONJ group. (Fig. 3) This, the low concentration of TRACP 5b may indicate abnormal suppression of bone remodeling, which can lead to the development of ONJ.

RANK/RANKL/OPG is a well-known system that is capable of regulating most aspects of osteoclast function, including proliferation, differentiation, activation, and apoptosis[20]. Abnormalities in this system have been implicated in the pathogenesis of various bone diseases[21]. Regarding circulating OPG and RANKL, the diagnostic and prognostic applications of the serum RANKL/OPG ratio in multiple myeloma and bone metastatic diseases showed promise for reflecting abnormal bone remodeling[22,23]. Interestingly, a prospective study of 906 patients reported significantly lower serum levels of RANKL as an independent predictor of non-traumatic fracture with a relative risk of 10[37]. Also, several *in vitro* and clinical studies revealed a direct influence of bisphosphonates on mRNA expression and serum level of RANKL and OPG[23,38-41]. Therefore RANKL may have an important role in bone turnover, which can be similarly interpreted to ONJ. However, several limitations, such as the relatively low bone-specificity and questionable capacity for reflecting the localized bone environment, should be resolved in order to determine whether RANKL has relevant clinical applications[23].

There have been increasing evidences that inactive mature osteoclasts play an important role in mediating bone remodeling through coupling process[15,18,42,43]. Coupling factors were demonstrated to be secreted normally in relatively inactive osteoclasts, and correlated to osteoclast number rather than activity[15,19]. These findings provide valuable insights into the biological interpretation of the results of this study. The significantly higher TRACP 5b and RANKL/OPG ratio in non-ONJ group without difference of bone resorption as expressed by CTx imply the role of inactive

osteoclasts for the osteoclast-mediated bone remodeling activity regardless of resorptive activity. Subsequently, these biomarkers could facilitate to reflect oversuppression of bone associated with BRONJ. Besides, this interpretation is corroborated by the biological performance of TRACP 5b, which is relatively more investigated; non-resorbing inactive osteoclasts were also able to secrete TRACP 5b albeit at a slightly lower level than their resorbing counterparts[44], and TRAP release occurs at an earlier stage of osteoclastogenesis and is not restricted to the bone resorption by mature osteoclasts[45].

In this study, serum TRACP 5b levels were significantly reduced in the ONJ group at the time of sacrifice, while the serum RANKL/OPG ratio was significantly reduced at both the time of sacrifice and the time of surgical intervention. Clinically, the time of sacrifice may be interpreted as having diagnostic capability, while the time of surgical intervention can be interpreted at having risk predictive ability. Because the current diagnostic criteria for ONJ are based only on ambiguous clinical information[11], diagnosis of ONJ through biomarkers may allow the establishment of clear diagnostic criteria and may enable the disease to be standardized. On the other hand, markers that are thought to have more clinical significance may be able to be applied for assessment of ONJ risk. When dental intervention is needed in patients receiving bisphosphonates, biomarkers that can determine the risk of ONJ development would be useful for the choice of treatment modalities by physicians and dentists. Considering that the term biomarker generally connote the predictability of disease progress, the significance of serum TRACP 5b only at the time of surgical intervention alludes the limited applicability of results of this study. Although the serum RANKL/OPG ratio differed significantly at the time of surgical intervention in this thesis, further validation in human studies is required.

Several clinical and in vivo studies have reported an association between ONJ development and the dose-dependent accumulation of bisphosphonates in the jaw[31,46]. Nevertheless, the various responses to bisphosphonates under similar conditions such as in animal studies may stem from different individual pharmacological responses[47,48]. This hypothesis is associated with another ONJ hypothesis: role of pharmacogenetic susceptibility[49]. It can be inferred that individuals who respond to bisphosphonates well may be at higher risk of ONJ development. This is consistent with the results of this thesis; lower levels of TRACP 5b and lower RANKL/OPG ratios were observed in the ONJ group. Further pharmacogenetic researches, such as determination of individual genetic profiles and pharmacological responses, are required.

In conclusion, our data demonstrated that lower serum TRACP 5b levels and reduced RANKL/OPG ratios were associated with ONJ. These findings are consistent with both the current hypothesis of ONJ and the molecular roles of biomarkers. After validation studies with human ONJ patients, these biomarkers may provide crucial tools for diagnosis and risk prediction of ONJ and insights into future directions for ONJ research.

References

1. Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. *J Oral Maxillofac Surg* 2003:61:1115-7.

2. Allen MR, Burr DB. The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. *J Oral Maxillofac Surg* 2009:67:61-70.

3. Subramanian G, Cohen HV, Quek SY. A model for the pathogenesis of bisphosphonate-associated osteonecrosis of the jaw and teriparatide's potential role in its resolution. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011:112:744-53.

4. Reid IR, Cornish J. Epidemiology and pathogenesis of osteonecrosis of the jaw. *Nat Rev Rheumatol* 2012:8:90-6.

5. Kim JW, Kong KA, Kim SJ, Choi SK, Cha IH, Kim MR. Prospective biomarker evaluation in patients with osteonecrosis of the jaw who received bisphosphonates. *Bone* 2013:57:201-5.

6. Marx RE, Cillo JE, Jr., Ulloa JJ. Oral bisphosphonate-induced osteonecrosis: risk factors, prediction of risk using serum CTX testing, prevention, and treatment. *J Oral Maxillofac Surg* 2007:65:2397-410.

7. Choi S-Y, An C-H, Kim S-Y, Kwon T-G. Bone turnover and inflammatory markers of bisphosphonate-related osteonecrosis of the jaw in female osteoporosis patients. *Journal of Oral and Maxillofacial Surgery, Medicine, and Pathology* 2012.

8.   Lehrer S, Montazem A, Ramanathan L, Pessin-Minsley M, Pfail J, Stock RG, et al. Bisphosphonate-induced osteonecrosis of the jaws, bone markers, and a hypothesized candidate gene. *J Oral Maxillofac Surg* 2009:67:159-61.

9.   Lazarovici TS, Mesilaty-Gross S, Vered I, Pariente C, Kanety H, Givol N, et al. Serologic bone markers for predicting development of osteonecrosis of the jaw in patients receiving bisphosphonates. *J Oral Maxillofac Surg* 2010:68:2241-7.

10.   Morris PG, Fazio M, Farooki A, Estilo C, Mallam D, Conlin A, et al. Serum N-telopeptide and bone-specific alkaline phosphatase levels in patients with osteonecrosis of the jaw receiving bisphosphonates for bone metastases. *J Oral Maxillofac Surg* 2012:70:2768-75.

11.   Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. 2014 AAOMS Position Paper on Medication-Related Osteonecrosis of the Jaw. Avalable at: http://www.aaoms.org/index.php.

12.   Szulc P, Delmas PD. Biochemical markers of bone turnover: potential use in the investigation and management of postmenopausal osteoporosis. *Osteoporos Int* 2008:19:1683-704.

13.   Koh AJ, Demiralp B, Neiva KG, Hooten J, Nohutcu RM, Shim H, et al. Cells of the osteoclast lineage as mediators of the anabolic actions of parathyroid hormone in bone. *Endocrinology* 2005:146:4584-96.

14.   Martin TJ, Sims NA. Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends Mol Med* 2005:11:76-81.

15.   Karsdal MA, Martin TJ, Bollerslev J, Christiansen C, Henriksen K. Are nonresorbing osteoclasts sources of bone anabolic activity? *J Bone Miner Res* 2007:22:487-94.

16. Rissanen JP, Suominen MI, Peng Z, Halleen JM. Secreted tartrate-resistant acid phosphatase 5b is a Marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. *Calcif Tissue Int* 2008:82:108-15.

17. Janckila AJ, Yam LT. Biology and clinical significance of tartrate-resistant acid phosphatases: new perspectives on an old enzyme. *Calcif Tissue Int* 2009:85:465-83.

18. Henriksen K, Tanko LB, Qvist P, Delmas PD, Christiansen C, Karsdal MA. Assessment of osteoclast number and function: application in the development of new and improved treatment modalities for bone diseases. *Osteoporos Int* 2007:18:681-5.

19. Karsdal MA, Henriksen K, Sorensen MG, Gram J, Schaller S, Dziegiel MH, et al. Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption. *Am J Pathol* 2005:166:467-76.

20. Hofbauer LC, Neubauer A, Heufelder AE. Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. *Cancer* 2001:92:460-70.

21. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. *JAMA* 2004:292:490-5.

22. Terpos E, Szydlo R, Apperley JF, Hatjiharissi E, Politou M, Meletis J, et al. Soluble receptor activator of nuclear factor kappaB ligand-osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. *Blood* 2003:102:1064-9.

23. Rogers A, Eastell R. Circulating osteoprotegerin and receptor activator for nuclear factor kappaB ligand: clinical utility in metabolic bone disease assessment. *J Clin Endocrinol Metab* 2005:90:6323-31.

24. Yamashita J, Koi K, Yang DY, McCauley LK. Effect of zoledronate on oral wound healing in rats. *Clin Cancer Res* 2011:17:1405-14.

25. Croucher PI, De Hendrik R, Perry MJ, Hijzen A, Shipman CM, Lippitt J, et al. Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. *J Bone Miner Res* 2003:18:482-92.

26. Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, et al. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 2013:28:2-17.

27. Hannon RA, Clowes JA, Eagleton AC, Al Hadari A, Eastell R, Blumsohn A. Clinical performance of immunoreactive tartrate-resistant acid phosphatase isoform 5b as a marker of bone resorption. *Bone* 2004:34:187-94.

28. Okamoto T, de Russo MC. Wound healing following tooth extraction. Histochemical study in rats. *Rev Fac Odontol Aracatuba* 1973:2:153-69.

29. Lin JH. Bisphosphonates: a review of their pharmacokinetic properties. *Bone* 1996:18:75-85.

30. Weiss HM, Pfaar U, Schweitzer A, Wiegand H, Skerjanec A, Schran H. Biodistribution and plasma protein binding of zoledronic acid. *Drug Metab Dispos* 2008:36:2043-9.

31. Hokugo A, Sun S, Park S, McKenna CE, Nishimura I. Equilibrium-dependent bisphosphonate interaction with crystalline bone mineral explains anti-resorptive pharmacokinetics and prevalence of osteonecrosis of the jaw in rats. *Bone* 2013:53:59-68.

32. Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws--2009 update. *J Oral Maxillofac Surg* 2009:67:2-12.

33. Chapurlat RD, Delmas PD. Drug insight: Bisphosphonates for postmenopausal osteoporosis. *Nat Clin Pract Endocrinol Metab* 2006:2:211-9; quiz following 38.

34. Khan AA, Sandor GK, Dore E, Morrison AD, Alsahli M, Amin F, et al. Bisphosphonate associated osteonecrosis of the jaw. *J Rheumatol* 2009:36:478-90.

35. Halleen JM, Tiitinen SL, Ylipahkala H, Fagerlund KM, Vaananen HK. Tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of bone resorption. *Clin Lab* 2006:52:499-509.

36. Chao TY, Wu YY, Janckila AJ. Tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) as a serum maker for cancer with bone metastasis. *Clin Chim Acta* 2010:411:1553-64.

37. Schett G, Kiechl S, Redlich K, Oberhollenzer F, Weger S, Egger G, et al. Soluble RANKL and risk of nontraumatic fracture. *Jama* 2004:291:1108-13.

38. Koch FP, Merkel C, Ziebart T, Smeets R, Walter C, Al-Nawas B. Influence of bisphosphonates on the osteoblast RANKL and OPG gene expression in vitro. *Clin Oral Investig* 2012:16:79-86.

39. Viereck V, Emons G, Lauck V, Frosch KH, Blaschke S, Grundker C, et al. Bisphosphonates pamidronate and zoledronic acid stimulate osteoprotegerin production by primary human osteoblasts. *Biochem Biophys Res Commun* 2002:291:680-6.

40. Valleala H, Mandelin J, Laasonen L, Koivula MK, Risteli J, Konttinen YT. Effect of cyclical intermittent etidronate therapy on circulating osteoprotegerin levels in patients with rheumatoid arthritis. *Eur J Endocrinol* 2003:148:527-30.

41. Alvarez L, Peris P, Guanabens N, Vidal S, Ros I, Pons F, et al. Serum osteoprotegerin and its ligand in Paget's disease of bone: relationship to disease activity and effect of treatment with bisphosphonates. *Arthritis Rheum* 2003:48:824-8.

42. Howard GA, Bottemiller BL, Turner RT, Rader JI, Baylink DJ. Parathyroid hormone stimulates bone formation and resorption in organ culture: evidence for a coupling mechanism. *Proc Natl Acad Sci U S A* 1981:78:3204-8.

43. Takeshita S, Fumoto T, Matsuoka K, Park KA, Aburatani H, Kato S, et al. Osteoclast-secreted CTHRC1 in the coupling of bone resorption to formation. *J Clin Invest* 2013:123:3914-24.

44. Henriksen K, Gram J, Schaller S, Dahl BH, Dziegiel MH, Bollerslev J, et al. Characterization of osteoclasts from patients harboring a G215R mutation in ClC-7 causing autosomal dominant osteopetrosis type II. *Am J Pathol* 2004:164:1537-45.

45. Karsdal MA, Hjorth P, Henriksen K, Kirkegaard T, Nielsen KL, Lou H, et al. Transforming growth factor-beta controls human osteoclastogenesis through

the p38 MAPK and regulation of RANK expression. *J Biol Chem* 2003:278:44975-87.

46. Otto S, Pautke C, Opelz C, Westphal I, Drosse I, Schwager J, et al. Osteonecrosis of the jaw: effect of bisphosphonate type, local concentration, and acidic milieu on the pathomechanism. *J Oral Maxillofac Surg* 2010:68:2837-45.

47. Zhou K, Pearson ER. Insights from genome-wide association studies of drug response. *Annu Rev Pharmacol Toxicol* 2013:53:299-310.

48. Sedghizadeh PP, Jones AC, LaVallee C, Jelliffe RW, Le AD, Lee P, et al. Population pharmacokinetic and pharmacodynamic modeling for assessing risk of bisphosphonate-related osteonecrosis of the jaw. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013:115:224-32.

49. Nicoletti P, Cartsos VM, Palaska PK, Shen Y, Floratos A, Zavras AI. Genomewide pharmacogenetics of bisphosphonate-induced osteonecrosis of the jaw: the role of RBMS3. *Oncologist* 2012:17:279-87.

Abstract (In Korean)

비스포스포네이트 관련 악골 괴사증의 바이오마커 탐지

김 진 우

연세대학교 대학원 치의학과

지도 교수   차 인 호

비스포스포네이트 사용과 관련된 심각한 합병증인 악골 괴사증의 진단과 예후 예측, 그리고 위험도 평가를 위해 여러 골 마커들이 제안되어 왔으나 많은 논란이 있어왔다. 본 연구의 목적은 동물 모델을 통해 비스포스포네이트 관련 악골 괴사증의 바이오마커를 발굴하기 위함이다.

총 48 마리의 Sparague-Dawley 쥐를 한 주에 한번씩 zoledronic acid 를 정맥 투여한 비스포스포네이트 그룹 (n=36)과 생리 식염수를 투여한 대조군 (n=12)으로 무작위 분류하였다. 투여 6 주 후, 외과적 처치로써 하악 좌측 제 1,2,3 대구치의 발거가 시행되었으며 그 후 8 주 동안 추가 투여를 시행하고 희생하였다. CTx, Glu-OC, TRACP 5b, RANKL, OPG 등의 바이오마커를 실험 시작 시 (T0), 외과적 처치 시 (T1), 희생 시 (T2)에 측정하였으며, 비스포스포네이트 그룹의 쥐들은 조직학적인 분석을 통하여 골괴사 발생 그룹 (ONJ)과 미발생 그룹 (non-ONJ) 그룹으로 분류하였다. 또한 파골세포의 정량화를 위한 조직계측학적 분석이 시행되었다.

반복측정 분산분석 결과, TRACP 5b 및 RANKL/OPG 비율이 시간에 따라 ONJ 그룹에서 non-ONJ 에 비해 유의하게 감소하였다 (P <0.05). T2 시점에서 TRACP 5b 의 곡선하면적 (area under curve)은 0.807 (컷 오프에서 민감도: 88.9%, 특이도: 66.7%) 이었으며, RANKL/OPG 비율은 0.765 (컷 오프에서 민감도: 77.8%, 특이도 62.9%)로써 만족스러운 진단학적인 가치를 보였다. TRACP 5b 는 RANKL/OPG 비율에 비하여 더 낮은 최소 유의 변화 (least significant change; 29.6%) 및 더 높은 signal-to-noise ratio (2.76)을 나타내어, TRACP 5b 가 더 높은 마커 정밀도를 나타내었다. 조직계측학적 분석 결과 ONJ 그룹은 non-ONJ 그룹에 비교하여 유의하게 파골세포 수가 더 적었다. (P <0.05)

위 연구 결과를 종합하여 볼 때, TRACP 5b 및 RANKL/OPG 비율이 비스포스포네이트 관련 악골 괴사증의 바이오마커로 사용될 수 있을 것이라 사료된다. 본 연구 결과를 뒷받침하기 위한 악골 괴사증이 발병한 인간 환자를 대상으로 임상 연구가 필요할 것으로 사료된다.