치의과학박사학위논문

Bisphosphonate-related Osteonecrosis of the Jaw: Cell-specific Responses and Clinical Treatment Outcomes

비스포스포네이트 관련 악골괴사
– 세포 특이적 반응과 임상적 치료 결과 연구

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- ABSTRACT -

Bisphosphonate-related Osteonecrosis of the Jaw: Cell-specific Responses and Clinical Treatment Outcomes

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Background and Purpose

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a challenging complication, the pathophysiology of which is not completely understood. The aims of this study were two folds: (1) to identify the risk factors associated with relapse or treatment failure after surgery for BRONJ in osteoporosis patients and (2) experimentally to investigate the effect of zoledronate on dental stem cells.

Part I. Clinical study

[Patients and methods] This was a retrospective cohort study of BRONJ in patients with osteoporosis who had undergone surgical procedures between 2004 and 2016 at the Department of Oral and Maxillofacial Surgery, Seoul National University Dental Hospital. The heterogeneous predictor variables were demographic (age and sex), anatomic (maxilla, mandible, maxilla plus mandible, affected location), clinical (disease stage, etiology,

comorbidities, and history of intravenous bisphosphonate intake), time-related (conservative treatment before surgery, bisphosphonate treatment before BRONJ development, discontinuation of the drug before surgery, interval to final follow-up, and interval to reoperation in the case of relapse or treatment failure), and perioperative variables (type of anesthesia and type of surgical procedure). The primary outcome variable was relapse after surgery that required reoperation (yes vs. no). Descriptive and bivariate statistics were computed to assess the relationships among the study variables and the outcome. To determine risk factors, a survival analysis was conducted using the Cox model.

[Results] The final sample consisted of 325 subjects with a median age of 75 years. Ninety-seven percent of the subjects were female. After surgery, 30% of the patients did not completely recuperate and received repeat surgeries. The time from the first surgery to reoperation ranged from 10 days to 5.6 years. Relapse or treatment failure occurred most often immediately after surgery. The type of surgical procedure and type of anesthesia were the most important factors for the treatment outcome. Drug holiday did not appear to influence the occurrence of relapse after surgery.

Part II. *In vitro* study

[Materials and methods] Bone marrow stem cells (BMSCs) and periodontal ligament stem cells (PDLSCs) treated with various concentrations of zoledronate were investigated with regard to proliferation and differentiation capacity. The osteogenic differentiation of dental stem cells treated with zoledronate was evaluated by alizarin red S staining and alkaline phosphatase activity staining. RT-PCR was performed to compare MSX1, osteocalcin, collagen type I, and

EF1a mRNA levels. Apoptosis was detected using FITC-Annexin V and PI staining assay. The dental stem cells were treated with bone morphogenetic protein (BMP)-2 to evaluate the possibility of overcoming the effects of zoledronate.

[Results] High concentrations of zoledronate suppressed PDLSC and BMSC viability. The apoptosis of PDLSCs and BMSCs was triggered by both zoledronate and BMP-2. Higher concentrations of zoledronate triggered apoptotic activity in BMSCs, but additional BMP-2 treatment did not affect the apoptotic activity triggered by zoledronate. Moreover, zoledronate treatment significantly suppressed the formation of calcium deposits on PDLSCs and BMSCs regardless of BMP-2 treatment. BMP-2 treatment did not overcome the inhibitory effects of zoledronate on the osteogenic differentiation of PDLSCs and BMSCs. The MSX1 gene, highly expressed during craniofacial development, was significantly suppressed by 10 μM of zoledronate during the osteogenic differentiation of PDLSCs and BMSCs.

Conclusions

Surgical treatment of BRONJ in osteoporosis patients may benefit from more careful and extensive surgical procedures rather than curettage under local anesthesia. Zoledronate at high doses inhibited the proliferation, osteogenic differentiation, and MSX1 expression of dental stem cells. These findings indicate that zoledronate has negative effects on the regenerative capacity of dental stem cells, which may also explain the disease progression of BRONJ.

Keywords: bisphosphonate, osteonecrosis, relapse, risk factor, zoledronate, dental stem cell, MSX1
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Introduction

Bisphosphonates inhibit bone resorption and treat bone metastasis, multiple myeloma, and Paget's disease.[1] They have also been used to reduce the risk of fracture and skeletal complications in osteoporosis patients. However, certain patients receiving bisphosphonates suffer from osteonecrosis of the jaw after invasive dental procedures, such as surgical extraction, as first reported by Marx.[2] Since bisphosphonate-related osteonecrosis of the jaw (BRONJ) was first defined,[3, 4] its highest reported occurrence has been in oncology patients.[5-10] For uneventful healing, invasive dental procedures must be followed by regeneration of alveolar bone and mucoperiosteal soft tissue. Mesenchymal stem cells (MSCs) seem to play a key role in this process. As bisphosphonates tend to accumulate in the bone, their effects on bone marrow stem cells (BMSCs) and periodontal ligament stem cells (PDLSCs) may be important to developing an understanding of the pathophysiology of

BRONJ. In addition to patients treated with high-dosage intravenous bisphosphonate, many BRONJ patients are osteoporosis patients treated with oral bisphosphonate.[4, 11-13]

Prevention may be the most important aspect of this condition.[6] Once BRONJ occurs, conservative approaches, such as palliative treatment of pain and infection, are recommended first.[3, 6, 8] If surgical treatment cannot be avoided, effective surgery should be considered to improve the BRONJ prognosis.[7, 12, 14-17] In cases of surgical intervention, the extent of surgery necessary has provoked the most debate.[16]

Despite debates on the surgical treatment of BRONJ, several authors have reported surgical cure rates from 59%[6, 7] to more than 90%,[7, 12, 14, 18, 19] which has encouraged doctors to continue to treat BRONJ surgically.[6, 17, 20, 21] Although the clinical features of BRONJ have been well documented and a variety of treatment protocols have been recommended in the past,[5, 14] a clear surgical guideline is still lacking and the prognosis after surgical intervention is uncertain.[22]

Part I of this study primarily addresses surgical treatment outcomes in BRONJ patients who do not have malignant diseases. The majority of BRONJ patients previously studied have suffered from malignant diseases, such as breast cancer, prostate cancer, multiple myeloma, and bone metastasis.[6, 8, 9] BRONJ is a serious side effect of bisphosphonate treatment for osteoporotic conditions, especially in the elderly.[11] BRONJ may be more prevalent than in the past due to increased life expectancy.

Survival analysis is a suitable method of analyzing data that have a principal end point. It has been a popular systematic statistical method of evaluating the clinical success or failure of

specific surgical procedures, such as implant survival.[23] However, only a few studies have applied survival analysis to the treatment efficacy of BRONJ patients.[6, 24] As most BRONJ patients are elderly, some subjects have been lost to follow-up and some have died of unrelated causes. Survival analysis solves these problems and can be applied irrespective of different follow-up schedules. In addition, when there are multiple factors that may jointly affect surgical outcomes, it is difficult to determine which ones are the most important. In this situation, a conditional inference tree structure combined with a survival function facilitates visualization.[23] In addition, survival analysis can provide clinicians a postoperative relapse pattern over time.

Part II of this study is an *in vitro* study investigating the effect of bisphosphonate on dental stem cells. As dental stem cells play a key role in maxillofacial bone regeneration, it may help broadening an understanding of the pathogenesis and treatment consequence of BRONJ.

According to a previous study, low bisphosphonate concentrations seem to improve the osteogenic differentiation of MSCs.[25] However, high bisphosphonate concentrations may lead to the development of BRONJ due to bisphosphonate accumulation. A recent study suggested that low levels of zoledronate suppress osteoblast differentiation via downregulation of bone morphogenetic protein (BMP)-2, but that high levels of zoledronate induce cytotoxicity in murine preosteoblast cell lines.[26] In addition to its bone-healing feature, bisphosphonate taken for its antiangiogenic effects may be a causal factor of BRONJ. One study reported the suppression of endothelial differentiation by bisphosphonates in human placental MSCs.[27]

Considering the lack of definitive treatment strategies for BRONJ, various clinical trials have been published. Although BMSCs have received attention in cell-based therapies of the craniofacial region,[28] treatment of BRONJ has been attempted via transplantation of BMSCs or their conditioned media and bone grafts including BMP-2.[29]

Part I of this study aimed to answer the following clinical questions: for BRONJ in osteoporosis patients, 1) which kind of surgical procedures might have produced better treatment outcomes and 2) did a shorter period of bisphosphonate intake or a longer period of discontinuation (drug holiday) lead to better results? I hypothesized that 1) more extensive surgical procedures might result in better BRONJ treatment outcomes in osteoporosis patients and 2) patients who have had shorter periods of bisphosphonate intake and/or longer periods of discontinuation before surgery may demonstrate better results, as the dose and type of bisphosphonate intake was believed to play a role in initiating BRONJ.[4, 10, 30-32] patients who have had a shorter period of bisphosphonate intake and/or a longer period of discontinuation before surgery may show a better result. The specific aim was to identify the risk factors associated with relapse or treatment failure after surgical treatment of BRONJ in osteoporosis patients. The clinical features of BRONJ patients and the postoperative relapse pattern over time were also estimated.

Part II of this study aimed to investigate the effects of zoledronate on dental stem cells. BRONJ commonly occurs after dental extraction in patients who receive bisphosphonates. So far, zoledronate is the most potent agent that can be administrated intravenously.[33] The hypothesis of this part was that high doses of bisphosphonate reduced osteogenesis and

increase apoptosis of dental stem cells. The specific aims were to investigate the response of dental stem cells to various concentrations of zoledronate, to estimate the role of BMP-2 in overcoming the adverse effects of zoledronate, and to identify the pathogenesis of BRONJ.

Part I. Clinical study

A. Patients and methods

1. Study design and sample

This study was a single-center retrospective cohort study. The entire study population consisted of patients who had presented to the Department of Oral and Maxillofacial Surgery at the Seoul National University Dental Hospital for evaluation and management of BRONJ between March 2004 and September 2016.

To be included in the study sample, patients had to be confirmed as having BRONJ according to the definition of the American Association of Oral and Maxillofacial Surgeons,[3, 4] which was in line with the eligibility criteria of previous studies.[6, 9] The inclusion criteria were Stage 2 and 3 BRONJ in need of surgical intervention. The exclusion criteria were history of malignant diseases and chemotherapy treatment. Patients were also excluded if their medical records were incomplete and it was not possible to identify the necessary variables.

The Institutional Review Board for Protection of Human Subjects reviewed and approved the research protocol (Institutional Review Board No. S-D 20140015).

2. Study variables

2.1 Predictor variables

The predictor variables consisted of a set of heterogeneous variables categorized as 1) demographic (age and sex), 2) anatomic (maxilla, mandible, maxilla plus mandible, and affected location), 3) clinical (disease stage; etiology; comorbidities, such as diabetes, hypertension, and steroid use; type of bisphosphonate; and history of intravenous bisphosphonate intake), 4) time-related (conservative treatment before surgery, bisphosphonate treatment before BRONJ development, discontinuation of the drug before surgery or drug holiday, interval to final follow-up, and interval to reoperation in the case of relapse or treatment failure), and 5) perioperative variables (where BRONJ was first diagnosed, type of anesthesia during surgery, and type of surgical procedure).

The surgical procedures conducted were curettage, sequestrectomy, saucerization, and mandibulectomy. Curettage is the removal of superficial inflammatory soft tissue and necrotic bone. It is a mild local intervention.[16] Sequestrectomy is the removal of infected and avascular pieces of bone. Saucerization is the removal of the adjacent cortical bone and formation of a saucer-like depression. Mandibulectomy is the marginal or segmental resection of the mandible, followed by reconstruction. It is the most radical type of surgical intervention.[16, 19] The surgical procedures were also reviewed whether performed under intravenous sedation or local or general anesthesia (**Table 1**).

2.2 Outcome variable

The primary outcome variable was relapse followed by surgical treatment (yes vs. no). The relapse group included the patients who did not heal or improve after the first surgery and thus

required surgical reintervention. To apply the survival analysis, relapse or failure of surgical intervention was coded as the event and the time elapsed from the first surgical procedure to the reoperation was measured.

3. Statistical analysis

Descriptive statistics were computed for each variable. Between the success and relapse groups, chi-square tests and Fisher's exact tests were conducted for count data. For the time-related variables, medians rather than means were computed and the Wilcoxon rank sum test was applied, as time-related variables have skewed distributions.[13] To identify the risk factors and the recurrence pattern over time, survival analysis using the Cox proportional hazard model was applied. To graphically reveal the most important factor for the treatment outcome, the conditional inference tree structure was depicted using the conditional inference tree package in the R Programming Language (R Foundation for Statistical Computing, Vienna, Austria).[34]

B. Results

1. Descriptive summary of study sample

From March 2004 to September 2016, 352 patients were screened for eligibility (**Table 1**). Among them, 27 patients were excluded because they had previously received chemotherapy treatment for malignant diseases. The final sample consisted of 325 osteoporosis patients with BRONJ. The majority of the patients were female (97%). The median age at which BRONJ

was diagnosed was 75 years. However, by the final follow-up visit, nine patients (with a median age of >83 years) had died of their underlying diseases. Before surgical intervention, the patients received conservative and supportive treatment, including the cessation of bisphosphonate treatment for a median of 51 days. After surgery, the median follow-up time was 6 months (**Fig. 1**). The number of patients with BRONJ has been increasing during the past decade (**Fig. 2**).

No significant difference was found between the ages of the female and male patients. The BRONJ lesions were more frequently located in the mandible than in the maxilla and most were in the molar and premolar regions. However, the patients who had lesions in the maxilla were significantly older than those with mandibular lesions.

The most frequent concurrent medications were those for hypertension (55%) and diabetes (25%). Most of the patients were taking an oral bisphosphonate and 16 patients (5%) were receiving intravenous injections. Alendronate was the most common type of bisphosphonate.

The median duration of bisphosphonate treatment before the development of BRONJ was 39.5 ± 57.0 months. Before surgical treatment of BRONJ lesions, the median number of months of drug discontinuation (i.e., the drug holiday period) was 4.0 ± 7.0 months.

Sequestrectomy was the most common surgical procedure used. Approximately one half of the surgical procedures were performed with patients under general anesthesia. After surgery, 228 patients (70%) had treatment success. However, 97 patients (30%) did not completely recuperate and required further surgical management to treat the relapsed lesion. Some of these

patients (19%) underwent surgical treatment again, 6% underwent reoperation twice, and 5% underwent more than three procedures (**Table 1**).

The interval from the first surgery to reoperation ranged from 10 days to 5.6 years. Relapse or treatment failure occurred more often immediately after surgery. The most frequent recurrences occurred within 9 months of the initial surgical treatment (**Fig. 3**).

2. Risk factors in the relapse/treatment failure group

The descriptive statistics are listed by outcome in **Table 2**. Patients with hypertension, longer conservative treatment times, or more extensive surgical treatments than curettage performed with local anesthesia demonstrated lower incidences of relapse or treatment failure after surgery. As these variables could be related confounding factors, multivariate analysis was necessary. The multivariate Cox proportional hazards regression analysis of the treatment outcomes revealed a significant risk factor. Thus, more extensive surgical treatment led to better results and lower risk of relapse necessitating reoperation (**Table 3**). The conditional inference tree combined with the survival function graph indicates that the type of surgical procedure and the type of anesthesia are the most important factors for clinical success after BRONJ surgery. The patients who underwent curettage under local anesthesia had the worst prognoses (**Fig. 4**).

Part II. *In vitro* study

A. Materials and methods

1. Human periodontal stem cell (PDLSC) and human bone marrow stem cell (BMSC) primary culture

PDLSCs from human teeth (surface of the root) and BMSCs from human maxilla were isolated and digested in a 3 mg/mL of type 1 collagenase (BioBasic, Toronto, ON, Canada) and 4 mg/mL of dispase II (GIBCO BRL, Waltham, MA) for 1 h by shaking at 37°C in a 5% CO_2 incubator. Primary cells were cultured with alpha minimum essential medium (α-MEM, GIBCO BRL) including 15% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX), 100 μM of L-ascorbic acid (BioBasic), 2 mM of L-glutamine (GIBCO BRL), and 100 U/mL of antibiotic-antimycotic solution (GIBCO BRL), which was renewed every 2 to 3 days. PDLSCs and BMSCs at passages of 2 to 5 were used for each experiment. This protocol was approved by the Institutional Review Board at the Seoul National University School of Dentistry (Institutional Review Board No. S-D20080009).

2. Proliferation assay

For direct cell counting, the PDLSCs and BMSCs were seeded with 10^4 cells into each 24 wells. After overnight incubation, 1, 5, and 10 □M of zoledronate (Novartis, Switzerland) and/or 200 ng/mL of recombinant human BMP-2 (rhBMP-2, Daewoong Pharmaceutical Co., Seongnam, Korea) were treated for days 1, 3, 5, and 7. Three plates for each group were used for cell counting with 1× Trypsin-EDTA (GIBCO BRL). Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution after 2 h of

incubation. The formazan crystals were dissolved in DMSO and the optical density was read at 540 nm with a microplate reader (Fluostar Optima, BMG LABTECH).

3. Apoptosis assay

The PDLSCs and BMSCs treated with 1, 5, and 10 □M of zoledronate for 5 days were collected to assess apoptosis. Approximately 2×10^5 cells were detected using an FITC-Annexin V Apoptosis Detection Kit I (BD Biosciences, NJ) following the suggested protocol. The stained cells were collected by FACSCAN (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FACSDiva software (Becton Dickinson).

4. Osteogenic differentiation

Approximately 10^5 PDLSCs and BMSCs were seeded into six well plates. After 50% to 60% confluency was established, the cells were incubated in a osteogenic differentiation medium consisted of α-MEM supplemented with 10% FBS (Equitech-Bio), 10 nM of dexamethasone (Sigma-Aldrich Co., St. Louis, MO), 5 mM of glycerol phosphate (Sigma-Aldrich), 100 U/mL of antibiotic-antimycotic solution (GIBCO BRL), and 100 μM of L-ascorbic acid (BioBasic). An QuantiChrom Alkaline Phosphatase (ALP) Assay Kit (BioAssay Systems, Hayward, CA) was used to determine the activity of ALP following the manufacturer′s directions. The proteins from each cell were lysed in 0.5% Triton X-100 in distilled water at room temperature. The value of optical density was measured at 405 nm using a microplate reader (Fluostar Optima, BMG LABTECH). After 2 to 3 weeks of

incubation in the osteogenic differentiation medium, the PDLSCs and BMSCs were stained with alizarin red S solution (40 mM, pH 4.2). The stained calcium deposits were dissolved with 20% methanol and 10% acetic acid. The optical density was detected and quantified with a spectrophotometer (Fluostar Optima, BMG LABTECH, Ortenberg, Germany) at 450 nm.

5. RT-PCR

RNA from each group was collected using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). Then, the first strand cDNA was synthesized using the SuperScript III First-Strand Kit (Invitrogen) and amplified with EF1α, osteocalcin (OC), collagen type 1 (ColI), and MSX1 primers (**Table 4**).

6. Statistical analysis

The data were presented as the means $\pm$ standard deviations of triplicate experiments. One-way ANOVA followed by post-hoc comparisons with the Tukey test were used to compare the differences between groups. $P < 0.05$ was considered as a statistically significant difference.

B. Results

1. Morphology of PDLSCs and BMSCs treated with zoledronate

To determine the effect of zoledronate on PDLSCs and BMSCs, primary stem cells isolated from periodontal ligaments and bone marrow of maxilla were cultured in normal conditional media with and without zoledronate. As shown in **Figs. 5** and **7**, the PDLSCs and BMSCs

showed a spike-like morphology on days 4 and 10 in the control group. However, certain morphological changes were observed in the PDLSCs and BMSCs treated with 10 μM of zoledronate. Compared to the control group, a large proportion of these zoledronate-treated PDLSCs and BMSCs presented as detached, round cells after washing. In addition, the proportion of the detached cells increased with zoledronate treatment on day 10.

2. Proliferation of PDLSCs and BMSCs treated with zoledronate and/or rhBMP-2

Cell viability was detected by direct cell counting and using the MTT assay on days 1, 3, 5, and 7. With 1, 5, and 10 □M of zoledronate, the number of PDLSCs was not significantly changed on day 1 (**Fig. 6**). Interestingly, cell counting showed increased proliferation of PDLSCs in 1 □M of zoledronate after day 3 and this enhanced proliferation was reduced lower than control in 200 ng/mL BMP-2 treatment (**Fig. 6A**). Treatment of 5 and 10 □M of zoledronate suppressed PDLSC proliferation on day 7. BMP-2 treatment showed inconsistent effects on cell proliferation. On the contrary, the MTT assay showed significant changes with zoledronate after day 5, which may not affected by BMP-2 treatment (**Fig. 6B**). Overall, high concentrations of zoledronate inhibited PDLSC proliferation.

The effects of zoledronate on the BMSCs were significant and partly similar to those on the PDLSCs. High concentrations of zoledronate suppressed BMSC viability whereas low concentration (1 □M) enhance cell proliferation (**Fig. 8**). However, 200 ng/mL of BMP-2 suppressed cell viability with 1 □M zoledronate (**Fig. 8A**). BMSC proliferation was affected by zoledronate and BMP-2, as determined by direct cell counting and the MTT assay (**Fig. 8**).

3. Apoptosis of PDLSCs and BMSCs treated with zoledronate and/or rhBMP-2

Flow cytometry was used to determine the apoptotic activity of PDLSCs and BMSCs treated with zoledronate for 5 days (**Fig. 9** and **10**). Zoledronate showed inconsistent effects on the apoptotic activity of the PDLSCs (**Fig. 9**). However, additional treatment of 200 ng/mL of BMP-2 showed clear increased apoptosis.

The BMSCs showed clear apoptotic effects of zoledronate in dose dependent manner. With or without BMP-2 treatment, higher concentrations of zoledronate triggered the apoptotic activity of the BMSCs (**Fig. 10**). Significantly, the percentage of apoptotic BMSCs increased from 11.20% to 19.09% with 10 □M of zoledronate. In contrast to PDLSC, the BMP-2-treated BMSCs did not show significantly increased apoptotic cells.

4. Effects of zoledronate and/or rhBMP-2 on osteogenic differentiation in PDLSCs and BMSCs

The evaluation of osteogenic differentiation was proven by ALP activity and the alizarin red S staining (**Figs. 11** and **12**). The zoledronate-treated PDLSCs demonstrated dose-dependent suppression of ALP activity on day 5 (**Fig. 11C**). With 10 μM of zoledronate treatment, ALP activity, an early osteogenic marker, was significantly suppressed. However, BMP-2 treatment increased ALP activity with 5 and 10 □M of zoledronate treatment of the PDLSCs. Overall, BMP-2 affected the early osteogenic differentiation suppressed by zoledronate on PDLSCs (**Fig. 11**). Although 10 uM of zoledronate suppressed ALP activity on the BMSCs,

ALP activity on the BMSCs was not changed with or without BMP-2 treatment. Alizarin red S staining, late-stage osteogenic markers, showed similar results on the PDLSCs (**Fig. 11AB**). BMP-2 treatment increased the calcium deposits on the control group PDLSCs. However, treatment with zoledronate significantly suppressed the formation of calcium deposits on the PDLSCs with or without BMP-2 treatment. Zoledronate also suppressed osteogenic differentiation of the BMSCs (**Fig. 12**). BMP-2 treatment did not overcome osteogenic PDLSC or BMSC differentiation. It only overcame early-stage osteogenic PDLSC differentiation.

5. Changes in gene expression by zoledronate and/or BMP-2 treatment

The RT-PCR results revealed that osteogenic markers were more suppressed by zoledronate (**Fig. 11D**). 1 □M of zoledronate increased osteocalcin and type I collagen gene expression. However, 10 □M of zoledronate suppressed the gene expression of both osteogenic markers, osteocalcin and type I collagen. Interestingly, the MSX1 gene, which functions in craniofacial development, was significantly suppressed by 10 □M of zoledronate during the osteogenic differentiation of the PDLSCs. BMP-2 did not increase osteogenic differentiation of the PDLSCs treated with zoledronate. The osteogenic markers on the BMSCs were more suppressed by zoledronate than were those on the PDLSCs (**Fig. 12D**). Zoledronate treatment decreased osteocalcin gene expression on the BMSCs in a dose-dependent manner in the 200-ng/mL BMP-2 treatment groups. In addition, MSX1 gene expression was higher in the BMSCs treated with BMP-2 than in the control group BMSCs. This increased MSX1 expression was

not detected in the BMSCs treated with 10 μM of zoledronate. Overall, the BMSCs were affected by zoledronate more than the PDLSCs during osteogenic differentiation.

Discussion

This study consisted of two parts. In Part I, the clinical features of BRONJ and its clinical treatment prognosis were investigated to determine the surgical outcomes and their possible risk factors for BRONJ in osteoporosis patients. As the treatment results for these patients were not always satisfactory and some of them had to undergo repeated surgeries, the risk factors affecting relapse or treatment failure were assessed in these patients after surgical intervention. The initial hypothesis was that the outcome would be better for patients who had had shorter periods of bisphosphonate intake or longer periods of drug holiday before surgical treatment of BRONJ.

However, drug holiday did not appear to affect the likelihood of relapse after surgery. Instead, patients who underwent curettage under local anesthesia showed the worst prognoses after surgery and required reoperation. No significant association was found between post-surgical prognosis and history of intravenous administration of bisphosphonate, duration of bisphosphonate use, or drug holiday before surgery. The risk of recurrence was greatest immediately after surgery, after which the probability of relapse decreased over time. Moreover, extensive surgical procedures resulted in better prognoses than less extensive treatment methods (**Table 3**; **Fig. 4**).

The number of patients in this single-center investigation was greater than that in almost every other study on this subject from 2003 to 2014.[35] During the past decade, an increase in the number of patients with BRONJ without malignant comorbidity was perceived at our institution. The number of patients with BRONJ increased from 2004 to 2016 (**Fig. 2**). Several

possible explanations exist. First, the increase may have occurred because our institution is an academic center. One would expect patients who undergo surgery for BRONJ to be concentrated in academic centers due to the complexity of the surgery. It is possible that patients who undergo surgery for BRONJ have been referred from surgeons in private practices to academic oral maxillofacial surgeons (OMSs) at university hospitals. In fact, 64% of patients were referred from private practitioners to the university's OMSs for treatment of their BRONJ lesions (**Table 1**). Second, the increase may have occurred because general dentists and private specialists do not have a good understanding of BRONJ treatment and may not be comfortable performing oral surgery on BRONJ patients.[36] The chance of a dentist encountering a patient with BRONJ in an average practice is very low (<1 in 1,000).[11] Third, socioeconomic changes may underlie the increasing number of BRONJ patients. Fleifel et al. conducted a systematic review of 97 studies from 2003 to 2014 that included 4,879 patients with BRONJ.[35] The mean duration of bisphosphonate administration in patients with BRONJ was similar to that cited in previous studies; the median duration was 3 years. However, the patients in this study were older than those in previous studies (74 ± 8 years in this study compared to 67 ± 5 years in the systematic review).[35] BRONJ is considered to be a side effect of bisphosphonate therapy, especially in the elderly, a population in which the frequency of BRONJ is higher. Given that older age is regarded as a potential risk factor (along with female gender), the number of BRONJ patients increase in line with extended life expectancy.

Most studies have included patients suffering from malignant diseases.[6, 8, 9, 37] All of the patients in this study had osteoporosis without malignant comorbidities. More than half of the

patients in a study from Italy used zoledronate, which was not the case for the patients in this study.[9] The most-used type of bisphosphonate (46%) was alendronate and oral administration prevailed (95%) over intravenous infusion.

Except for the older age of the patients and the type of bisphosphonate intake, my BRONJ patients shared features with those in other studies. As reported by others, a predilection for the mandible was evident. Predilections for the molar and premolar regions were also observed, which matches previous descriptions of BRONJ characteristics.[8, 9, 17]

As the origin of BRONJ is connected to the use of bisphosphonate, is most prevalent in the elderly and in women, most commonly affects the mandible, it is hypothesized that these variables would be related to post-surgical prognosis. However, the aforementioned factors were not associated with relapse. These results were similar to those of previous investigators. For example, gender and age had no significant effect on the outcome of surgical treatment,[6, 17]nor did the duration of preoperative bisphosphonate discontinuation (i.e., drug holiday).[6]

Several of my findings differed from those of previous studies. First, a previous evaluation of surgical success in 88 patients with BRONJ found that BRONJ in the maxilla necessitated reoperation significantly earlier than BRONJ in the mandible.[6] However, there was no significant difference on the frequency of relapse and the time from the first surgical treatment to the repeat surgery between the maxilla and the mandible (median 3.8 months).

Second, the incidence of BRONJ is greater in oncology patients who are treated with high-dosage intravenous bisphosphonate.[10, 31] A recent report compared the prognosis of BRONJ patients in oral and intravenous bisphosphonate administration groups, and found that the oral

intake group had far better treatment outcomes.[10] However, a recent study on the efficacy of surgical therapy found that neither the number of applications nor the type of bisphosphonate influenced treatment response.[18] The results of this study do not suggest a relationship between post-surgical prognosis and history of intravenous administration or duration of bisphosphonate use.

Third, discontinuation of bisphosphonate treatment (i.e., drug holiday) was found to improve surgical outcomes.[7] However, it did not appear to affect the likelihood of post-surgical relapse. The decision of whether to continue or suspend bisphosphonate therapy before surgery remains controversial.[16] The uncertainty may be due in part to the fact that prospective or controlled studies are difficult to perform.

It has been well established that BRONJ occurs more frequently in the mandible than in the maxilla. The predilection of BRONJ lesions for the mandible has been repeatedly reported and is thought to be almost beyond doubt. In this study, older patients had more BRONJ lesions in the maxilla than average. The incidence in the maxilla increased with age (40s and 50s, 0%; 60s, 14.5%; 70s, 23.9%; 80s, 32.9%; and 90s, 40%). As previously suggested, this finding may have occurred because maxillary lesions are more apt to remain hidden and unnoticed.[31] Maxillary lesions have more severe BRONJ stages upon initial diagnosis.[31] Furthermore, BMSCs from the maxilla and mandible have different properties, which may influence the progress of BRONJ in these locations.[17] Bisphosphonates are known to prevent resorption of bone and reduce its turnover.[20] The pathogenesis of BRONJ has been suggested to be related to local infection of previously treated intraoral lesions.[8] In animal models with intentional

initiation of BRONJ lesions, the administration of bisphosphonate alone does not trigger pathogenesis.[38] Genetic factors and environmental risk factors are also likely to play a role.[37] The greater frequency of BRONJ lesions in the maxilla in older patients is difficult to interpret clearly, as no solid biological or clinical explanation exists.[5] In addition, despite the severity of the disease, little is known about the treatment protocol. A more detailed investigation is necessary to confirm the findings.

It is true that treatment should be as conservative as possible. However, in clear surgical cases, treatment protocols return superior results.[12, 14] An extensive surgical approach was once defined as segmental resection of the jaw.[21] In the same article, the authors reviewed extensive surgical approaches that had resulted in the highest healing rates (over 80%). When surgical resection of the jaw was performed in cancer patients with refractory BRONJ, the surgical outcome was reported to be favorable with little morbidity and good survival. The recurrence rate after mandibulectomy has been reported to be 3% and 9% at 3 and 6 months, respectively.[19] In this study, jaw resection cases did not show a recurrence.

The risk of recurrence was greatest immediately after surgery, after which the probability of relapse decreased over time. The data in **Fig. 3** suggest that the 9-month period after surgery may be critical. This implies the importance of special care for BRONJ patients, especially shortly after surgery.

The goal of surgical treatment of BRONJ patients should be prevention of relapse after surgery. More extensive surgical procedures than curettage produced better surgical outcomes in terms of lower incidence of relapse and repeat surgery. Extensive surgical procedures

appeared to have better prognoses than less extensive treatments. Moreover, intravenous sedation or general anesthesia was associated with better results than local anesthesia. It seems that deeper anesthesia enables surgeons to remove more lesions more extensively. This may be because hypertension modifies OPG, RANK, and RANKL expression and bone repair during the healing period after surgery.[39, 40] In addition, considering the greater post-surgical risk, more frequent and careful follow-ups with OMSs, especially immediately after the procedure, may be advisable. Although most recurrences develop soon after the surgical treatment of BRONJ, all healthcare providers should remember that recurrence could also develop long after surgery.

One strength of this study was that the data included a large number of BRONJ cases in patients with osteoporosis. This was a single-center investigation. An overwhelming majority of the subjects were women (95%), which prevented sex differences from being properly studied. In addition, the focus of this study was the surgical treatment of BRONJ. Its results do not expand our understanding of the pathogenesis of BRONJ, which remains uncertain and elusive.[8, 41] It was not assessed the role of health-threatening habits, such as smoking[31, 32, 42] and drinking alcohol, as predisposing factors. Furthermore, as a retrospective study, it might have had some limitations, such as operator bias, various extents of affected bone, and ambiguous definitions for surgical methods.

In Part II, the effects of zoledronate on the representative maxillofacial stem cells, the PDLSCs and BMSCs, were evaluated. MSCs are considered to play a pivotal role in the

regeneration of craniofacial bone.[28] Thus, the effects of bisphosphonates on MSCs must be elucidated to understand the nature and pathophysiology of BRONJ.

Bisphosphonate has a high affinity for hydroxyapatite and preferentially deposits on bone mineral. The amount of bisphosphonate retained in bone is estimated to be 75 mg/2 kg mineral after 10 years of treatment with 10 mg/day of alendronate.[43] Moreover, bisphosphonates are not homogenously distributed in the body and seem to be more abundant in regions with greater bone turnover. The maxillofacial region is vulnerable to infection due to the existence of resident microorganisms and microtrauma. Therefore, bony remodeling occurs continually in this region, consequently leading to a high bone turnover rate. As such, the jaw bones seem to be more susceptible to the effects of bisphosphonates.[44]

The effects of zoledronate on representative maxillofacial stem cells were evaluated. It was hypothesized that high bisphosphonate concentrations reduce osteogenesis and increase apoptosis of MSCs. The specific aims of the Part II of this study were to (1) evaluate the responses of PDLSCs and BMSCs to various concentrations of zoledronate, (2) evaluate the effects of BMP-2 on the effects of zoledronate, and (3) identify unique properties related to the jaw-specific pathogenesis of BRONJ.

High concentrations of zoledronate suppressed PDLSC and BMSC viability. Both zoledronate and BMP-2 increased apoptotic activity. Moreover, zoledronate treatment significantly suppressed the formation of calcium deposits on PDLSCs and BMSCs with or without BMP-2 treatment. Furthermore, BMP-2 treatment did not overcome the inhibitory effects of zoledronate on osteogenic differentiation. BMP-2 only overcame the suppression of

early-stage osteogenic differentiation of the PDLSCs treated with high concentrations (>5 μM) of zoledronate. Interestingly, the MSX1 gene, which functions in craniofacial development, was significantly suppressed by 10 μM of zoledronate during osteogenic PDLSC differentiation. The osteogenic markers on the BMSCs were more suppressed by zoledronate than the PDLSCs. Overall, the BMSCs were influenced by zoledronate more than the PDLSCs during osteogenic differentiation.

Pharmacokinetic studies have reported that peak plasma concentrations of zoledronate after intravenous administration ranges from 1 to 10 μM, with bioassays revealing concentration levels ranging from 0.4 to 4.5 μM in bone and saliva samples from patients with BRONJ.[45] Thus, zoledronate was tested at concentrations ranging from 1 to 10 μM in this study. Evaluations of human BMSCs have revealed that proliferation and differentiation are induced at low concentrations of zoledronate (from 10^{-4} to 1 μM).[25] Murine preosteoblast cell lines have revealed similar results at low concentrations of zoledronate (from 10^{-2} to 1 μM) along with inhibition of cell proliferation at concentrations over 10 μM. However, mineralization is suppressed following treatment of zoledronate at concentrations over 10^{-2} μM.[26] When treated by less potent bisphosphonates, such as alendronate, BMSCs have also demonstrated increased proliferation and osteogenic differentiation at the same concentrations as those in this experiment.[46] In this study, 1 μM of zoledronate enhanced proliferation but suppressed osteogenic differentiation. This osteogenic suppression was detected on every concentration of zoledronate used in this study, possibly because the original sites that the human BMSCs came from were different.

Certain studies have reported that BMSCs possess site-specific characteristics and responses to bisphosphonates and, thus, BMSCs from the mandible and iliac crest may possess different properties.[47, 48] In this study, human PDLSCs and BMSCs showed varying results on zoledronate treatment in terms of proliferation and osteogenesis.

Apoptosis of various mesenchymal cells following bisphosphonate treatment has been proposed to increase or decrease depending on the bisphosphonate concentration used. In murine preosteoblast cell line (MC3T3-E1) cells, higher concentrations of zoledronate (>10 M) induce apoptosis of osteoblasts, whereas lower concentrations of zoledronate suppress osteoblastic differentiation through downregulation of BMP-2.[26] In murine long bone-derived osteocytic cell line (MLO-Y4) cells, low concentrations of bisphosphonate inhibit, whereas high concentrations (>1 μM) enhance, apoptotic activity in these cells.[17, 49] This inhibitory effect on apoptosis was not detected even at low concentrations of zoledronate (1 μM).

BMPs are members of the transforming growth factor-ß (TGF-ß) superfamily and approximately 20 types are known. BMP-2 is one of these types and induces bone formation through the Smad 1/5/8 signaling pathway.[50] Studies have shown that BMP-2 enhances bone formation of MSCs.[51, 52] Using BMP-2 with MSCs in bone repair results in good bone regeneration. In an extension of these studies, the use of BMP-2 as a treatment solution for BRONJ has been attempted. Kim et al. introduced the use of bone grafts with BMP-2 to the treatment of refractory BRONJ patients.[53] However, in this study, BMP-2 did not overcome the reduced osteogenic properties of human dental stem cells.

MSX1 is known to exist transiently in long extracranial bones during fracture.[54] However, it exists permanently in the adult jawbone.[55] MSX1 suppression by bisphosphonates may be related to the pathogenesis of BRONJ. Increased MSX1 expression is noted in cherubism and giant cell granulomas that appear only in the jawbone and are characterized by hyperproliferation and lack of mineralization.[56] In contrast, another study reported the hypermineralization of alveolar bone exposed to bisphosphonates.[57]

In concordance with my results, Wehrhan et al. reported suppressed MSX1 gene expression in oral mucoperiosteal specimens collected from patients with BRONJ.[58] Moreover, their subsequent study using jawbone specimens from patients with BRONJ revealed similar results (i.e., suppression of MSX1 gene expression).[59] In contrast, Koch et al. reported that compared to the control group, zoledronate led to an approximately 40-fold increase in MSX1 gene expression in human hip-bone osteoblasts at a concentration of 50 μM.[60] This seems highly doubtful, as cell survival was not guaranteed in cells treated with 10 μM of zoledronate in this study.

In this study, the effect of bisphosphonate was shown on stem cells from the human maxillofacial area, which is most relevant to the current clinical scenario of BRONJ. However, this study had certain limitations. As the study design was limited to an *in vitro* model, it was difficult to simulate the consequences of long-term zoledronate exposure. In addition, BRONJ may be induced by various bisphosphonates, only zoledronate was tested as the most potent drug. Moreover, only maxillary BMSCs were used, but no samples from the mandible which is the most dominant site of BRONJ.

Conclusions

Extensive surgical procedures resulted in better treatment outcomes than less extensive treatments, such as curettage with patients under local anesthesia. The risk of recurrence was greatest immediately after surgery, emphasizing the importance of special care and frequent follow-up visits, especially in the period shortly after surgical intervention. The results of this study indicate that discontinuation of bisphosphonate intake before surgery can be omitted. A study with a prospective experimental design may provide a clearer understanding of BRONJ in patients with osteoporosis.

On the cellular level, zoledronate inhibited PDLSC and BMSC proliferation and osteogenic differentiation. Apoptosis or necrosis increased at higher concentrations. Furthermore, high doses of zoledronate (10 μM) reduced the MSX1 expression of MSCs. Additional BMP-2 had no reversal role over adverse effect of zoledronate. These findings suggest that zoledronate has negative effects on the regenerative capacity of MSCs, which may explain the progression of BRONJ.

References

1. Russell RGG. Bisphosphonates: the first 40 years. Bone 2011;49:2-19.
2. Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. J Oral Maxillofac Surg 2003;61:1115-7.
3. Advisory Task Force on Bisphosphonate-Related Osteonecrosis of the Jaws AAoO, Maxillofacial S. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg 2007;65:369-76.
4. 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 Maxillofac Surg 2014;72:1938-56.
5. Spanou A, Lyritis GP, Chronopoulos E, Tournis S. Management of bisphosphonate-related osteonecrosis of the jaw: a literature review. Oral Diseases 2015;8:927-36.
6. Holzinger D, Seemann R, Klug C, et al. Long-term success of surgery in bisphosphonate-related osteonecrosis of the jaws (BRONJs). Oral Oncol 2013;49:66-70.
7. Wutzl A, Pohl S, Sulzbacher I, et al. Factors influencing surgical treatment of bisphosphonate-related osteonecrosis of the jaws. Head Neck 2012;34:194-200.
8. Otto S, Schreyer C, Hafner S, et al. Bisphosphonate-related osteonecrosis of the jaws - characteristics, risk factors, clinical features, localization and impact on oncological treatment. J Craniomaxillofac Surg 2012;40:303-9.

9. Vescovi P, Campisi G, Fusco V, et al. Surgery-triggered and non-surgery triggered bisphosphonate-related osteonecrosis of the jaws (BRONJ): a retrospective analysis of 567 cases in an Italian multicenter study. Oral Oncol 2011;47:191-4.

10. Shintani T, Hayashido Y, Mukasa H, et al. Comparison of the prognosis of bisphosphonate-related osteonecrosis of the jaw caused by oral and intravenous bisphosphonates. Int J Oral Maxillofac Surg 2015;44:840-4.

11. Ulmner M, Jarnbring F, Torring O. Osteonecrosis of the jaw in Sweden associated with the oral use of bisphosphonate. J Oral Maxillofac Surg 2014;72:76-82.

12. Carlson ER, Basile JD. The role of surgical resection in the management of bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg 2009;67:85-95.

13. Lee CH, Park HH, Seo BM, Lee SJ. Modern trends in Class III orthognathic treatment: a time series analysis. Angle Orthod 2017;87:269-78.

14. Ristow O, Otto S, Troeltzsch M, Hohlweg-Majert B, Pautke C. Treatment perspectives for medication-related osteonecrosis of the jaw (MRONJ). J Craniomaxillofac Surg 2015;43:290-3.

15. Jabbour Z, El-Hakim M, Mesbah-Ardakani P, Henderson JE, Albuquerque R, Jr. The outcomes of conservative and surgical treatment of stage 2 bisphosphonate-related osteonecrosis of the jaws: a case series. Int J Oral Maxillofac Surg 2012;41:1404-9.

16. McLeod NM, Patel V, Kusanale A, Rogers SN, Brennan PA. Bisphosphonate osteonecrosis of the jaw: a literature review of UK policies versus international

policies on the management of bisphosphonate osteonecrosis of the jaw. Br J Oral Maxillofac Surg 2011;49:335-42.

17. Ruggiero SL, Kohn N. Disease stage and mode of therapy are important determinants of treatment outcomes for medication-related osteonecrosis of the jaw. J Oral Maxillofac Surg 2015;73:S94-100.

18. Lopes RN, Rabelo GD, Rocha AC, Carvalho PA, Alves FA. Surgical therapy for bisphosphonate-related osteonecrosis of the jaw: six-year experience of a single institution. J Oral Maxillofac Surg 2015;73:1288-95.

19. Bedogni A, Saia G, Bettini G, et al. Long-term outcomes of surgical resection of the jaws in cancer patients with bisphosphonate-related osteonecrosis. Oral Oncol 2011;47:420-4.

20. Lopez-Cedrun JL, Sanroman JF, Garcia A, et al. Oral bisphosphonate-related osteonecrosis of the jaws in dental implant patients: a case series. Br J Oral Maxillofac Surg 2013;51:874-9.

21. Rupel K, Ottaviani G, Gobbo M, et al. A systematic review of therapeutical approaches in bisphosphonates-related osteonecrosis of the jaw (BRONJ). Oral Oncol 2014;50:1049-57.

22. Rollason V, Laverriere A, MacDonald LC, et al. Interventions for treating bisphosphonate-related osteonecrosis of the jaw (BRONJ). Cochrane Database Syst Rev 2016;2:Cd008455.

23. Lee SJ, Lin L, Kim SH, Chung KR, Donatelli RE. Survival analysis of a miniplate and tube device designed to provide skeletal anchorage. Am J Orthod Dentofacial Orthop 2013;144:349-56.

24. Ferlito S, Puzzo S, Palermo F, Verzi P. Treatment of bisphosphonate-related osteonecrosis of the jaws: presentation of a protocol and an observational longitudinal study of an Italian series of cases. Br J Oral Maxillofac Surg 2012;50:425-9.

25. Ribeiro V, Garcia M, Oliveira R, et al. Bisphosphonates induce the osteogenic gene expression in co-cultured human endothelial and mesenchymal stem cells. J Cell Mol Med 2014;18:27-37.

26. Huang X, Huang S, Guo F, et al. Dose-dependent inhibitory effects of zoledronic acid on osteoblast viability and function in vitro. Mol Med Rep 2016;13:613-22.

27. Sharma D, Hamlet SM, Petcu EB, Ivanovski S. The effect of bisphosphonates on the endothelial differentiation of mesenchymal stem cells. Sci Rep 2016;6:20580.

28. Shi S, Bartold PM, Miura M, et al. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. Orthod Craniofac Res 2005;8:191-9.

29. Ogata K, Katagiri W, Osugi M, et al. Evaluation of the therapeutic effects of conditioned media from mesenchymal stem cells in a rat bisphosphonate-related osteonecrosis of the jaw-like model. Bone 2015;74:95-105.

30. Ruggiero SL, Dodson TB, Assael LA, 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.

31. Nisi M, La Ferla F, Karapetsa D, et al. Risk factors influencing BRONJ staging in patients receiving intravenous bisphosphonates: a multivariate analysis. Int J Oral Maxillofac Surg 2015;44:586-91.

32. Watters AL, Hansen HJ, Williams T, et al. Intravenous bisphosphonate-related osteonecrosis of the jaw: long-term follow-up of 109 patients. Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:192-200.

33. Dodson TB. Intravenous bisphosphonate therapy and bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg 2009;67:44-52.

34. R Development Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2016.

35. Fliefel R, Troltzsch M, Kuhnisch J, Ehrenfeld M, Otto S. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int J Oral Maxillofac Surg 2015;44:568-85.

36. Alhussain A, Peel S, Dempster L, Clokie C, Azarpazhooh A. Knowledge, practices, and opinions of ontario dentists when treating patients receiving bisphosphonates. J Oral Maxillofac Surg 2015;73:1095-105.

37. Sarasquete ME, González M, San Miguel JF, García-Sanz R. Bisphosphonate-related osteonecrosis: genetic and acquired risk factors. Oral Diseases 2009;15:382-7.

38. Sharma D, Hamlet S, Petcu E, Ivanovski S. Animal models for bisphosphonate-related osteonecrosis of the jaws - an appraisal. Oral Diseases 2013;19:747-54.

39. Manrique N, Pereira CC, Luvizuto ER, et al. Hypertension modifies OPG, RANK, and RANKL expression during the dental socket bone healing process in spontaneously hypertensive rats. Clin Oral Investig 2015;19:1319-27.

40. Gealh WC, Pereira CC, Luvizuto ER, et al. Healing process of autogenous bone graft in spontaneously hypertensive rats treated with losartan: an immunohistochemical and histomorphometric study. J Oral Maxillofac Surg 2014;72:2569-81.

41. Ruggiero SL. Emerging concepts in the management and treatment of osteonecrosis of the jaw. Oral Maxillofac Surg Clin North Am 2013;25:11-20.

42. Lee LW, Hsiao SH, Chen LK. Clinical treatment outcomes for 40 patients with bisphosphonates-related osteonecrosis of the jaws. J Formos Med Assoc 2014;113:166-72.

43. Rodan G, Reszka A, Golub E, Rizzoli R. Bone safety of long-term bisphosphonate treatment. Curr Med Res Opin 2004;20:1291-300.

44. 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 Maxillofac Surg 2005;63:1567-75.

45. Scheper MA, Badros A, Salama AR, et al. A novel bioassay model to determine clinically significant bisphosphonate levels. Support Care Cancer 2009;17:1553-7.

46. Kim HK, Kim JH, Abbas AA, Yoon TR. Alendronate enhances osteogenic differentiation of bone marrow stromal cells: a preliminary study. Clin Orthop Relat Res 2009;467:3121-8.

47. Akintoye SO, Lam T, Shi S, et al. Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals. Bone 2006;38:758-68.

48. Stefanik D, Sarin J, Lam T, et al. Disparate osteogenic response of mandible and iliac crest bone marrow stromal cells to pamidronate. Oral Dis 2008;14:465-71.

49. Plotkin LI, Weinstein RS, Parfitt AM, et al. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J Clin Invest 1999;104:1363-74.

50. Scarfì S, Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair. World J Stem Cells 2016;8:1-12.

51. Edgar CM, Chakravarthy V, Barnes G, et al. Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. Bone 2007;40:1389-98.

52. Kang Q, Song WX, Luo Q, et al. A comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. Stem Cells Dev 2009;18:545-59.

53. Kim J-W, Kim S-J, Kim M-R. Simultaneous application of bone morphogenetic protein-2 and platelet-rich fibrin for the treatment of bisphosphonate-related osteonecrosis of jaw. J Oral Implantol 2016;42:205-8.

54. Gersch RP, Lombardo F, McGovern SC, Hadjiargyrou M. Reactivation of Hox gene expression during bone regeneration. J Orthop Res 2005;23:882-90.

55. Berdal A, Molla M, Hotton D, et al. Differential impact of MSX1 and MSX2 homeogenes on mouse maxillofacial skeleton. Cells Tissues Organs 2009;189:126-32.

56. Hyckel P, Berndt A, Schleier P, et al. Cherubism - new hypotheses on pathogenesis and therapeutic consequences. J Craniomaxillofac Surg 2005;33:61-8.

57. Favia G, Pilolli GP, Maiorano E. Histologic and histomorphometric features of bisphosphonate-related osteonecrosis of the jaws: an analysis of 31 cases with confocal laser scanning microscopy. Bone 2009;45:406-13.

58. Wehrhan F, Hyckel P, Ries J, et al. Expression of Msx-1 is suppressed in bisphosphonate associated osteonecrosis related jaw tissue-etiopathology considerations respecting jaw developmental biology-related unique features. J Transl Med 2010;8:96.

59. Wehrhan F, Hyckel P, Amann K, et al. Msx-1 is suppressed in bisphosphonate-exposed jaw bone analysis of bone turnover-related cell signalling after bisphosphonate treatment. Oral Dis 2011;17:433-42.

60. Koch FP, Merkel C, Al-Nawas B, et al. Zoledronate, ibandronate and clodronate enhance osteoblast differentiation in a dose dependent manner--a quantitative in vitro gene expression analysis of Dlx5, Runx2, OCN, MSX1 and MSX2. J Craniomaxillofac Surg 2011;39:562-9.

Table 1. Descriptive summary of study sample

Study Variables		Descriptive statistics
ample size		325 (100)
Gender	Female	314 (96.6)
Age (years)		75.0 ± 10.0
Anatomic location	Maxilla	72 (22.2)
	Mandible	239 (73.5)
	Maxilla + Mandible	14 (4.3)
Affected location	Incisor region	30 (9.2)
	Premolar region	32 (9.8)
	Molar region	181 (55.7)
	Premolar – molar region	60 (18.5)
	Incisor – premolar region	13 (4.0)
	Incisor – premolar – molar	9 (2.8)
Disease stage	2	31 (9.5)
	3	294 (90.5)
Initiating event (etiology)	Extraction	205 (63.1)
	Implant	52 (16.0)
	Prosthodontics	30 (9.2)
	Spontaneous	38 (11.7)
Co-morbidities	Diabetes	80 (24.6)
	Hypertension	189 (58.2)
	Steroid use	21 (6.5)
Intravenous bisphosphonate intake		16 (4.9)
Conservative treatment time (days)		51.0 ± 97.0
Bisphosphonate treatment time (months)		39.5 ± 57.0
Drug holiday before surgery (months)		4.0 ± 7.0
Time to final follow-up (months)		6.0 ± 14.9
Referral from private practitioner		207 (63.7)
Anesthesia	Local anesthesia	136 (41.8)
	Intravenous sedation	27 (8.3)
	General anesthesia	162 (49.8)
Surgical treatment	Curettage	35 (10.8)
	Sequestrectomy	207 (63.7)
	Saucerization	78 (24.0)
	Mandibulectomy	5 (1.5)
Number of relapse	None (treatment success)	228 (70.2)
	Once	62 (19.1)
	Twice	20 (6.2)
	>Three times	15 (4.6)
Time to relapse re-operation (months)		4.1 ± 8.6

Data presented as n (%) or median ± interquartile range.

Table 2. Study variables versus outcome variable

Study Variables		Success/Healed	Failure/Relapse	Hazard Ratio*	*P* Value
Sample size		228 (70.2)	97 (29.8)		
Gender	Female	220 (70.1)	94 (29.9)	1.04 (0.33-3.27)	0.953
	Male†	8 (72.7)	3 (27.3)		
Age (years)		75.0 ± 9.0	75.0 ± 11.0	1.01 (0.98-1.03)	0.597
Anatomic location	Maxilla	58 (80.6)	14 (19.4)	0.63 (0.36-1.13)	0.119
	Mandible†	166 (69.5)	73 (30.5)		
	Maxilla + Mandible	4 (28.6)	10 (71.4)	1.73 (0.89-3.37)	0.105
Affected location	Incisor region†	21 (70.0)	9 (30.0)		
	Premolar region	26 (81.2)	6 (18.8)	0.59 (0.21-1.67)	0.320
	Molar region	130 (71.8)	51 (28.2)	0.89 (0.44-1.81)	0.746
	Premolar – molar region	35 (58.3)	25 (41.7)	1.43 (0.67-3.08)	0.355
	Incisor – premolar region	11 (84.6)	2 (15.4)	0.45 (0.10-2.08)	0.305
	Incisor – premolar – molar	5 (55.6)	4 (44.4)	1.60 (0.49-5.24)	0.435
Disease stage	2†	24 (77.4)	7 (22.6)		
	3	204 (69.4)	90 (30.6)	1.18 (0.55-2.55)	0.673
Initiating event (etiology)	Extraction†	138 (67.3)	67 (32.7)		
	Implant	37 (71.2)	15 (28.8)	1.10 (0.63-1.93)	0.733
	Prosthodontics	19 (63.3)	11 (36.7)	1.12 (0.59-2.12)	0.727
	Spontaneous	34 (89.5)	4 (10.5)	0.36 (0.13-1.00)	0.050
Co-morbidities	Diabetes	50 (62.6)	30 (37.5)	1.30 (0.85-2.00)	0.240
	Hypertension	124 (65.5)	65 (34.4)	1.54 (1.01-2.35)	0.046
	Steroid use	15 (71.4)	6 (28.6)	1.08 (0.47-2.47)	0.853
Intravenous bisphosphonate intake		14 (87.5)	2 (12.5)	1.71 (0.42-6.95)	0.412
Conservative treatment time (days)		61.5 ± 97.0	30.0 ± 75.0	1.00 (1.00-1.00)	0.093
Bisphosphonate treatment time (months)		36.0 ± 60.0	45.5 ± 40.5	0.99 (0.99-1.00)	0.093
Drug holiday before surgery (months)		4.0 ± 6.0	2.0 ± 7.0	0.98 (0.95-1.01)	0.087
Referral from private practitioner		147 (71.0)	60 (29.0)	0.70 (0.46-1.07)	0.101
Anesthesia	Local anesthesia	77 (56.6)	59 (43.4)	3.43 (2.22-5.30)	<0.001
	Intravenous sedation	21 (77.8)	6 (22.2)	1.25 (0.52-2.98)	0.620
	General anesthesia†	130 (80.2)	32 (19.8)		
Surgical treatment	Curettage†	15 (42.9)	20 (57.1)		
	Sequestrectomy	147 (71.0)	60 (29.0)	0.50 (0.30-0.83)	0.007
	Saucerization	61 (78.2)	17 (21.8)	0.27 (0.14-0.53)	<0.001
	Mandibulectomy	5 (100)	0 (0)	0.00 (0.00-0.00)	1.000

Data presented as n (%) or median ± interquartile range.

*Hazard ratio was calculated from the univariate Cox proportional hazards regression model. The 95% confidence interval of the hazards ratio is in the parenthesis.

†Reference groups.

Table 3. Summary of survival analysis using the Cox regression model

Study Variables		Hazard Ratio	95% Confidence Interval	*P* Value*
Gender				
(reference, male)	Female	1.00	(0.29-3.44)	0.9947
Age (1-year increments)		1.00	(0.97-1.03)	0.8679
Anatomic location				
(reference, mandible)	Maxilla	0.68	(0.37-1.24)	0.2081
	Maxilla + Mandible	1.64	(0.81-3.33)	0.1675
Co-morbidities				
(reference, none)	Hypertension	1.50	(0.94-2.39)	0.0858
Conservative treatment		1.00	(1.00-1.00)	0.0860
Drug holiday		0.99	(0.96-1.02)	0.1109
Mode of anesthesia				
(reference, general)	Local anesthesia	2.91	(1.83-4.62)	<0.0001
Surgical treatment				
(reference, curettage)	Extensive surgeries except curettage	0.51	(0.30-0.87)	0.0145

*Result of the multivariate Cox proportional hazards regression model.

Table 4. Sequence of primers

Gene		Primer
EF1 α	forward	5′ -AGGTGATTATCCTGAACCATCC-3′
	reverse	5′ -AAAGGTGGATAGTCTGAGAAGC-3′
Osteocalcin	forward	5′ -CATGAGAGCCCTCACA-3′
	reverse	5′ -AGAGCGACACCCTAGAC-3′
Collagen type 1	forward	5′ -CAAAGAGTCTACATGTCTAG-3′
	reverse	5′ -CATGGGGCCAGGCACGGAAA-3′
MSX1	forward	5′ -CTCCTCAAGCTGCCAGAAGAT-3′
	reverse	5′ -GCTTACGGTTCGTCTTGTGTT-3′

FIGURES

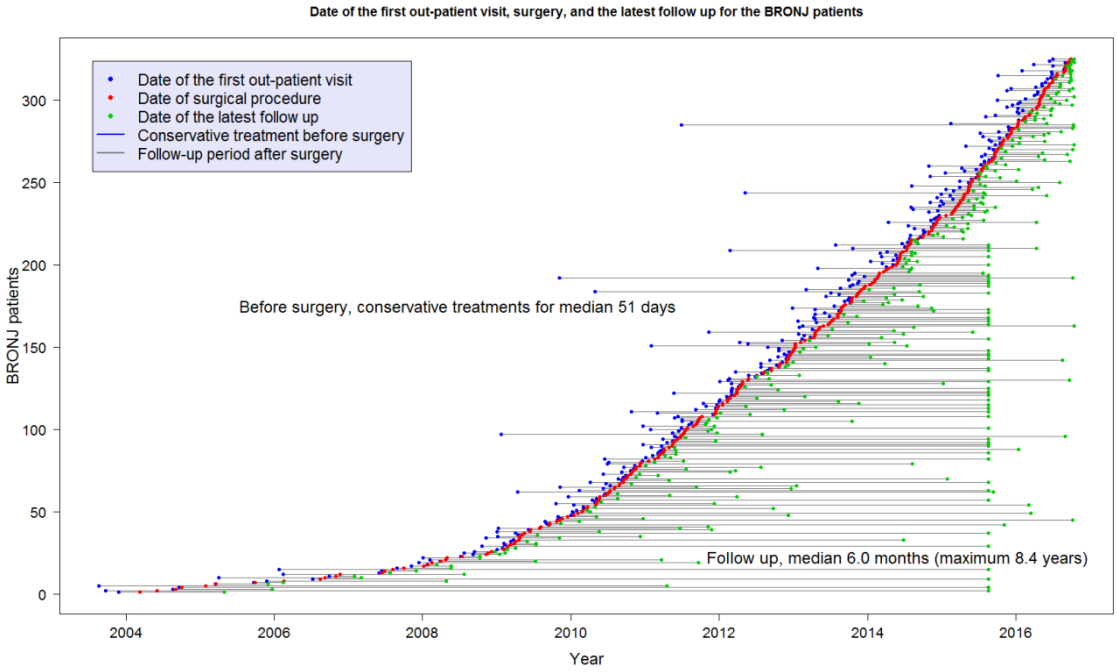

Figure 1. Distribution of treatment and follow-up time for 325 patients with BRONJ from 2004 to 2016. The patients are sorted on the y-axis according to date of first surgery on the x-axis. Before undergoing surgical treatment, the patients received conservative and supportive treatment for a median of 51 days. After surgery, the median follow-up time was 6 months.

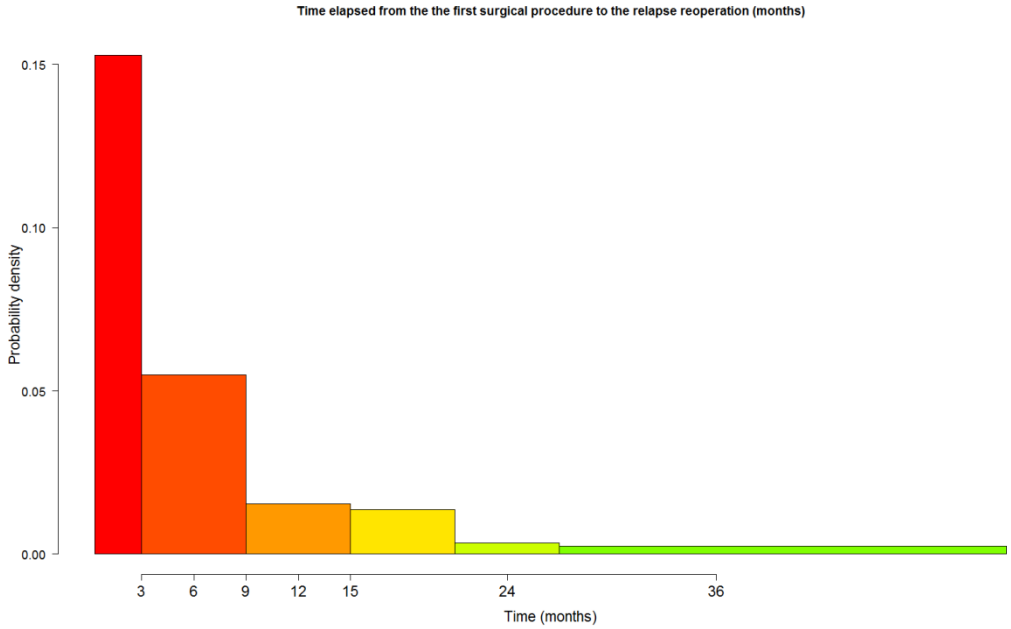

Figure 2. Time from first surgical procedure to relapse operation. Despite careful surgical procedures, 97 patients (30%) did not completely recuperate and required repeated surgical management to treat their relapsed lesions. The time from the first surgery to the relapse reoperation ranged from approximately 10 days to 5.6 years. The probability of recurrence was greatest immediately after surgery and decreased over time. The most frequent recurrences developed within 9 months of the initial surgical treatment.

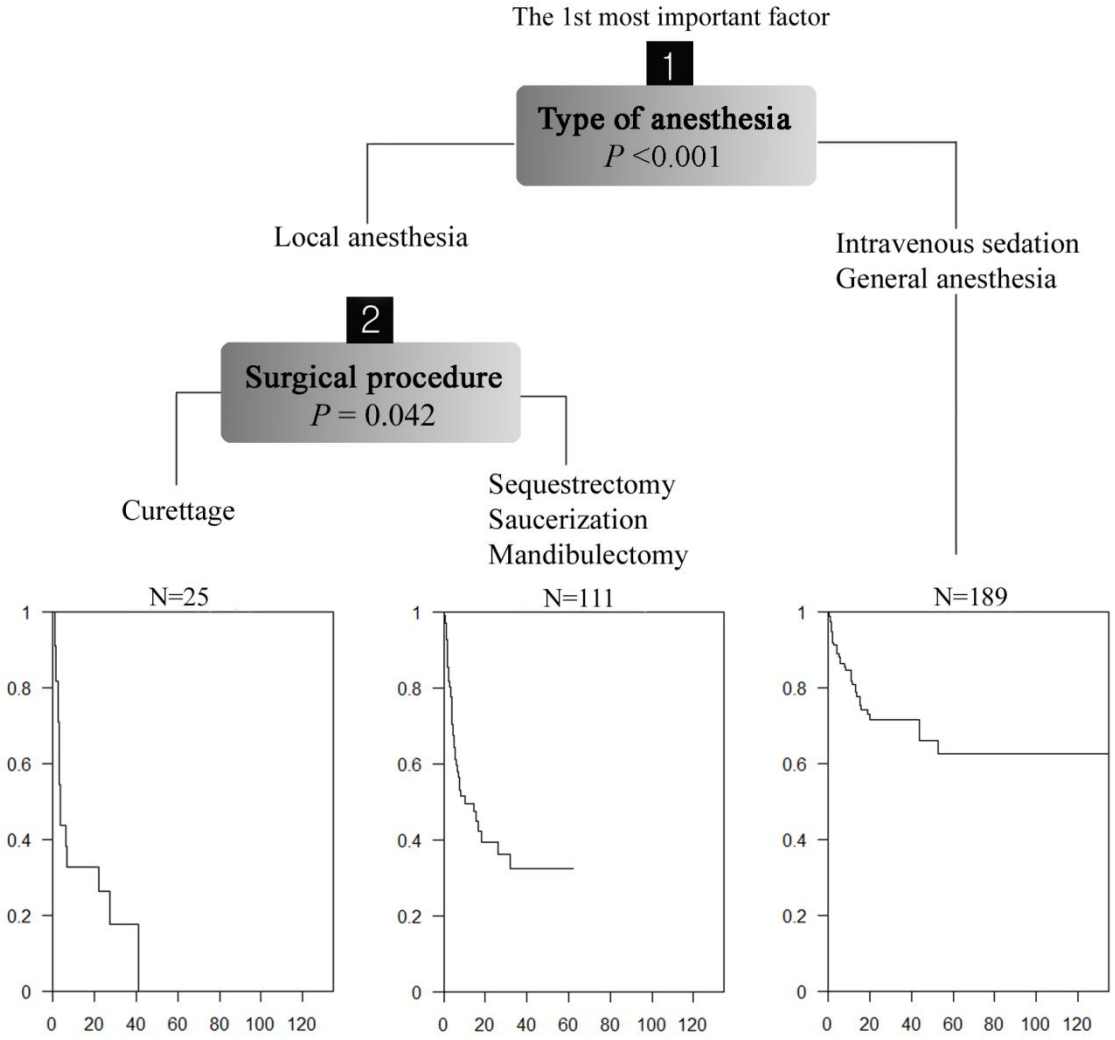

Figure 3. Conditional inference tree combined with Kaplan-Meier survival function. The type of surgical procedure was the most important variable for clinical success after surgery for BRONJ. The patients who underwent curettage had the worst prognoses. The patients who received sequestrectomy and saucerization under general anesthesia or intravenous sedation had the best treatment outcomes over time.

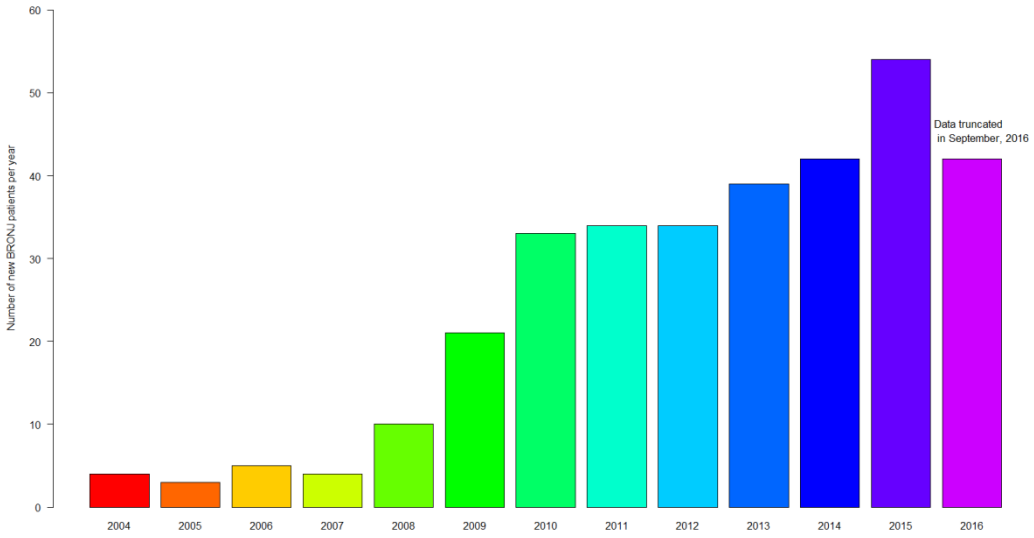

Figure 4. The number of new patients with BRONJ requiring surgical treatment in our institution has been increasing over the past decade.

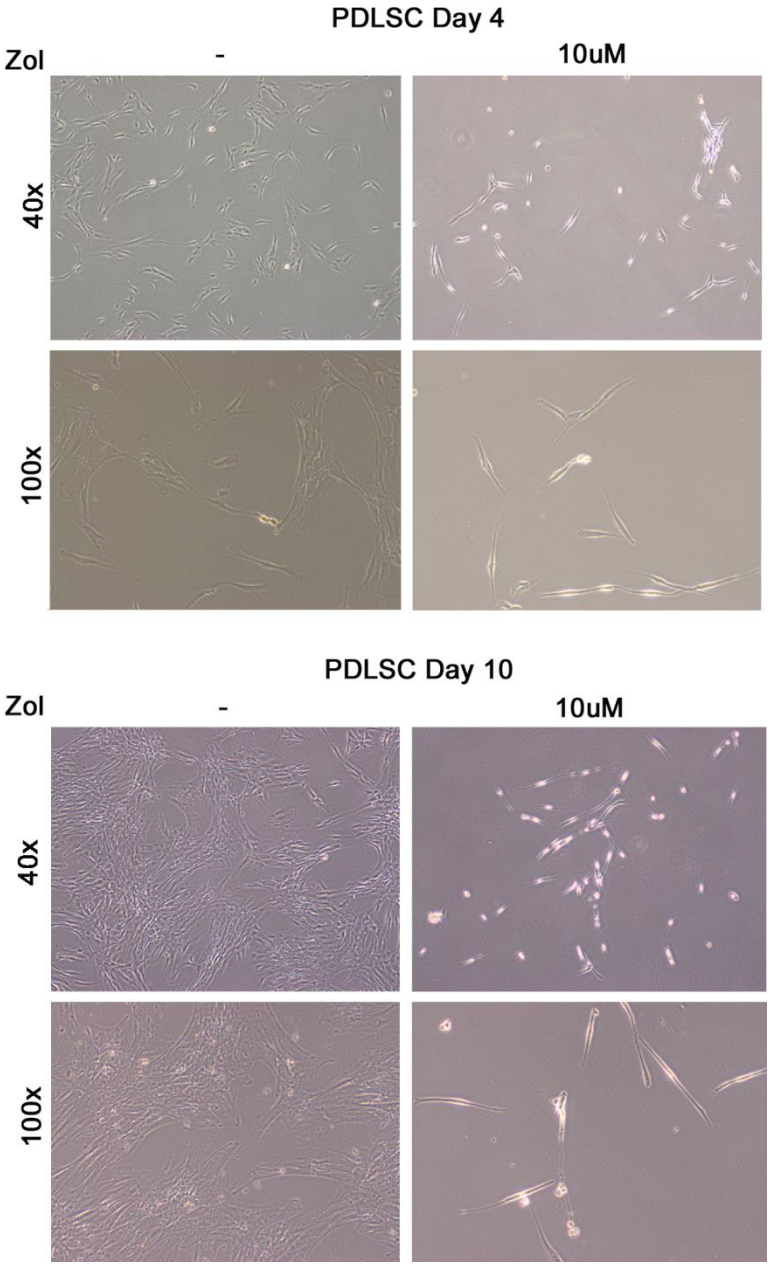

Figure 5. Morphology of PDLSCs treated with zoledronate. PDLSCs showed a spike-like morphology on days 4 and 10 in the control group. Cell death was found in a large proportion of the PDLSCs treated with zoledronate.

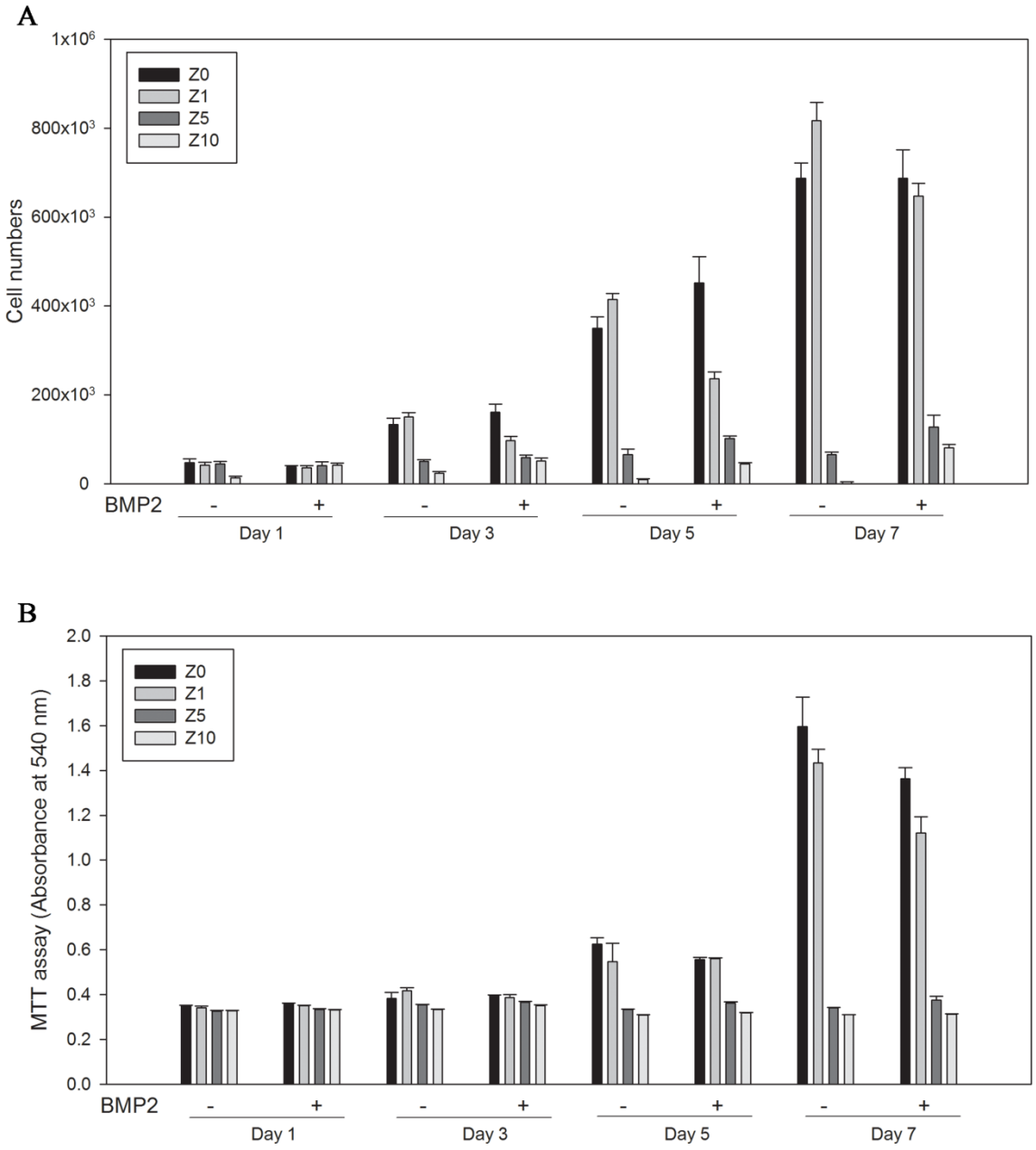

Figure 6. PDLSC proliferation and viability were detected by (A) direct cell counting and (B) the MTT assay on days 1, 3, 5, and 7 with 1, 5, and 10 μM of zoledronate. PDLSC proliferation and viability were suppressed by high concentration of zoledronate treatment.

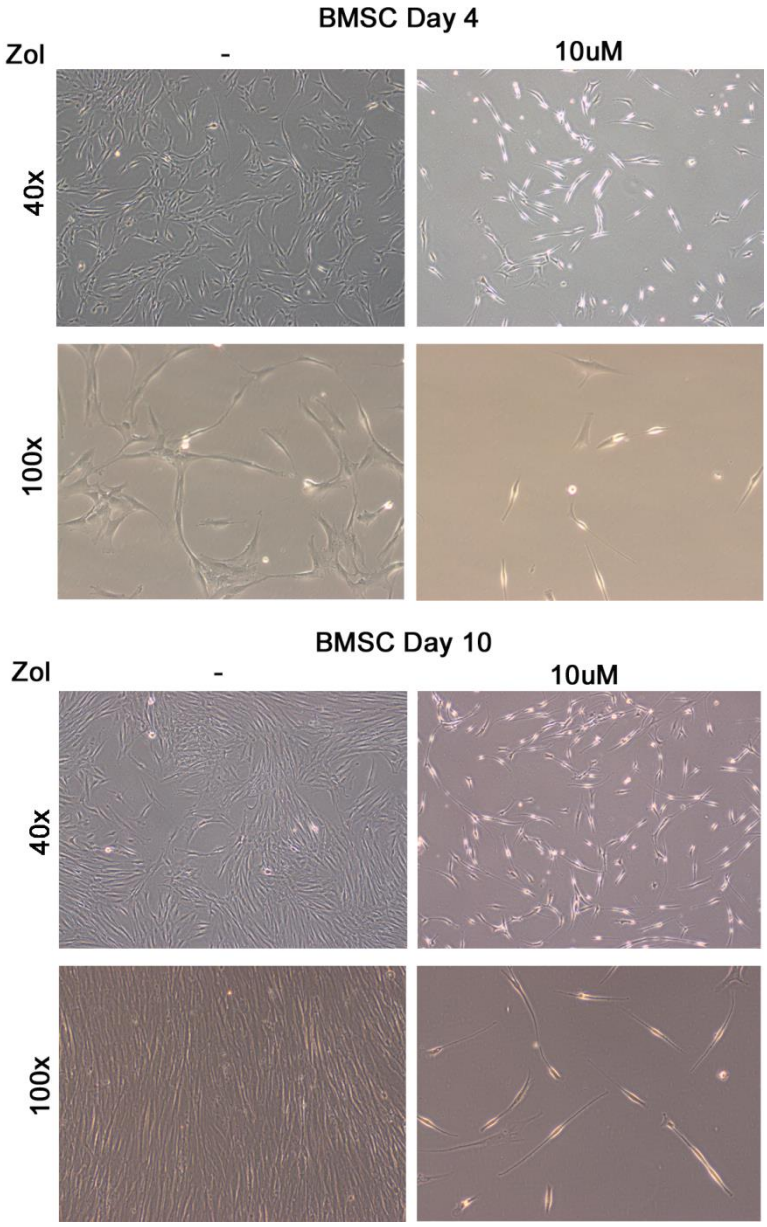

Figure7. Morphology of BMSCs treated with zoledronate. Cell death was found in a large proportion of the BMSCs treated with zoledronate. A large proportion of zoledronate-treated BMSCs presented as detached, round cells after washing.

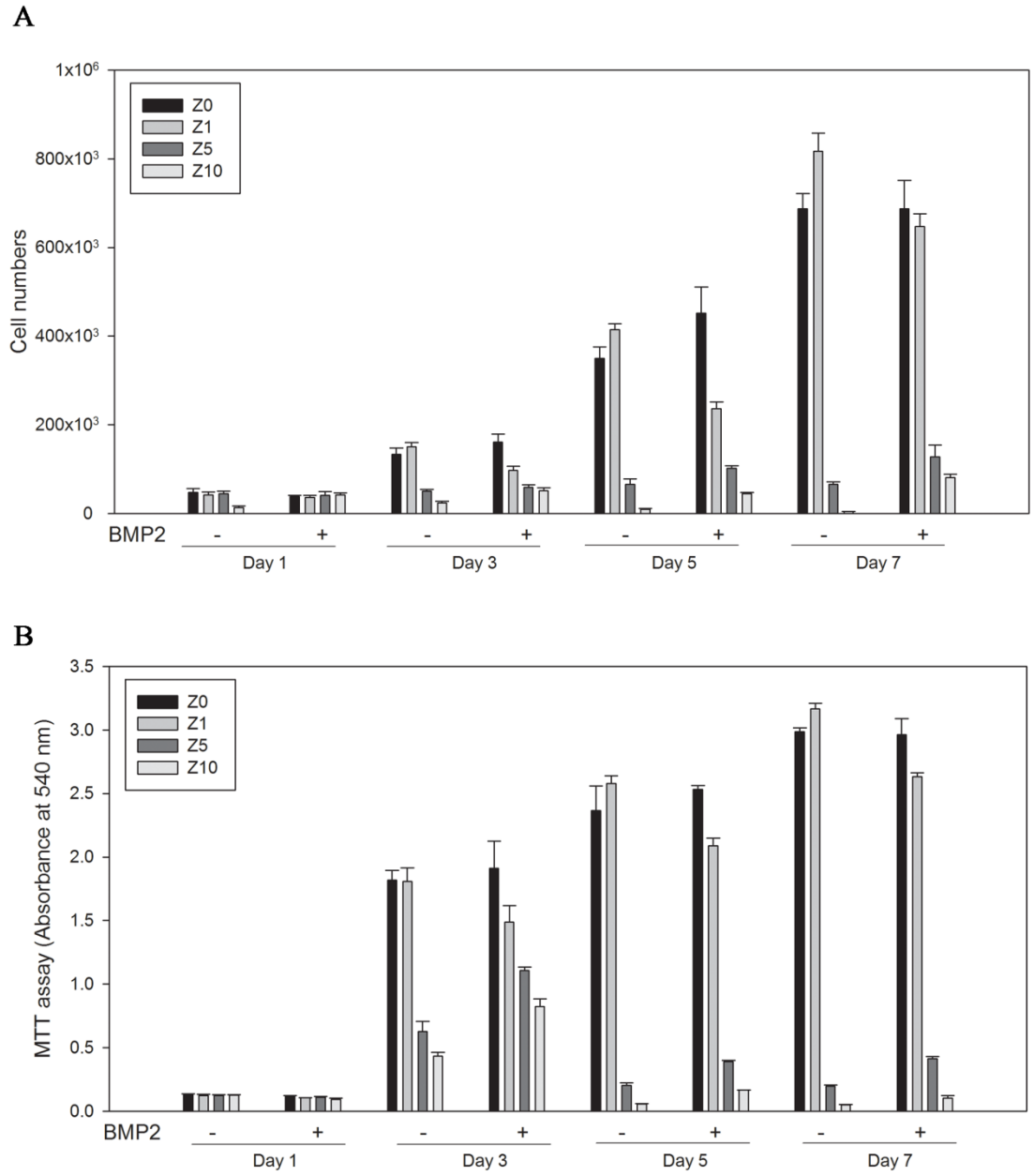

Figure 8. BMSC viability was detected by (A) direct cell counting and (B) the MTT assay on days 1, 3, 5, and 7 with 1, 5, and 10 μM of zoledronate. High concentrations of zoledronate suppressed the cell viability on BMSCs, also, 200 ng/mL of BMP-2 suppressed the cell viability in 1μM zoledronate

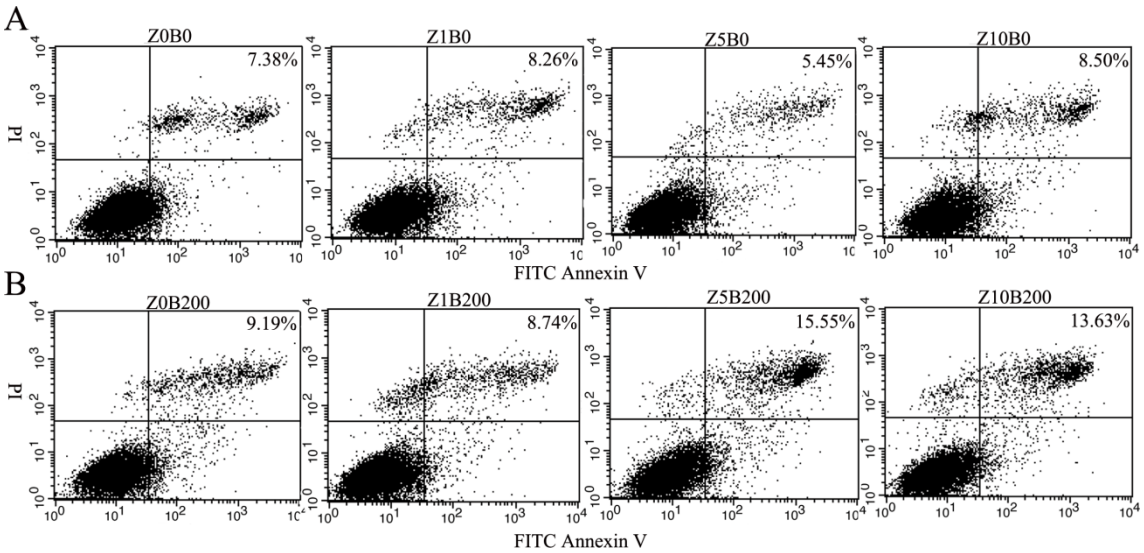

Figure 9. Flow cytometry determined the apoptotic activity of PDLSCs treated with zoledronate over 5 days. Both zoledronate and BMP-2 affected the apoptotic activity of the PDLSCs. Not only PDLSC viability was not only suppressed by zoledronate treatment, but also by BMP-2 treatment.

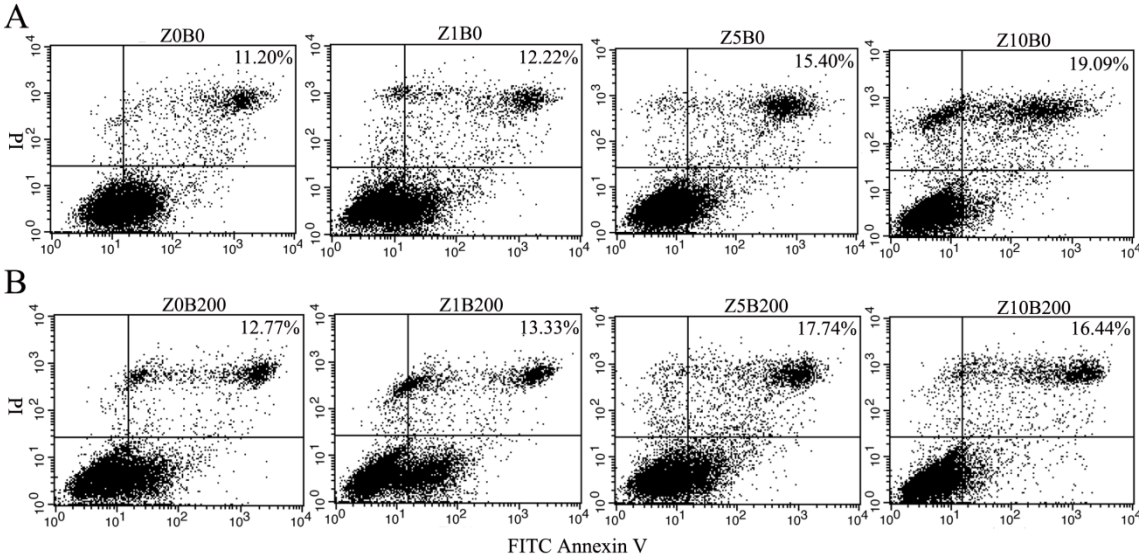

Figure 10. Flow cytometry determined the apoptotic activity of BMSCs treated with zoledronate over 5 days. Zoledronate affected the apoptotic activity of the BMSCs in dose dependent manner. Additional BMP-2 did not show significant enhanced effect on BMSCs apoptosis influenced by zoledronate.

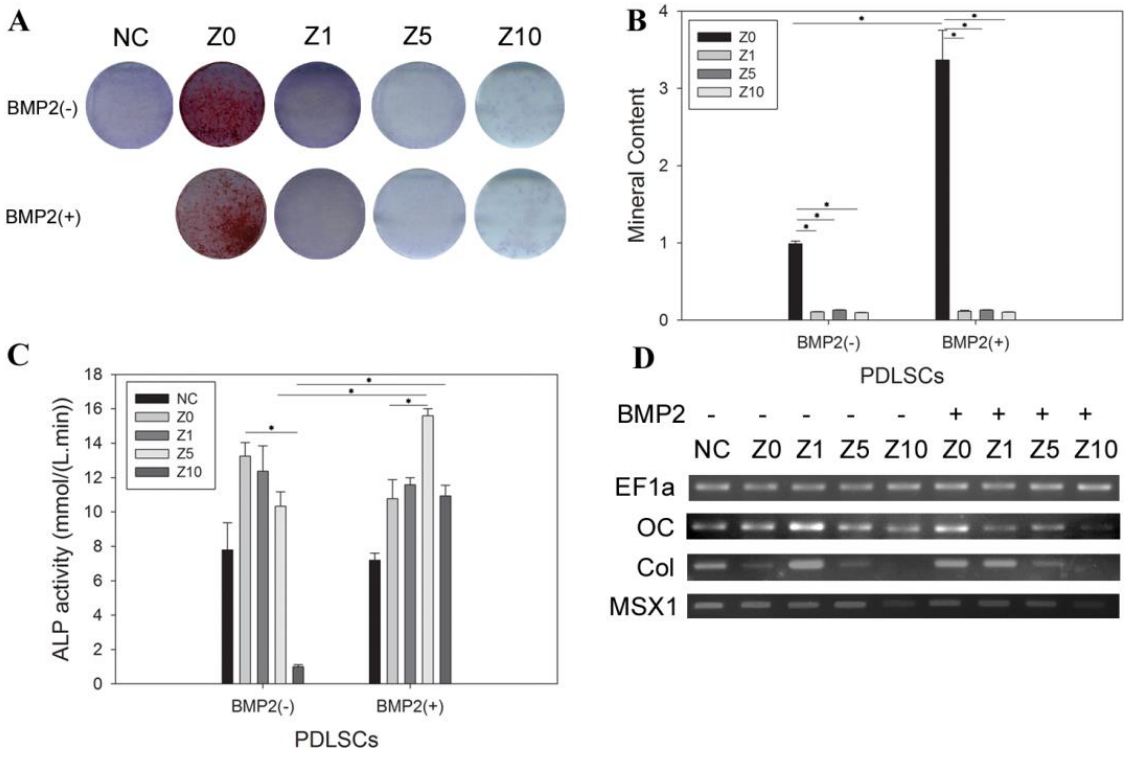

Figure 11. Osteogenic differentiation of PDLSCs was evaluated by (A, B) alizarin red S staining and (C) ALP activity. (D) RT-PCR showed that zoledronate and BMP-2 did not have significant effects on the osteogenic markers, osteocalcin and type I collagen.

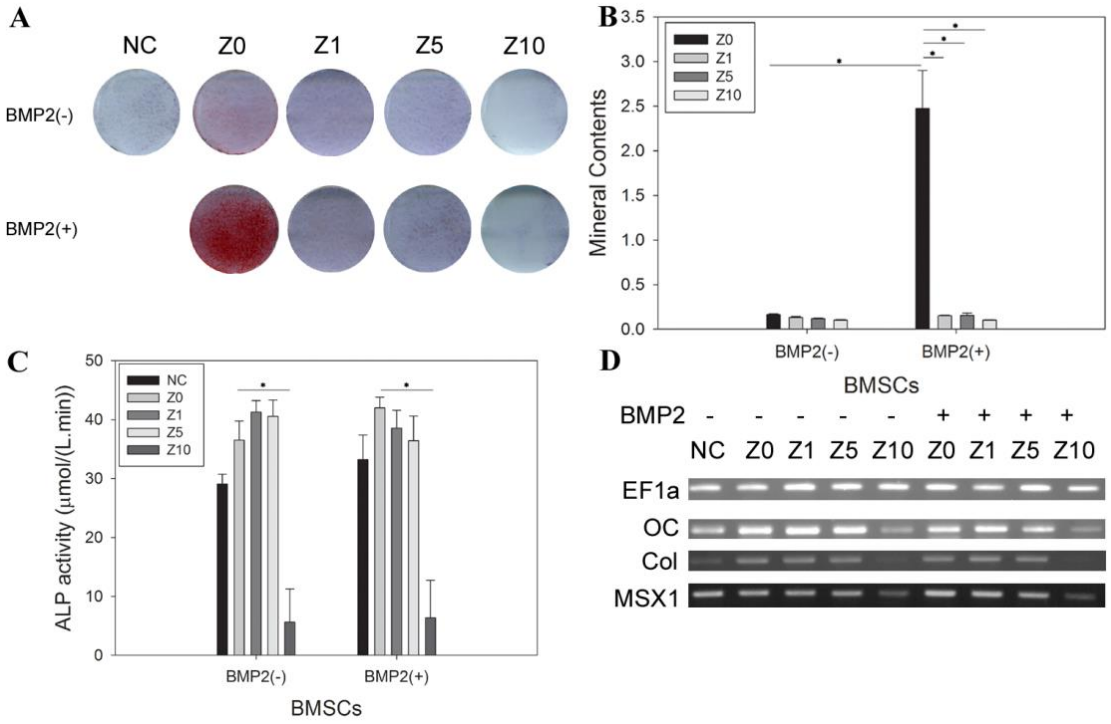

Figure 12. Osteogenic differentiation of BMSCs was evaluated by (A, B) alizarin red S staining and (C) ALP activity. (D) RT-PCR showed that gene expression of osteocalcin on BMSCs was decreased by zoledronate treatment in dose-dependent manners in the 200-ng/mL BMP-2 treatment groups.

국문초록

비스포스포네이트 관련 악골괴사

– 세포 특이적 반응과 임상적 치료 결과 연구

김 희 영

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연구 목적

비스포스포네이트 관련 악골괴사는 그 기전이 잘 밝혀지지 않은 약물 투약과 관련된 합병증이다. 이 연구의 목적은 임상적으로는 비스포스포네이트 관련 악골괴사(BRONJ)의 수술적 치료 후에 재발이나 치료 실패를 유발하는 위험인자를 밝혀내고자 하였고, 실험적으로는 대표적인 비스포스포네이트 제제인 졸레드로네이트가 치아 줄기세포에 미치는 영향을 살펴보아 악골에 선택적으로 나타나는 원인을 찾아보고자 하였다.

Part I. 임상 연구

[재료 및 방법]

이 후향적 코호트 연구는 2004년에서 2016년 사이에 서울대학교치과병원 구강악안면외과에서 수술적 치료를 시행 받은 BRONJ 환자들 중 골다공증을 이유로 비스포스포네이트를 투약받은 이들을 대상으로 하였다. 예측변수들은 한 세트의 비균질한 변수로서 다음의 사항을 조사 하였다; 인구통계학적 변수 (성별, 나이); 해부학적 변수(상악이나 하악 혹은 양쪽 모두, 이환된 위치); 임상적 변수(질환 기수, 병인, 동시이환, 비스포스포네이트의 종류, 정주로 비스포스포네이트를 투약 받은 병력); 시간 (수술전 보존적 치료, BRONJ 발병 이전에 비스포스포네이트의 투약, 수술전 투약중지 기간, 마지막 내원일까지의 시간, 재수술이나 치료실패의 경우 재수술까지 걸린 시간); 그리고 수술 중 변수 (마취의 종류, 수술 술식의 종류). 일차 결과 변수는 수술 후에 재발이 발생해서 재수술이 필요했는지 여부이다. 위험 인자를 결정하기 위해 Cox 모델을 이용하여 생존분석을 적용하였다.

[연구결과]

최종 표본은 325명으로 평균 연령 75세이고 97%가 여성이었다. 수술 후에 30%의 환자들은 완전히 치유가 되지 않아 재수술을 받게 되었다. 처음 수술로부터 재수술까지 걸리는 기간은 10일에서 5.6년으로 다양하였다. 재발이나 치료의 실패는 수술 직후에 가장 빈번하게 나타났다. 수술 방법과 마취의 종류는 치료

결과에서 가장 중요한 요소였다. 약물휴지기는 수술 후 재발이 일어나는데 영향을 미치는 요소로 나타나지 않았다.

Part II. 세포 실험

[재료 및 방법]

골수줄기세포와 치주인대줄기세포를 다양한 농도의 졸레드로네이트로 처리하여 연구하였다. 졸레드로네이트에 영향을 받은 치아줄기세포의 골형성 분화를 알리자린 레드 염색과 알칼리성 인산가수 분해효소 활성 염색을 통해 보았다. RT-PCR을 시행하여 MSX-1, 오스테오칼신, 콜라겐 타입1, EF1a의 mRNA 수치를 비교하였다. 세포자멸은 FITC 아넥신 V 와 PI 염색 분석으로 검출하였다. 골형성단백질-2(BMP-2)도 치아줄기세포에 처리하여 졸레드로네이트의 영향을 극복할 수 있는지 보고자 하였다.

[연구결과]

고농도의 졸레드로네이트는 치주인대줄기세포와 골수줄기세포의 세포 생존능력을 억제하였다. 졸레드로네이트 뿐만 아니라 BMP-2 역시 치주인대세포의 세포자멸을 야기하였다. 고종도의 졸레드로네이트는 골수줄기세포의 세포자멸을 야기하였지만, BMP-2는 졸레드로네이트에 의해 야기된 세포자멸에 영향을 미치지 않았다. 또한, 졸레드로네이트의 처리는 치주인대줄기세포와 골수줄기세포의 칼슘 침착을

억제하였는데, 이는 BMP-2의 처리와는 무관하였다. BMP-2의 처리로 치주인대세포와 골수줄기세포의 골형성 분화를 극복할 수 없었다. 악안면 발생에서 영향을 미치는 MSX1 유전자가 졸레드로네이트 10 μM을 처리한 치주인대줄기세포의 골형성 분화를 유의미하게 억제하는 것으로 나타났다.

결론

골다공증 환자에 있어 BRONJ 치료는 국소마취하에서 소파술을 시행하는 것보다는 광범위한 수술적 치료가 더 좋은 결과를 가져올 수 있다. 고농도의 졸레드로네이트는 치아줄기세포의 증식, 골형성 분화와 MSX1의 발현을 억제하는 것으로 나타났다. 이 결과는 졸레드로네이트가 치아줄기세포의 증식억제와 분화억제를 보임으로써 BRONJ의 발생에 기여요인으로 작용할 것으로 분석된다.

주요어 : 비스포스포네이트, 골괴사, 치료, 재발, 위험요소, 치아줄기세포, MSX1

학번 : 2012-30595