BCL-XL EXPRESSION IN OSTEOCLASTS OF PATIENTS WITH MEDICATION-RELATED OSTEONECROSIS OF THE JAW: A COMPARISON WITH OSTEORADIONECROSIS AND OSTEOMYELITIS

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การศึกษาเปรียบเทียบการแสดงออกของบีซีแอล-เอ็กซ์แอลในเซลล์ทำลายกระดูกระหว่างกระดูกขากรรไกรตายจากยา กระดูกขากรรไกรตายจากรังสีรักษา และ กระดูกขากรรไกรอักเสบ

น.ส.วิ่ เจิน หง็อก ทรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	BCL-XL EXPRESSION IN OSTEOCLASTS OF PATIENTS WITH MEDICATION-RELATED OSTEONECROSIS OF THE JAW: A COMPARISON WITH OSTEORADIONECROSIS AND OSTEOMYELITIS
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Field of Study	Oral Biology
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วี่ เจิน หง็อก ทรี : การศึกษาเปรียบเทียบการแสดงออกของบีซีแอล-เอ็กซ์แอลในเซลล์ทำลายกระดูกระหว่างกระดูกขากรรไกรตายจากยา กระดูกขากรรไกรตายจากรังสีรักษา และ กระดูกขากรรไกรอักเสบ. (BCL-XL EXPRESSION IN OSTEOCLASTS OF PATIENTS WITH MEDICATION-RELATED OSTEONECROSIS OF THE JAW: A COMPARISON WITH OSTEORADIONECROSIS AND OSTEOMYELITIS) อ.ที่ปรึกษาหลัก : รศ.ทพญ.ดร.ริสา ชัยศุภรัตน์, อ.ที่ปรึกษาร่วม : รศ.ทพญ. ดร.นีรชา สารชวนะกิจ

วัตถุประสงค์: กลไกการเกิดของกระดูกขากรรไกรตายจากยายังไม่ทราบแน่ชัด ออสติโอคลาสต์เป็นเซลล์ที่มีความเกี่ยวข้องกับกลไกการเกิดของรอยโรคนี้ วัตถุประสงค์ของวิจัยนี้เพื่อศึกษาลักษณะทางจุลพยาธิวิทยาในแง่ลักษณะรูปร่างและจำนวนของออสติโอคลาสต์ และการแสดงออกของของบีซีแอล-เอ็กซ์แอลในออสติโอคลาสต์ในรอยโรคกระดูกขากรรไกรตายจากยาเปรียบเทียบกับกระดูกขากรรไกรตายจากรังสีรักษา กระดูกขากรรไกรอักเสบ และกระดูกปกติ

วิธีการทดลอง: การวิจัยนี้ศึกษาผู้ป่วยจำนวน 57 ราย ประกอบด้วย ผู้ป่วยที่ได้รับการวินิจฉัยเป็นโรคกระดูกขากรรไกรตายจากยา กระดูกขากรรไกรตายจากรังสีรักษา กระดูกขากรรไกรอักเสบและกระดูกปกติ ทำการศึกษาลักษณะทางจุลพยาธิวิทยาจากสไลด์ย้อมฮีมาทอกซีลินและอีโอซินของทั้ง 57 รายเพื่อศึกษาลักษณะทางจุลพยาธิวิทยาของออสติโอคลาสต์ และศึกษาทางอิมมูโนฮีสโตเคมีของทีอาร์เอพีและบีซีแอล-เอ็กซ์แอล หลังจากนั้นทำการวิเคราะห์ทางสถิติเพื่อดูความสัมพันธ์ระหว่างลักษณะต่างๆของออสติโอคลาสต์และลักษณะทางจุลพยาธิวิทยาของรอยโรค

ผลการทดลอง: จากการศึกษาลักษณะทางจุลพยาธิวิทยา พบลักษณะร่วมกันของทั้ง 3 รอยโรคคือ กระดูกตาย ลักษณะทางจุลพยาธิวิทยาที่มีความแตกต่างกันระหว่างกระดูกตายจากยาและกระดูกตายจากรังสีรักษาคือ พบเนื้อเยื่อไฟบรัส ลักษณะทางจุลพยาธิวิทยาที่มีความแตกต่างกันระหว่างกระดูกตายจากยาและกระดูกอักเสบคือ กลุ่มแบคทีเรีย พบว่าออสติโอคลาสต์ในกระดูกตายจากยามีการเพิ่มขนาดและจำนวนอย่างมีนัยสำคัญ นอกจานี้พบว่ามีความสัมพันธ์ระหว่างออสติบลาส เซลล์อักเสบ และกลุ่มแบคทีเรีย กับ รูปร่างและจำนวนของออสติโอคลาสอย่างมีนัยสำคัญ อย่างไรก็ตามไม่พบความแตกต่างของการแสดงออกของทีอาร์เอพีและบีซีแอล-เอ็กซ์แอลในออสติคลาสในกระดูกตายจากยากับรอยโรคกระดูกตายจารังสีและกระดูกอักเสบ

สรุปผลการทดลอง: การที่พบออสติโอคลาสต์ในกระดูกขากรรไกรตายจากยาที่มีจำนวนเพิ่มขึ้นและมีขนาดที่ใหญ่ขึ้นมีความสัมพันธ์กับการพบออสติบลาส เซลล์อักเสบ และกลุ่มแบคทีเรียเป็นอาจหลักฐานสนับสนุนความเกี่ยวข้องของออสติคลาสในกลไกการเกิดกระดูกขากรรไกรตายจากยา

สาขาวิชา	ชีววิทยาช่องปาก	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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KEYWORD: Medication-Related Osteonecrosis Of The Jaw, Histology, Osteoclasts

Vy Tran Ngoc Thuy : BCL-XL EXPRESSION IN OSTEOCLASTS OF PATIENTS WITH MEDICATION-RELATED OSTEONECROSIS OF THE JAW: A COMPARISON WITH OSTEORADIONECROSIS AND OSTEOMYELITIS. Advisor: Assoc. Prof. RISA CHAISUPARAT, Ph.D. Co-advisor: Assoc. Prof. Neeracha Sanchavanakit, Ph.D.

Objective: The pathological mechanism of medication-related osteonecrosis of the jaw (MRONJ) is still unknown now. Osteoclasts are cells directly influenced by MRONJ, which might be the key mediator of pathological mechanism. This study aimed to evaluate the histological features of MRONJ, investigate the morphology and quantity of osteoclasts in MRONJ as well as expression of Bcl-xl, and compare it with ORN, OM, and normal jaw bone.

Methods: In this study, 57 subjects, including patients with MRONJ, osteoradionecrosis of the jaw (ORN), osteomyelitis of the jaw (OM), and normal jaw bone were studied. Hematoxylin and eosin-stained slides of these diagnosed cases were reviewed to investigate the histologic features and osteoclasts' characteristics. Immunohistochemistry was performed to observed the function (TRAP staining) and Bcl-xL expression of osteoclasts. These characteristics of osteoclasts were also evaluated in the relationship with the histological features using statistical analysis.

Results: The results showed that MRONJ, ORN, and OM shared the characteristic feature of necrotic bone. The significant difference found between MRONJ and ORN was the presence of fibrous tissue ($p<0.05$), and between MRONJ and OM was the status of bacterial colonies ($p<0.05$). Osteoclasts in MRONJ enhance activity by increasing the size and the quantity ($p<0.05$). The regression analysis showed a strong correlation between the presence of osteoblasts, inflammatory cells, and bacterial colonies with the change in morphology and the number of osteoclasts ($p<0.05$). However, the TRAP-positive mean number and the TRAP intensity of osteoclasts in MRONJ did not show a significant difference with those in other groups ($p>0.05$); and Bcl-xL did not express in osteoclasts of MRONJ.

Conclusion: Osteoclasts in MRONJ showed an enhanced response to increase size and number that might relate to the presence of osteoblasts, inflammation and bacteria. This finding supports the idea that osteoclasts might be the main key to investigate MRONJ pathogenesis.

Field of Study:	Oral Biology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

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Vy Tran Ngoc Thuy

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Chapter 1 Introduction

Osteonecrosis of the jaw is a pathological condition that is characterized clinically by chronic exposed jaw bone. Among many types of osteonecrosis of the jaw, medication-related osteonecrosis of the jaw (MRONJ) is a serious side effect of drugs administrated to the patients with bone diseases [1]. Patients treatment with anti-resorptive agents and several other bone-related drugs showed a high prevalence of necrotic bone development of the jaw, especially following invasive dental treatments. Besides MRONJ, osteoradionecrosis (ORN) and osteomyelitis (OM) of the jaw have been more frequently found in the recent years [2]. All 3 diseases share similar clinical characteristics and radiographic images despite differences in cause and pathophysiology [3]. Until now, the management of MRONJ is difficult and is not always successful because of the undetermined mechanisms.

MRONJ was first reported in 2003 by Marx [4] as bisphosphonate-related osteonecrosis of the jaw, after which it was renamed to MRONJ as it is now [1]. Although only detecting in the past 20 years, MRONJ has been a topic of attraction due to the high utilization of medications indicated in many bone diseases. The pathophysiology of MRONJ has not yet been fully elucidated until now. There are several suggested hypotheses put forward to explain the emergence of MRONJ, it still could not deny the central role of the inhibition of bone resorption [1]. Osteoclast, the only cell capable of bone degradation, might be the key mediator of MRONJ.

The response of osteoclasts in MRONJ is so complicated. It was broadly believed that the ultimate goal of treatment of osteoporosis and other bone diseases would be achieved when the number of osteoclasts was reduced due to the potent effect

of drug-induced osteoclasts apoptosis [5, 6]. But in the last ten years, there have been numerous studies showing that the response of osteoclasts to anti-resorptive agents is not always as expected. The number of osteoclasts did not decrease but also increased although they inactivated [7-10]. Additionally, an abnormal morphology as giant hypernucleated osteoclasts are also observed and reported to increase in long-term treatment of bisphosphonates [7-9]. Interestingly, giant hypernucleated protracted apoptotic osteoclast only appeared in MRONJ but not ORN or OM [7].

The behavior of bone cells, particularly osteoclasts, in MRONJ is still under controversy. Many studies have been done examining signal pathways in osteoclasts of MRONJ, however, the exact role of osteoclast in MRONJ remains missing. By looking at the detail of osteoclast, we might find something worth investigating MRONJ's pathogenesis. Thus, evaluating the detail of osteoclast's profile in humans is necessary to investigate the relationship between osteoclast and pathophysiology of MRONJ. The objective of this study was to evaluate the histological features of MRONJ, investigate the morphology and quantity of osteoclasts in MRONJ, and compare it with ORN, OM, and normal jaw bone.

Research question

Are osteoclasts in MRONJ different from those in ORN, OM and normal jaw bone?

Objectives and hypothesis

Objective 1

To evaluate the histological features of MRONJ in comparision with those of ORN, OM, and normal jaw bone group

Experimental design

Formalin-fixed paraffin-embedded (FFPE) tissue blocks of MRONJ, ORN, OM and normal bone (control group) were retrieved from the Surgical Pathology archive of the Faculty of Dentistry of Chulalongkorn University of Bangkok, Thailand. Patient information of all cases was reviewed from biopsy reports accordingly for sample selection. After case review, hematoxylin and eosin (H&E) slides of selected cases were retrived for histologic analysis. Histologic features were evaluated involving soft tissue, hard tissue, inflammation and bacterial colony.

Objective 2

To analyse the osteoclast morphology and quantity in MRONJ, ORN, OM, and normal jaw bone.

Experimental design

The regions of interest (ROIs) in H&E slides were detected under a light microscope at magnification of x20. The number of osteoclasts in ROIs was counted and expressed per bone length and per medullary areas. The morphology of osteoclasts was also determined and analyzed. Specimens of MRONJ, ORN, OM and control group were

incubated with an antibody against tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin for revealing the osteoclasts activation. The number of positively labeled osteoclasts were also calculated.

Objective 3

To investigate the expression of Bcl-xL by osteoclasts in MRONJ, ORN, OM and normal jaw bone.

Hypothesis

Osteoclasts in MRONJ, ORN, OM and normal jaw bone do not express Bcl-xL.

Experimental design

All cases with diagnosis of MRONJ, ORN, OM and control samples were further analyzed for Bcl-xL expression. The target protein was detected by incubating sections with an anti-Bcl-xL-antibody, followed by a nuclear counterstaining with hematoxylin. Positive and negative controls were included in each staining series. The number of positively labeled and non-labeled osteoclasts were detected.

Expected benefit

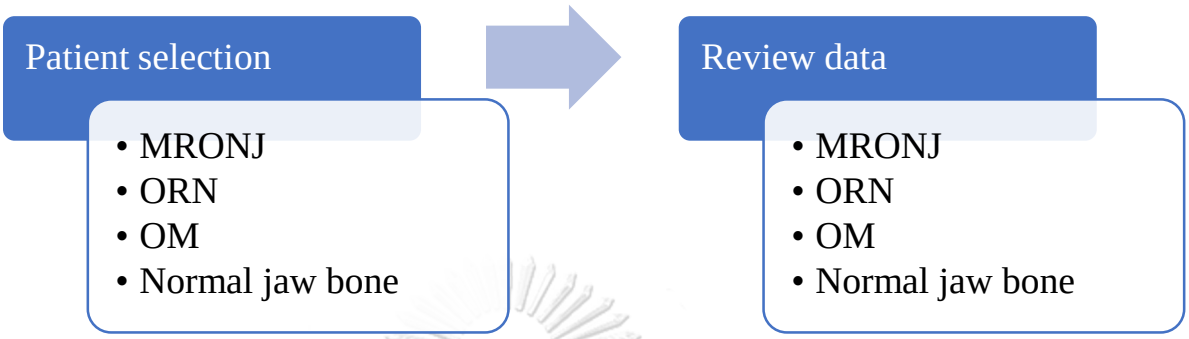
The knowledge gained from this study will contribute to the comprehension of pathogenesis of MRONJ as well as to the understanding of the mechanism induced by anti-resorptive agents and anti-angiogenesis agents. The impacts of medications on osteoclast survival as well as how osteoclasts of the jaw respond to medications might benefit the diagnosis, prognosis of MRONJ and drive exploration of new therapeutic concepts and approaches.

Research design

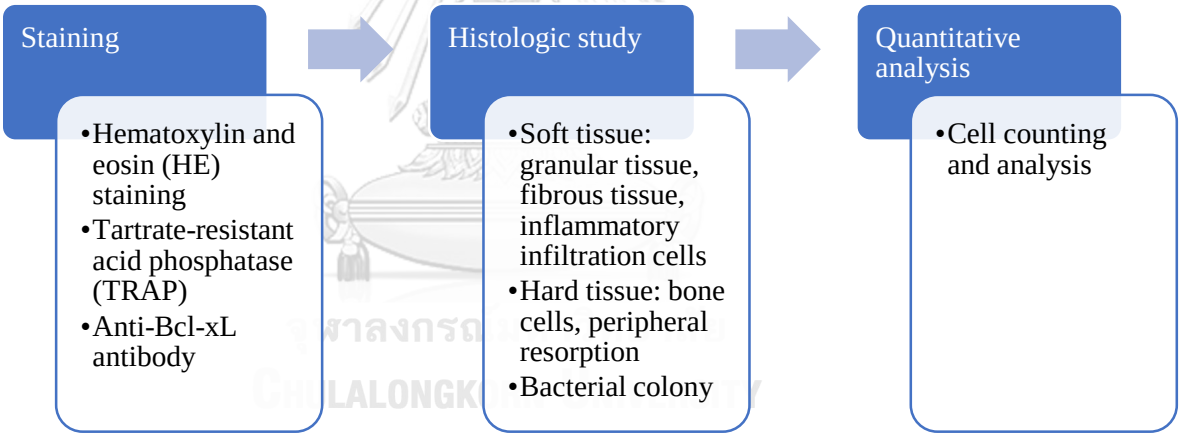
Laboratory experimental research

Conceptual framework

1. Data collection



2. Laboratory work



3. Analysis the result by statistic

The results are expressed as the mean, standard deviation (SD), median, interquartile (IQR), minimum (Min), and maximum (Max). Statistical analysis was performed using SPSS 26.

Chapter 2 Literature review

2.1 Osteonecrosis of the jaw

Osteonecrosis of the jaw (ONJ), which is histologically characterized by osteocyte death and marked by the appearance of empty lacunae in the dense bone, is a disease occurring in the maxillofacial region of patient [11]. To reach an optimal results in treatment, a clear pathophysiology is essential. In fact, there are many types of ONJ with different causes and related pathophysiology [12]. Among them, particular attention is given to medication-related osteonecrosis of the jaw (MRONJ), osteoradionecrosis of the jaw (ORN) and osteomyelitis of the jaw (OM) which show high prelevance and proved difficult to differentiate due to some similarities in clinical symptoms and histological finding [3, 13, 14].

2.1.1 Medication-related osteonecrosis of the jaw

Medication-related osteonecrosis of the jaw (MRONJ) is primarily a serious side effect of anti-resorptive agents used to manage skeletal events, comprising osteoporosis, multiple myeloma and bone metastases [1]. MRONJ was first reported in 2003 by Robert Marx as one of the most serious side effects of bisphosphonates (BPs) therapy and it was called “Bisphosphonate-related osteonecrosis of the jaw” [4]. After that, other drugs have been reported to relate with this serious disease thus it was renamed medication-related osteonecrosis of the jaw (MRONJ) [1]. MRONJ is termed as exposed bone in the maxillofacial region that can be probed through at least one intraoral or extraoral fistula for at least 8 weeks; The patients have a history of received treatment with bisphosphonates or denosumab or anti-angiogenic therapy and are

without being exposed to radiation in radiation therapy [1]. The diagnosis of MRONJ in cases where there is no evidence of exposed bone or necrotic bone is also considered [15].

The prevalence of MRONJ is varied from 0.01 to 15%, basing on the underlying medical condition, cumulative dose and further risk factors [16]. Histologic examination of the bone specimen is not required for a conclusive diagnosis of the MRONJ. However, microscopic examination of the biopsy specimen from the suspected MRONJ could be complementary to the clinical diagnosis. Histology of the MRONJ of human specimens shows empty lacunae where lack of osteocytes, absence of osteoblasts along new bone, and a decreased osteoclast activity [17]. Moreover, giant round shaped osteoclasts with pyknotic nuclei being were also reported [7-9]. The formation of complex biofilms on the surface of exposed bone has also been reported, which suggest that MRONJ might be asscociated with a secondary infection [18, 19].

2.1.2 Osteoradionecrosis of the jaw

Osteoradionecrosis (ORN) is a severe complication of radiotherapy used to manage head and neck cancer [13]. ORN was first published in 1922 and it still is a clinical challenge until now [13]. The prevalence of ORN varies widely in the literature but the most frequently reported prevalence rate is 5–15% [13]. The variability in prevalence of ORN depends on many factors such as total radiation dose, oral hygiene, dental extractions, property of tumor, as well as chemotherapy [13]. According to the literature, ORN was defined as exposed irradiated bone that failed to heal for at least 3 months and are without evidence of persistent or recurrent tumor [13]. In spite of difference in cause and pathophysiology, ORN still shares a similar histology with

MRONJ such as empty osteocyte lacunae, lack of osteoblasts, and a reduced osteoclast activitiy [3, 14]. However, ORN is predominantly found in the mandible [13]. ORN lesions were also described to be more homogenous with extended completely empty lacunae whereas MRONJ (BP) lesions showed a diffuse pattern of empty lacunae and viable osteocytes [20]; ORN is a condition characterized by increased fibrosis while MRONJ is characterized by architectural disruption of the normal bone [21].

2.1.3 Osteomyelitis of the jaw

Osteomyelitis (OM) is one of the oldest known inflammatory diseases [22]. The most common cause of OM of the jaw is supposed to be induced by polymicrobial odontogenic infection [23]. Osteomyelitis is defined as an inflammatory condition of the bone involving the medullary cavity, haversian systems and periosteum of the bone [23]. In clinical treatment, OM usually shows symptons such as swelling, suppuration, fistula formation and bone sequestration but not an exposed necrotic bone [24]. Histologically, as with MRONJ and ORN, in OM necrotic bone is identified as evidenced by empty lacunae, absence of osteoblastic lining [3]. However, osteoclasts were noted to be actively digesting necrotic bone [3]. As with ORN, the distribution of osteomyelitis shows a clear predominance for the mandible [24].

2.2 Pathophysiology

2.2.1 Medication-related osteonecrosis of the jaw

Anti-resorptive agents are potent inhibitors of bone resorption and are used commonly nowadays [1]. Therefore, most research studies emphasize on the mechanism of anti-resorptive agents to find out the pathophysiology of the MRONJ. The widely used anti-

resorptive drugs group consists of two main types which are bisphosphonate and denosumab. Other types of medications are reported to associate with ONJ involves anti-angiogenetic drugs [1]. This supports the multi-factorial views of MRONJ's pathogenesis.

Bisphosphonates (BPs) are highly potent inhibitors of osteoclast that plays a crucial role in controlling bone breakdown. BPs' pronounced affinity for mineralized tissues, in particular bone [25]. BPs have been reported to adhere to the bone surface at the active remodeling site and be ingested by osteoclasts through endocytosis [26]. Once being inside the osteoclasts, BPs interfere osteoclasts' biological system then inhibit OC's bone resorption activity. In clinical practice, there are 2 different large groups of BPs characterized by the presence of a nitrogen-containing side chain lead to a distinct mechanisms, including: 1) nonnitrogen containing class (including clodronate, tiludronate, and etidronate) and 2) nitrogen-containing class (pamidronate, alendronate, ibandronate, riserdronate, and zoledronate) [27]. Both classes of BPs are synthetic analogs of pyrophosphate. The nonnitrogen containing BPs was generated earlier. They inhibit bone resorption based on an ability to incorporate into ATP, interfere with mitochondrial function and then induce osteoclasts apoptosis [28]. The decrease in the amount of osteoclasts caused by apoptosis helps to prevent bone resorption, therefore achieve the goal of treatment. Meanwhile, the second generation of BPs is modified by adding nitrogen inside the structure, thus increases the ability to inhibit bone resorption [27]. Although it has the same effect as inhibiting bone resorption, the mechanism of nitrogen-containing BPs (N-BPs) action is completely different [27]. Instead of inducing osteoclasts apoptosis directly, N-BPs interfere mevalonate pathway which is known as the metabolic pathway that plays an important

role in biosynthesis of cholesterol and isoprenoid lipids. Particularly, N-BPs were reported to inhibit farnesyl diphosphate synthase (FDPS) which is the key enzyme in the mevalonate pathway. As a result, inhibition in the biosynthesis of cholesterol and small GTPases, a class of isoprenoid lipids, leading to the suppression of function and survival of osteoclasts [25].

Denosumab (Dsm) is a new antiresorptive agent that has been usded recently and has been shown better effects comparing with BPs [29]. Dsm is a human monoclonal antibody that can target the Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) [29]. Inside human body, bone remodeling depends on a balance between RANKL and osteoprotegerin (OPG). RANKL binds to its receptor (RANK) present on osteoclasts precursor and mature osteoclasts, then enhance their differentiation, function and survival. OPG and Dsm share the similar mechanism to bind to RANKL, then block its interaction with RANK, suppressing the osteoclast differentiation and survival, thus decrease bone resorption [29]. Interestingly, ONJ in patients treated with Dsm reached similar prevalence rates compared with patients treated with BPs. Although having the same side effect of MRONJ but the mechanism by which BPs and denosumab exert their function are totally different [5]. This supports the view that inhibition of osteoclastic function might play a centrol role in the pathogenesis of MRONJ.

Anti-angiogenegic agents are also reported to be associated with ONJ [1]. These drugs are vascular endothelial growth factor (VEGF) antagonists and might be classify into two groups: monoclonal antibodies group and small molecule tyrosine kinase inhibitors (TKIs) group [30]. Monoclonal antibodies group can bind to VEGF then deactivates its biological activity. Meanwhile, TKIs group block the VEGF receptor

and its downstream signaling pathways. They suppress the formation of blood vessels, thus affect the healing process as well as increase the risk of becoming necrotic [1]. ONJ is also considered as an avascular necrosis, therefore it is not surprising that angiogenesis suppression is one proposed mechanism of pathophysiology of ONJ [1]. However, a recent study showed that anti-angiogenesis alone is not the main cause of ONJ, there might be a contributor to the severity of the disease if using in combination with anti-resorptive agents [31]. Inhibitors of mammalian target of rapamycin (mTOR) are recent medicinal agents which are reported as MRONJ inducers also [30].

Until now, the mechanisms underlying the occurrence of MRONJ are still unexclusive. Several hypotheses regarding the etiology of MRONJ have been proposed. These involve supression of osteoclastic bone resorption and remodeling, occurrence of inflammation and infection, inhibition of angiogenesis, microtrauma or immune cell dysfunction [1]. However, two theories are emerging called “inside-outside” and “outside-inside” theories providing an inside about the general pathophysiology of osteonecrosis of the jaw related to bisphosphonates, a main representative among medications used to treat skeletal-related events (Figure 2.1) [30]. The inside-outside theory demonstrates exposed bone as a result of bone death caused by bone turnover inhibition and bone homeostasis breakdown. In this theory, microtrauma of the jaw as well as an invasion of microorganism plays a role as a trigger that lead to activation of the inflammatory cascade. Meanwhile, outside-inside theory indicates that an occurrence of exposed bone due to dental lesion in the background of bone turnover suppression is the main cause inducing bone death. The underlying mechanism is supposed to be the spread of infection to the bone and thus cause osteonecrosis. In any case, when looking at the common point of the two theories, the central role of

osteoclasts is clear since these cells are directly affected by anti-resorptive agents. Moreover, with different mechanisms of action but BPs and Dsm both induce MRONJ. This fact emphasized the central role of bone resorption inhibition. Thus, osteoclasts become a pivotal point to investigate the pathogenesis of MRONJ.

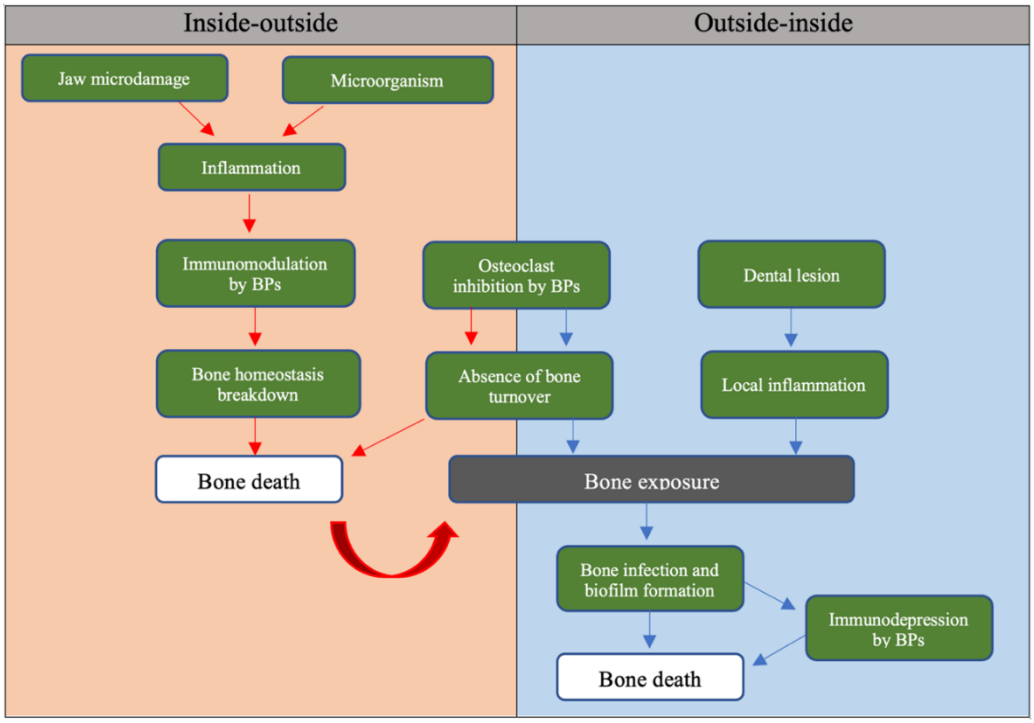


Figure 2.1. Theories of etiology of bisphosphonate-related osteonecrosis of the jaw (modified from [30]).

The question why the jaw but not other bones are affected is still not entirely answered and subject to speculation [32]. In the human skeleton, the jaw is a high-risk area for infection when it is covered only by a thin layer of oral mucosa, whereas relatively thick skin protect other bones. In addition, jaw is subjected to repeated microtraumas because of the force of mastication during daily function. Indeed, the alveolar bone turnover has higher bone turnover comparing with other long bones, thus it is not surprising that alveolar bone could incorporate more BPs than other skeleton sites [33, 34]. Another proposed theory suggested that drugs target the jaw could be due

to the pH change caused by dentoalveolar infections or surgeries in complicated oral environment and it could be a profound contributor to development and aggravation of MRONJ [35-37]. On the other hand, the jaw has a different embryologic development origin arising from neural-crest cells, not from the mesoderm like other bone in the body [38]. And finally, a site-specific function of osteoclasts as well as their response appears to be vary [39, 40]. A distinc response to BPs of osteoclasts at different bone sites has been compared and reported [41-43]. These differences between bones might help to explain why osteonecrosis is dominant in the jaw following anti-resorptive treatment.

2.2.2 Osteoradionecrosis of the jaw

Radiaotherapy is an effective tool using high physical energy to destroys tumor cells [44]. At the moment, radiation treatment is one of effective methods that is widely used in cancer treatment. Basically, this treatment is based on the theory that rapidly growing cancer cells are more sensitive to DNA damage than normal cells. However, normal cells adjacent to the tumor certainly receive significant amounts of ionizing radiation [44]. Obviously, additional side effects may appear later, such as ORN.

In normal living cells, DNA repair is a biological process that corrects error during DNA replication or repairs DNA damage that can be caused by reactive oxygen species (ROS) [44]. In order to maintain the integrity of genome, cells can identify damage signals to conduct repair or programmatic death in case of severe damage. Once damaged DNA is repaired properly, the cell can survive. However, an insufficient or incorrect DNA repaired may continue to exist. Based on this principle, radiotherapy uses radiation to induce damage in DNA strands, either by breaking down DNA strands

directly by ionization or indirectly by the formation of free radicals that damage the DNA. The direct effect is caused by high linear energy transfer radiation which causes directly complex damage in the helical turns of the DNA molecule. Meanwhile, indirect effect is named for low linear energy transfer radiation which can generates free radical and ROS. As a result, ROS can oxidates biological macromolecules and activates intracellular signaling pathways which enhance cell apoptosis and cell cycle arrest. Moreover, irradiated cells have been shown to affect neighboring non-irradiated cells, causing instability in the genome, resulting in stress response and altered programmed cell death or cell proliferation [44].

The mechanism of cellular organization response to radiation is complex, including DNA damage repair, cell death, inflammation, angiogenesis and matrix remodeling. These factors depend on the radiation dose and the duration of exposure [44]. Radiotherapy often seems to have caused the side effect as ORN. It affects the small blood vessels of bone, induces inflammation and formation of small blood clots that lead to disruption of tissue perfusion [45]. In addition, radiation therapy creates free radicals and alters collagen synthesis leads to the losses of normal cellularity, and undergoes fibrosis-atrophy of bone, resulting in a decrease of the capacity to repair and remodeling. In that case, even minimal external injury can cause an ulcer, facilitating bone infection followed bone necrosis. A number of theories have been proposed to explain its mechanism, including histamine released, radiation absorption, injury and infection. Until recently, hypoxia, hypovascularity and hypocellularity is the most widely accepted theory [45]. Radiation-induced fibrosis is a new accepted theory that explains the damage to normal tissues by inducing fibrosis (Figure 2.2) [46]. This

theory suggests that the key event for ORN to occur is the dysregulation of fibroblastic activity, which in turn results in atrophic tissue in the irradiated area.

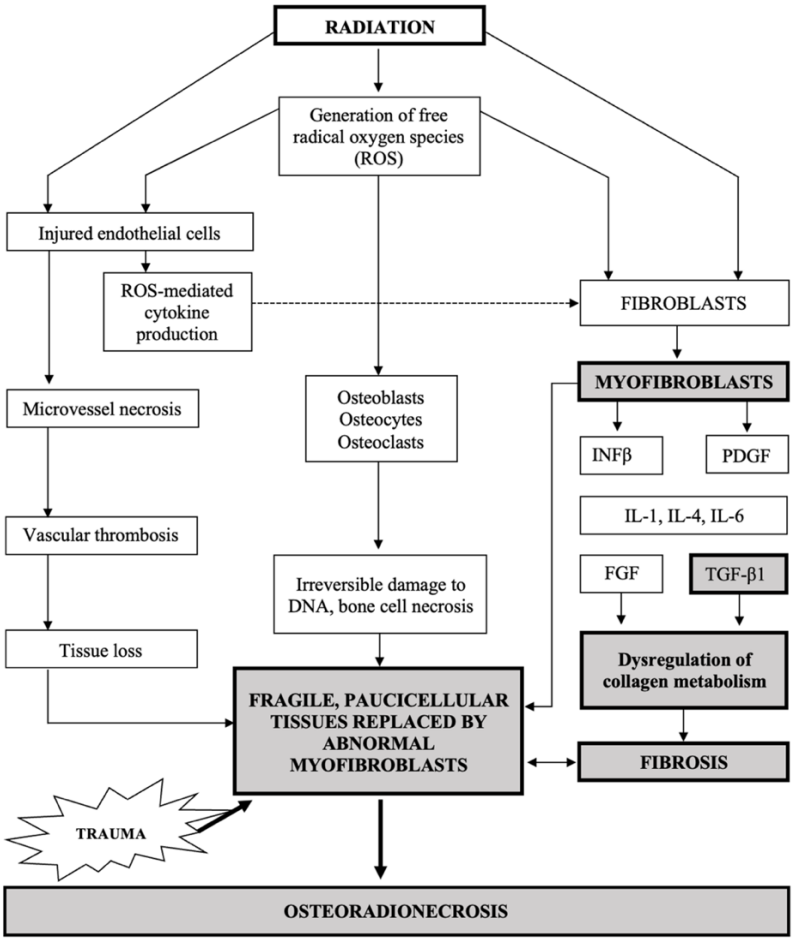


Figure 2.2. Hypothesis of etiology of radiation induce osteonecrosis of the jaw (modified from [46]).

2.2.3 Osteomyelitis of the jaw

The majority of osteomyelitis cases involving the jaws are usually caused by infection. The penetration of bacteria causes a cascade of immune response in the host body then increasing capillary permeability, inducing hyperemia and local inflammation. Proteolytic enzymes released during this immune reaction exacerbate bacterial destruction and create necrotic tissue. As a results, pus accumulates inside the

medullary cavity therefore increases intramedullary pressure, which leads to vascular collapse (Figure 2.3) [24]. Moreover, pus passes through the haversian and accumulates beneath the periosteum, thus futher reducing the blood supply.

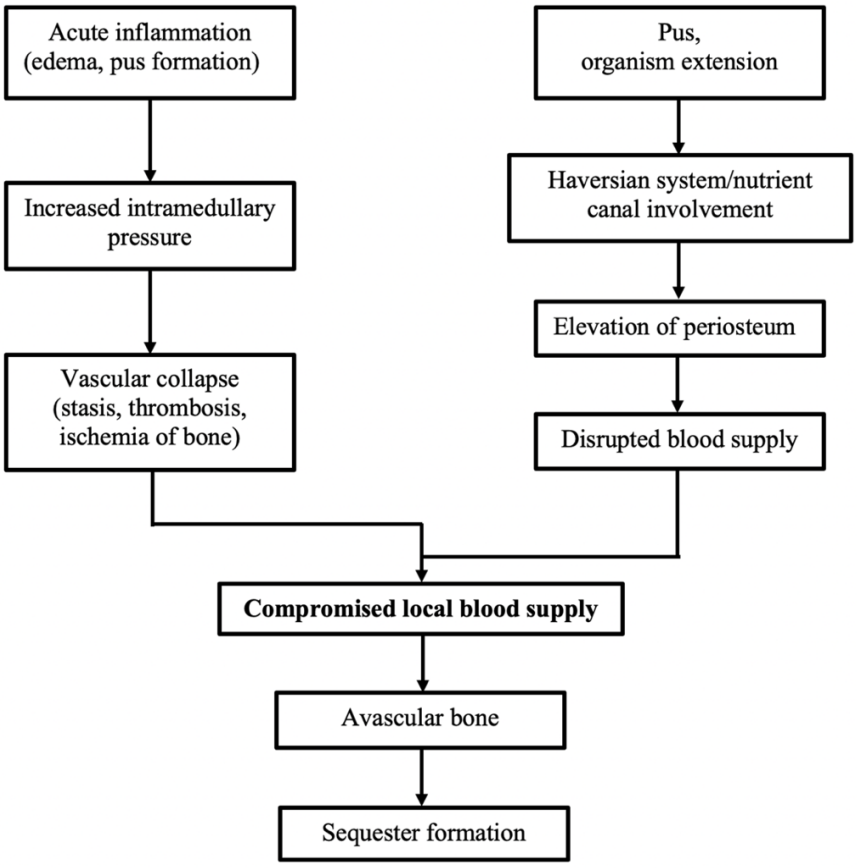


Figure 2.3. Etiology of osteomyelitis (modified from [24]).

2.3 Osteoclasts

Osteoclasts are specialized multinucleated cells which have a unique capacity to resorb bone. Coupling with osteoblasts, osteoclasts play a vital role in bone remodeling [47]. However, in many pathological conditions, the dominant bone resorptive activity leads to uncontrolled skeletal destruction [48]. As the only cells definitively shown to have the capacity to digest bone, osteoclasts are key mediators of skeletal diseases.

Osteoclasts are often found on the outer layer of bone to perform their bone-eating function. In order to perform their function as bone resorbing cells, osteoclasts have an ability to degrade both inorganic and organic components contained in bones by the polarized secretion of proteolytic enzymes such as cathepsin K and protons [47]. That polarized secrection comes from two important structures: a ruffled border and the sealing zone. The sealing zone delineates the area of bone resorption from the rest of the environment. Meanwhile, the ruffled border acts as a transporter of protons and proteolytic enzymes into the area of the resorption comparment to demineralize the bone and digest the bone matrix proteins. The process of digesting bone ends with the transport of the disintegrated products out by transcytosis through a vesicular process.

Osteoclasts develop and derive from monocytes, a common origin shared with macrophages [49]. Osteoclast develops and ends the differentiation process by cell-cell fusion of mononuclear pre-osteoclasts to form a multinucleated mature cells [47]. The differentiation of osteoclasts requires two important factors: the monocyte/ macrophage colony stimulating factor (M-CSF) and receptor activation of nuclear factor kappa B ligand (RANKL) which activate many signaling pathways that follow. M-CSF promotes proliferation of osteoclast precursors while RANKL is an indispensable element to guide development and differentiation of osteoclasts. In addition, a soluble decoy receptor for RANKL, osteoprotegrin (OPG), inhibits RANKL functions by competing with RANK. The RANKL/RANK/OPG system plays a main role in osteoclast generation. In addition, other signaling molecules as transcription factors and cytokines also play a crucial role in the process of development and differentiation of osteoclasts. After executing their function, osteoclast enter a programmed cell dead process termed as apoptosis.

The life span of osteoclasts of human might last to several weeks to months [50]. However, comparing to other bone cells, osteoclasts are relatively rare cells that their quantity remain 1/10 of osteoblasts and 1/100 of osteocytes. The limitation of determination and observation of apoptotic osteoclasts in the bone sections is due to the low number of osteoclasts.

There are many factors involved in the apoptotic pathway of osteoclasts, being an intrinsic and/or extrinsic pathway (Figure 2.4) [50]. Fas ligand stimulates osteoclast apoptosis via an extrinsic pathway [51, 52], whereas the participation of pro-apoptotic molecules BH3-only protein BIM induce osteoclast apoptosis via an intrinsic pathway [53]. A high extracellular calcium concentration can also induce osteoclast apoptosis [54]. Finally, detachment of osteoclasts also induces apoptosis, presumably by disrupting integrin-mediated survival signaling [55].

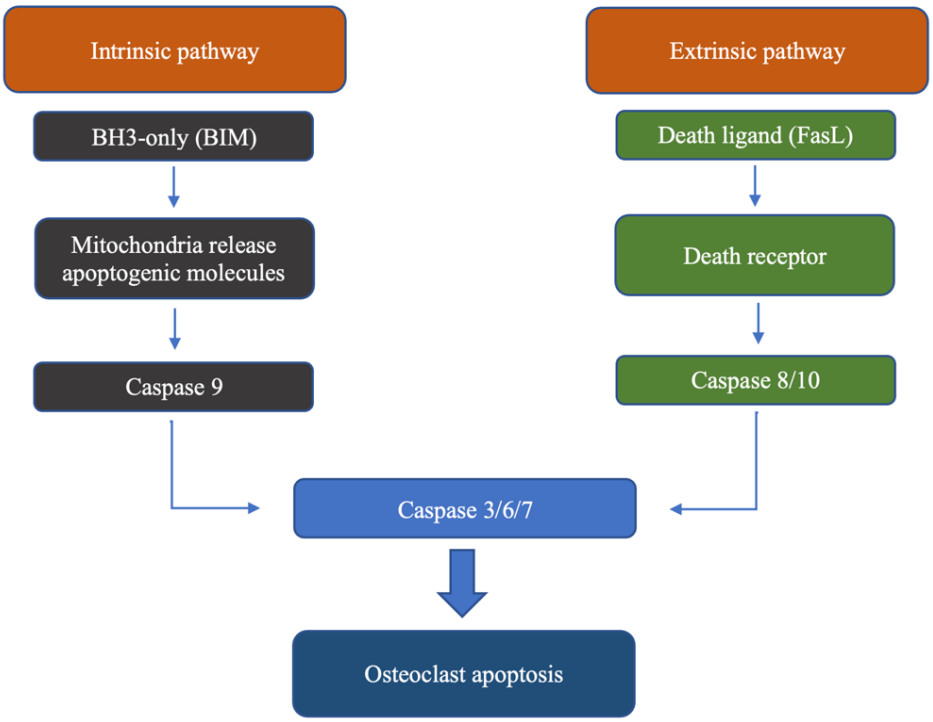


Figure 2.4. Intrinsic and extrinsic pathway of osteoclast apoptosis.

Beside pro-apoptosis signaling, pro-survival proteins also play a role in regulating osteoclast apoptosis [50]. RANKL, TNF-α, IL-1 and M-CSF, each activate activate antiapoptosis signaling in osteoclasts via extracellular signal-regulated kinases (ERKs), PI3K activity and the transcription factor NF-κB. The activation of Mammalian target of rapamycin (mTOR) is also required for the antiapoptotic actions of M-CSF, RANKL, and TNF-α in osteoclasts. Moreover, the transcription factor NF-κB which is activated by RANKL, TNF-α, and IL-1, has been widely known to prevent apoptosis by transactivating the expression of antiapoptotic genes such as Bcl-2 and Bcl-xL in many cell types [56]. Thus it might also suppress apoptosis in mature osteoclasts. However, osteoclast precursors lacking NFκB subunits have survive normally. The role of Bcl-2 and Bcl-xL in regulating osteoclast resist programmed cell death has also been emphasized through numerous studies [57-61].

2.4 Osteoclasts in patient with MRONJ

In clinical practice, it was broadly believed that the outcome of treatment osteoporosis and other bone diseases would be achieved when the number of osteoclasts was reduced due to the potent effect of drug-induced osteoclasts apoptosis [5, 6, 29]. But in the last ten years, there have been numerous studies showing that the response of osteoclasts to anti-resorptive agents is not always as expected. Writing on The New England of Medicine in 2009, Robert SW reported that long-term treatment of alendronate is associated with an increase in the number of osteoclasts. Moreover, giant hypernucleated osteoclasts that detach from the bone surface were also observed [9]. After that, similar findings were reported in other clinical treatments [7, 8, 10]. Another noteworthy point is that osteoclast precursors are reported to be effective but in the long term [62]. Many assumptions then were made but still could not explain clearly the increase in number of osteoclasts [9]. The dysfunction of cytoskeleton arrangement and loss of ruffle bordered due to pharmacodynamics might explain the abnormal shape as well as detachment of osteoclasts but not for their delay apoptosis as well as an increase in the number.

To find out an answer, many proposed mechanisms are established depending on the way of how osteoclast survive [58, 60, 63-65]. However, the exact mechanism by which osteoclasts increase their number remains unclear and depends on the fact that there are many signaling pathways and the interaction of other cell types as osteoblasts and osteocytes, regulating survival of osteoclast in the human body [50]. The interfere of the immune system and the effect of foreign elements also change the pH of the environment as well as increase inflammatory cytokines thus impact the

response of osteoclasts to medications [65, 66]. Moreover, the diverse effects of drugs on osteoclast also vary on the duration of treatment, the dosage of the medication and the position of osteoclast on different bone sites [9, 42].

2.5 Expression of survival protein on osteoclast in researchs

Osteoclasts have been observed and reported to increase in number in long-term treatment (Figure 2.5) [7-10]. It is hypothesized that antiresorptive treatment not only induces osteoclast apoptosis but also prolongs their lifespan and increases their number [9].

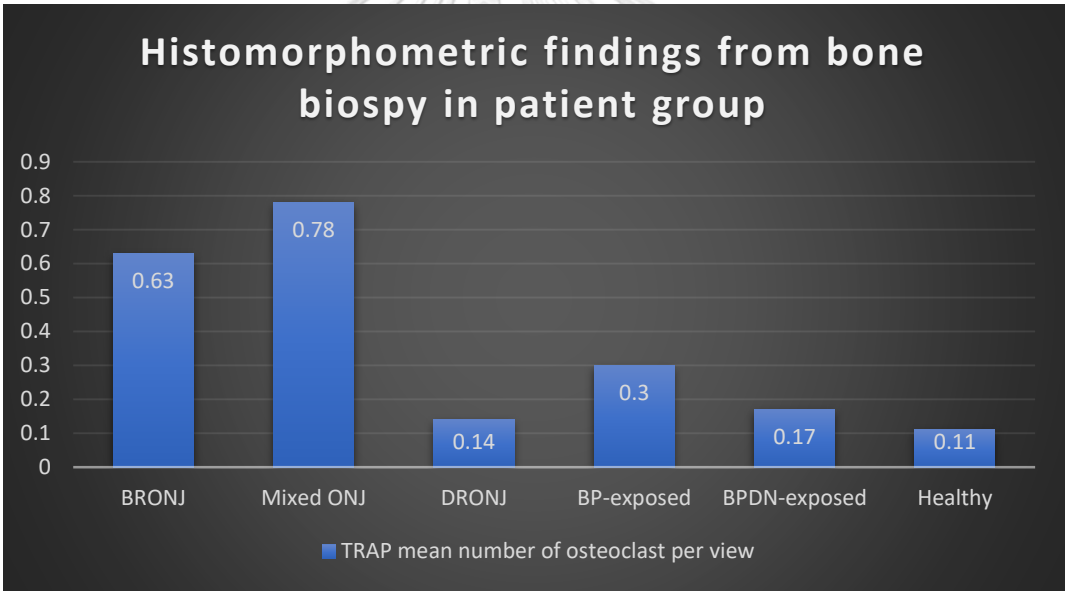


Figure 2.5. Histomorphometric finding from bone biopsies in patient group (created by author from data in [10]).

BP-exposed, bisphosphonate-exposed bone; BPDN-exposed, bisphosphonate and denosumab-exposed bone; BRONJ, bisphosphonate-related osteonecrosis of the jaw; DRONJ, denosumab-related osteonecrosis of the jaw; mixed ONJ, mixed osteonecrosis of the jaw.

In general, there are two main different signaling pathways controlling cell apoptosis: one is extrinsic pathway and the other is intrinsic pathway [67]. Bisphosphonate-induced apoptosis via intrinsic pathway as a consequence of disrupted cholesterol biosynthesis and reactive oxygen species (ROS) activation [25, 68]. Denosumab-induced apoptosis is also generated by the intrinsic pathway as a result of blocking downstream effectors of prosurvival signaling when binding to RANKL [50, 69]. Effects of anti-angiogenesis agents and inhibitors of mTOR are considered to associate with the mitochondrial apoptosis pathway [50, 70]. In osteoclasts, this pathway is regulated by the Bcl-2 family of proteins. Bcl-2 family play an important role in capturing pro-apoptotic factors, thereby preventing the secrection of cytochrome c, leading to the activation of caspases 9 and caspases 3 followed by cells apoptosis [61].

2.5.1 Bcl-xL

Bcl-xL is one of main pro-survival members of Bcl-2 family that is the key regulator of the intrinsic apoptotic pathway [61]. This pro-survival protein is reported to play an important role in osteoclast survival and function [71]. Osteoclasts expressed higher levels of Bcl-xL than Bcl-2, therefore Bcl-xL was supposed to be a critical regulator of apoptosis in osteoclasts [43, 58, 60]. Therefore several pathways of Bcl-xL expression that promote osteoclast's survival under BPs treatment have been proposed. The expression of Bcl-xL was regulated by Ets-2 [72]. Local high levels of TNF up-regulated Ets-2 expression by osteoclasts, which in turn stimulated Bcl-xL expression and reduced their sensitivity to bisphosphonate-induced apoptosis [60]. An

upregulation of inflammatory cytokines, in particular TNF-a, has been demonstrated following BPs treatment [65]. Other studies showed that treatment with ZA activated p38 MAPK, thereby increasing Bcl-xL expression [58]. Although several studies have shown the expression of Bcl-xL in osteoclasts after treating with anti-resorptive agents, there have not been any studies conducted to investigate the Bcl-xL expression in human osteoclasts from MRONJ's patients. Therefore, a futher study to investigate the Bcl-xL expression in human osteoclasts treated with antiresorptive agents is needed.

2.5.2 Other survival protein expression

In skeletal tissues, Bcl-2 was expressed in osteoclasts, osteoblasts and osteocytes [59, 61]. These data suggested that Bcl-2 might promotes the activity and survival of both osteoblasts and osteoclasts. However, research showed that Bcl-2 not be affect by BPs treatment [58, 60, 63]**.** Thus Bcl-2 might not play a major role in osteoclast to resist antiresorptive agents-induced apoptosis.

Mcl-1 is also a main member of prosurvival proteins. It is reported that an increase of Mcl-1 protects osteoclast from apoptosis-inducing and anti-resorptive effects of bisphosphonates in vitro [73]. Therefore, Mcl-1 is also a potential protein to investigate the mechanism of resistance to antiresorptive agents-induced apoptosis of osteoclast.

Chapter 3 Materials and Methods

3.1 Patients selection and tissue sections retrieved

A total of 106 formalin-fixed paraffin-embedded (FFPE) tissue blocks from 57 patients with 17 MRONJ patients, 15 ORN patients, 15 OM patients, and 10 normal jaw bone specimens as control were retrieved from the Surgical Pathology archive of the Department of Oral Pathology, Faculty of Dentistry, Chulalongkorn University of Bangkok, Thailand between the years 2010 to 2020. Patient information of all cases including age, gender, and lesion locations was reviewed from biopsy reports accordingly. For samples selection, samples have to meet the clinical criteria as follows:

1) MRONJ: clinical evidence of devitalized and exposed jaw bone; history of using antiresorptive or antiangiogenic agents; and no radiotherapy.

2) ORN: evidence of devitalized and exposed jaw bone; history of radiotherapy; no evidence of persisting or recurrent tumor; no documented therapy with antiresorptive or antiangiogenic agents.

3) OM: evidence of chronic inflammatory processes in the jaw bone that showed symptoms of swelling, suppuration, fistula formation, and bone sequestration in clinical presentation; no documented therapy with antiresorptive or antiangiogenic agents; and no radiotherapy.

4) Control: no documented therapy with antiresorptive, antiangiogenic agents; no local radiation exposure; and did not suffer from intraoral inflammation. The control group were obtained from bone specimens diagnosed with normal jaw bones which were parts of bone diseases that meet the criteria of sample selection.

3.2 Histologic analysis

After case review, Hematoxylin and eosin (H&E) slides of selected cases were retrived for histologic analysis. All sections were analyzed under a bright-field microscope (at a magnification of 40× to 400×). To study histopathologic profile, general histologic features was evaluated involving soft tissue and hard tissue. In soft tissue, the presence of granulation tissue, fibrous tissue and inflammatory cells infiltration were evaluated. In hard tissue, bony features such as bone cells (osteoblast, osteoclast, osteocyte) and bony peripheral resorptions were examined. Bacterial colony was also a trait of interest and assessment. The details of studied parameters in histologic examination is shown in Table 3.1.

Table 3.1. Histologic examination of studied parameters

Observation target
Hard tissue (Osteocyte, osteoblast, osteoclast, peripheral resorption)
Soft tissue (Granulation tissue, fibrous tissue)
Inflammatory cells (Plasma cell, lymphocytes, neutrophil)
Bacterial colony (Likert scale)

3.3 Immunohistochemical staining

3.3.1 Tartrate-resistant acid phosphatase (TRAP) staining

The immunohistochemical technique used for labeling osteoclasts in MRONJ, ORN, OM and control group is mouse monoclonal antibody against tartrate-resistant acid phosphatase (TRAP) of human origin (Santa Cruz Biotechnology sc-376875) at a

concentration of 1:250. For antigen retrieval, citrate buffer (10mM, pH 6.0) in a microwave at 700W and 100^0C for 10 minutes was applied. Incubation the sections with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. The target protein was incubated with TRAP at 4^0C overnight. For negative control, 1% BSA were added in the section instead of primary antibody. The normal jaw bone specimen containing osteoclasts was used as positive control. The slides were then incubated with Dako Envision + System-HRP Labeled Polymer Anti-mouse (K4001, Dako) for 60 minutes at room temperature. Incubated the slides with Dako Envision + System-HRP Labeled Polymer Anti-mouse (K4001, Dako) for 60 minutes at room temperature. The slides were then incubated with Liquid DAB+ Substrate Chromogen System (K3468, Dako) for 2 minutes and counterstained with hematoxylin for 1 minute.

Procedure detail:

1. Adhere the sections onto the microscope slides

-FFPE tissue blocks were cut into 1- μm sections and mounted on positive charged glass slides.

-The slides were dehydrated on the slide warmer at 60^0C for 60 minutes and cool down.

2. Deparafinization and rehydration:

-Incubate slides in Xylene 4x2 minnutes

-Incubate slides in 100% ethanol 2x1 mininutes

-Incubate slides in 95% ethanol 2x1 minutes

3. Wash slides in dH_2O 2x5 minutes

4. Antigen retrieval:

-Place slides in a Citrate Buffer solution (pH 6) in a microwave at 700W and 100^0C for 10 minutes

5. Blocking endogenous enzymes:

-Wash slides in dH_2O 2x5 minutes

-Incubate slides in 3% hydrogen peroxide for 10 minutes

-Wash slides in dH_2O 2x5 minutes

-Wash slides in 1X PBS for 5 minutes

6. Adding primary antibody or negative control reagent

-Use Liquid Blocker Mini PAP Pen to circle tissue sections

-Incubate section with primary antibody against TRAP with dilution at 1:250 or BSA 1% for negative control

-Incubate in wet chamber overnight at 4^0C

7. Adding secondary antibody and signal amplification

-Remove primary antibody and wash in 1X PBS 3x5 minutes

-Incubate section with goat anti-mouse IgG secondary antibody for 60 minutes at room temperature

-Remove secondary antibody and wash in 1X PBS 2x5 minutes

-Add DAB substrate chromogen to each section and incubate for 2 minutes

-Rinse in tap water for 5 minustes

8. Hematoxylin counterstain

-Counterstain with Hematoxylin for 1 minutes

-Rinse in tap water

-Incubate in Scott's water (Blueing solution) rapidly

-Rinse in tap water

9. Dehydration

-Incubate slides in 95% ethanol 2x5 seconds

-Incubate slide in 100% ethanol 2x5 seconds

-Incubate slide in Xylene 2x5 seconds

10. Stabilizing

-Add mounting medium to the surface of slides

-Tip the coverslip onto the mounting medium

11. Viewing the staining under the microscope

3.3.2 Anti Bcl-xL antibody staining

Immunohistochemical staining was performed using a rabbit anti–Bcl-xL antibody (cat. no. ab32370, Abcam, UK) dilluted at 1:100. For antigen retrieval, citrate buffer (10mM, pH 6.0) in a microwave at 700W and 100^0C for 10 minutes was applied. Next, the sections were incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. These sections were then incubated overnight with rabbit anti–Bcl-xL primary antibody at 4^0C in humidity chamber. Positive and negative control were included in each IHC running. For negative control, 1% BSA were added in the section instead of primary antibody. Slide case of squamous cell carcinoma of the tongue was used as positive control. After washing three times in 1X PBS for 15 minutes, the slides were incubated with secondary antibody Polyclonal Goat Anti-Rabit IgG HRP (P0448, Dako) at 1:100 dilution for 60 minutes at room temperature. the slides were incubated with Dako Envision + System-HRP Labeled Polymer Anti-mouse (K4001, Dako) for 60 minutes at room temperature. The slides were then incubated with Liquid DAB+ Substrate Chromogen System (K3468, Dako) for 5 minutes and counterstained with hematoxylin for 1 minute.

Procedure detail:

1. Adhere the sections to the microscope slides

-FFPE tissue blocks were cut into 1- μm sections and mounted on positive charged glass slides.

-The slides were dehydrated on the slide warmer at 60^0C for 60 minutes and cool down.

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-Incubate slides in 100% ethanol 2x1 mininutes

-Incubate slides in 95% ethanol 2x1 minutes

3. Wash slides in dH_2O 2x5 minutes

4. Antigen retrieval:

-Place slides in a Citrate Buffer solution (pH 6) in a microwave at 700W and 100^0C for 10 minutes

5. Blocking endogenous enzymes:

-Wash slides in dH_2O 2x5 minutes

-Incubate slides in 3% hydrogen peroxide for 10 minutes

-Wash slides in dH_2O 2x5 minutes

-Wash slides in 1X PBS for 5 minutes

6. Adding primary antibody or negative control reagent

-Use Liquid Blocker Mini PAP Pen to circle tissue sections

-Incubate section with rabit anti-Bcl-xL antibody with dilution at 1:100 or BSA 1% for negative control

-Incubate in wet chamber overnight at 4^0C

7. Adding secondary antibody and signal amplification

-Incubate section with goat anti-rabbit secondary antibody for 60 minutes at room temperature

-Remove secondary antibody and wash in 1X PBS 2x5 minutes

-Add DAB substrate chromogen to each section and incubate for 5 minutes

-Rinse in tap water for 5 minustes

8. Hematoxylin counterstain

-Counterstain with Hematoxylin for 1 minutes

-Rinse in tap water

-Incubate in Scott's water (Blueing solution) rapidly

-Rinse in tap water

9. Dehydration

-Incubate slides in 95% ethanol 2x5 seconds

-Incubate slide in 100% ethanol 2x5 seconds

-Incubate slide in Xylene 2x5 seconds

10. Stabilizing

-Add mounting medium to the surface of slides

-Tip the coverslip onto the mounting medium

11. Viewing the staining under the microscope

3.4 Osteoclasts morphology and quantitative analysis

Based on the distribution of osteoclasts, regions of interest (ROI) were detected. Cells counting were performed only in ROI. In each specimen, two visual fields (at x20 magnification) with a high probability for the presence of osteoclasts were detected and the medullary areas was defined as region of interest (Figure 3.1). If the visual size

exceed the section size, only one visual size was used. Specimens must have at least one cell meet the criteria of osteoclast: multinuclearity (have at least 2 nuclei); large size (cell body larger than two mononuclear cells after fusion); direct contact with bone or adjacent to the bone; and no foreign particles or granulomatous foci in cytoplasm. Due to limitation in FFPE tissue blocks of the source, only one section each specimen was used to analyze the osteoclast profile.

Osteoclast morphology and quantitative analysis was performed with ImageJ software (version 1.53, National Institutes of Health, Bethesda, USA). For morphology analysis, diameter and nuclerity of osteoclasts within visual fields were measured. The avarage index of each property was calculated accordingly for each section. For quantitative analysis, the number of cells that meet the criteria for osteoclasts was counted and expressed as the number per millimeter of bone perimeter and number per millimeter square of medullary area.

3.5 Immunohistochemical analysis

In each ROI, TRAP-positive cells containing at least two nuclei was considered to be TRAP-positive osteoclast. The number of TRAP-positive osteoclasts per view was counted. The TRAP stain intensity was also evaluated Likert score (0-3). For immunohistochemical expression of Bcl-xL, Bcl-xL positive cells that meet criteria of osteoclast were considered to be Bcl-xL positive osteoclasts.

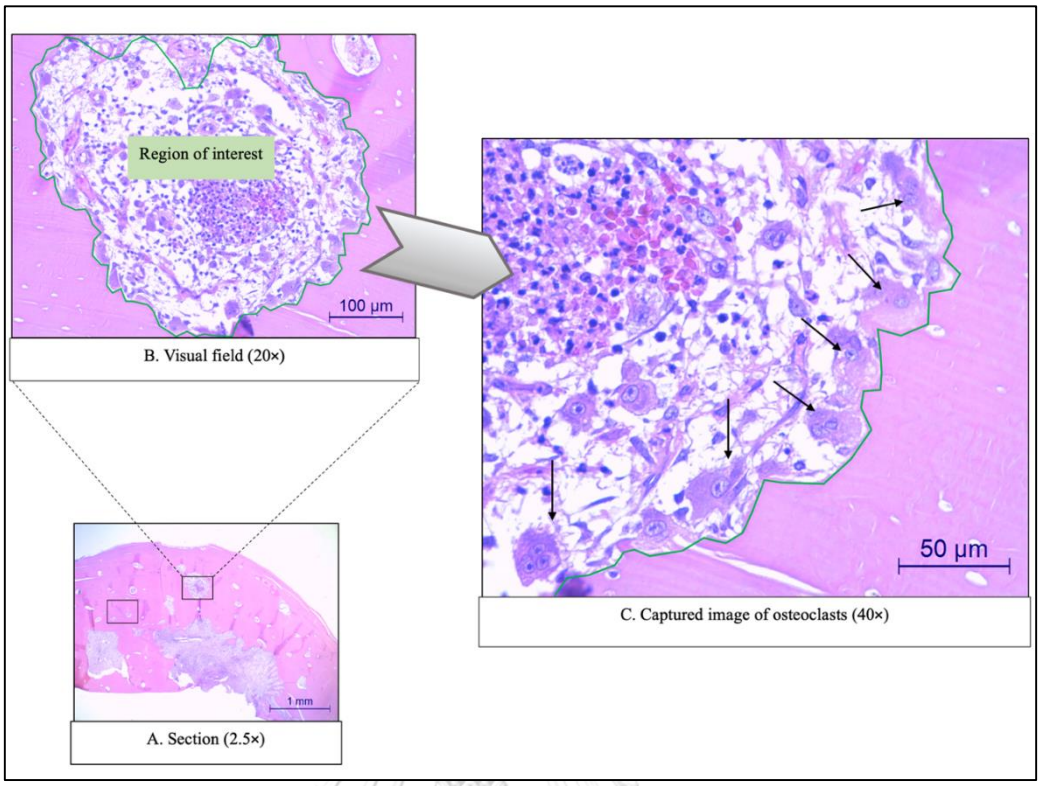


Figure 3.1. Regions of interest determination. The figure illustrates an H&E stained section contains mainly vital bone of MRONJ bone sample. (A) Two visual fields (rectangals) with a high probability for the presence of osteoclasts were detected in the section. (B) Regions of interest (ROI) were determined in visual field at objective magnification 20×. (C) Captured image within visual field shows numerous osteoclasts (black arrow) within the ROI.

3.6 Statistical analysis

Histological features of 4 groups were compared in pairs using Pearson's chi-square test and Fishers' exact test when appropriate. Logistic regression was fitted with multiple covariates to evaluated the relationship of relative factors include gender, age, location, and histologic variables. Numerical data was expressed as the minimum (Min), maximum (Max), mean, median, interquartile range (IQR), and standard

deviation (SD). The distribution of numerical data was verified with Shapiro-Wilk test. For data with non-normal distribution, Kruskal-Wallis H test was used. For data with normal distribution, groups were compared using one-way analysis of variance (ANOVA) followed by Tukey HSD post-hoc test. Spearman's correlation was also performed to explore the association between features of osteoclasts. Regression analysis was then applied to estimate the strength and character of relationship between those features. SPSS (version 26, IBM, New York, USA) was used. A p-value of less than 0.05 is defined as statistically significant.

3.7 Ethical consideration

This study was approved by The Human Research Ethics Committee of Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand prior to the onset of the study (060/2020).

Chapter 4 Results

Patient data of MRONJ, ORN, OM, and control group

Demographic information of 57 patient samples with MRONJ (17), ORN (15), OM (15), and normal jaw bone (10) was collected based on biopsy reports. Female accounts for 88% of MRONJ patients while the majority of ORN patients are men (80%). The OM group is also predominantly female. The average age of the MRONJ group is higher than the ORN and OM groups. Most lesion specimens collected in each group come from lower jaw bones (> 70%). Patients suffuring from MRONJ, ORN, and OM had the same characteristic clinical feature as bony exposure and other unspecific feature such as pus discharge, pain and tenderness. Radiographic feature of them were also similar as most lesion show radiopaque with ill-defined radiolucent rim. (Table 4.1)

Table 4.1. Patient data

	MRONJ	**ORN**	**OM**	**Control**
Number	17 82.4% N-BP, 17.6% Denosumab	15	15	10
Sex	88.2% women	80% men	66.7% women	60% women
Age	74.7 ± 10.67	55.9 ± 11.78	54.7 ± 19.83	40.7 ± 17.32
Primary diagnosis	52.94% osteoporosis, 11.76% multiple myeloma, 11.76% breast cancer, 5.9% lung cancer, 5.9% verrucous carcinoma, 5.9% SCCA	40% SCCA nasopharynx, 20% SCCA oropharynx, 20% SCCA oral cavity, 6.67% CA cervical lymph nodes, 6.67% non-Hodgkin's lymphoma	86.6% Chronic osteomyelitis, 13.3% Acute osteomyelitis	Normal jaw bone
Location	70.6% mandible, 29.4% maxilla	86.7% mandible, 13.3% maxilla	73.3% mandible, 36.7% maxilla	80% mandible, 20% maxilla
Clinical feature	**Bony exposure**, pus discharge, pain, tenderness, swelling soft tissue			
X-ray	Radiopaque lesion with ill-defined radiolucent rim			

Similarities in hard tissue of MRONJ, ORN, and OM

All 17 cases of MRONJ were confirmed to have necrotic bone which was characterized by empty osteocytic lacunae. 16/17 cases (94%) of MRONJ showed bones with peripheral resorptions showing irregular outline. The presence of osteoblasts was identified in 7/17 cases (41%) and the presence of osteoclasts was identified in 11/17 cases (65%) of MRONJ. Meanwhile, necrotic bones were also seen in 15 cases (100%) of ORN and 13/15 cases (86%) of OM. 13/15 cases (86%) of both ORN and OM showed scalloped bone borders. The presence of osteoblasts and osteoclasts of ORN was identified in 4/15 cases (26%) and 9/15 (60%), respectively. In OM, the presence of osteoblasts and osteoclasts was detected in 8/15 cases (53%) and 9/15 (60%), respectively. Bone specimens of MRONJ showed similar characteristics with ORN and OM with the characteristic feature of necrotic bone as empty lacunae, absence of osteoblastic rimming, and border resorptions representing empty Howship's lacunae. Though border resorptions were observed in the bone specimens of ORN and OM, peripheral resorptions appeared to be more pronounced in the MRONJ as more abnormal bone margins were found in the MRONJ specimens. These histological features were completely different when compared with normal jaw bone as the control group showing no necrotic bone. All 10 cases of the normal bone showed vital bones with osteoblastic rimming presence and smooth bone surface without a sign of bone resorption. The histological features of the hard tissue of the four groups are shown in Figure 4.1.

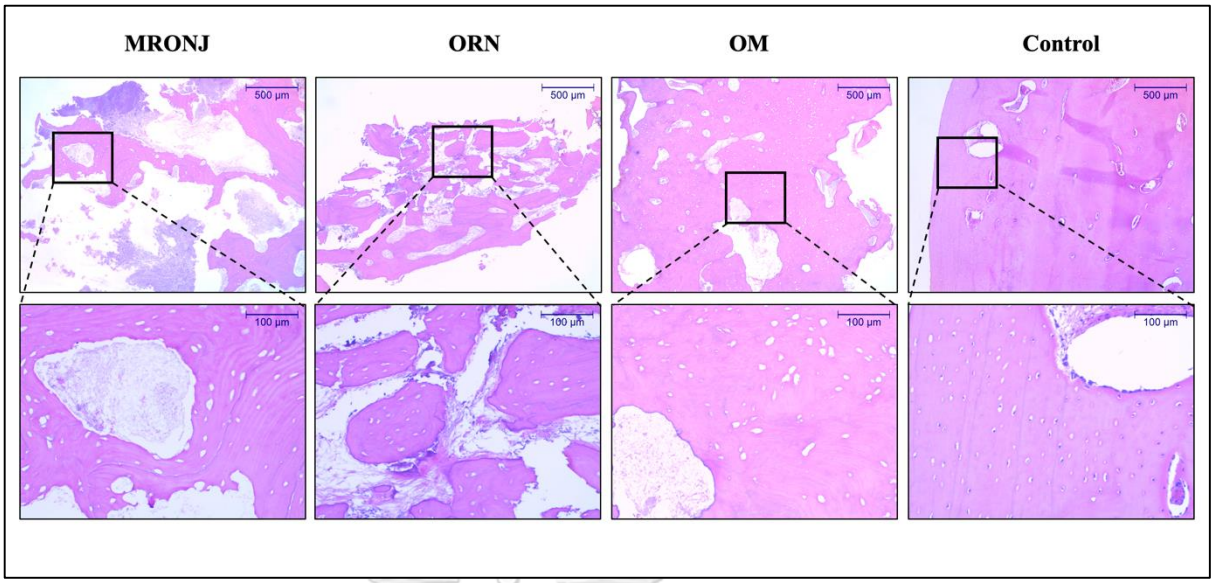


Figure 4.1. Histological features of necrotic bone comparing with normal jaw bone. Representative images are showed at 2 magnifications. Necrotic bone of MRONJ, ORN and OM show empty lacunae, lack of osteoblastic rimming and border resorption. Normal jaw bone with osteocyte inside lacunae, osteoblastic lining and smooth border bone line.

The difference in the soft tissue of MRONJ and ORN

Soft tissue observations in MRONJ showed that 9/17 cases (52%) identified the presence of granulation tissue and 3/17 cases (17%) identified the presence of fibrous tissue. Inflammatory infiltration was observed in 15/17 cases (88%) with mainly mixed inflammatory cells. Meanwhile, the presence of granulation tissue and fibrous tissue observed in ORN were 7/15 cases (46%) and 9/15 cases (60%), respectively. Soft tissue evaluation in OM indicated 5/15 cases (33%) to have granulation tissue and 3/15 cases (20%) to have fibrous tissue. Inflammation was identified in 13/15 cases (86%) of ORN and 14/15 cases (93%) of OM. The control group showed occasionally granulation tissue in 1 case (10%), fibrous tissue in 1 case (10%), and inflammation in 2 cases (20%). As shown in Figure 4.2B, the analyzed result indicated a significant difference in the presence of granulation tissue between MRONJ and the control group ($p<0.05$). ORN showed a significant difference in marrow fibrosis compared with others ($p<0.05$). Inflammatory infiltration was significantly increased in the necrotic group diseases compared with the normal jaw bone ($p<0.01$), however, the inflammation was equally found in all three necrotic bone groups when compare in pair ($p>0.05$). The presence of neutrophils and lymphocytes was observed in all three necrotic bone groups and there was no difference between them ($p> 0.05$). The histological features of soft tissue of four groups is shown in Figure 4.2.

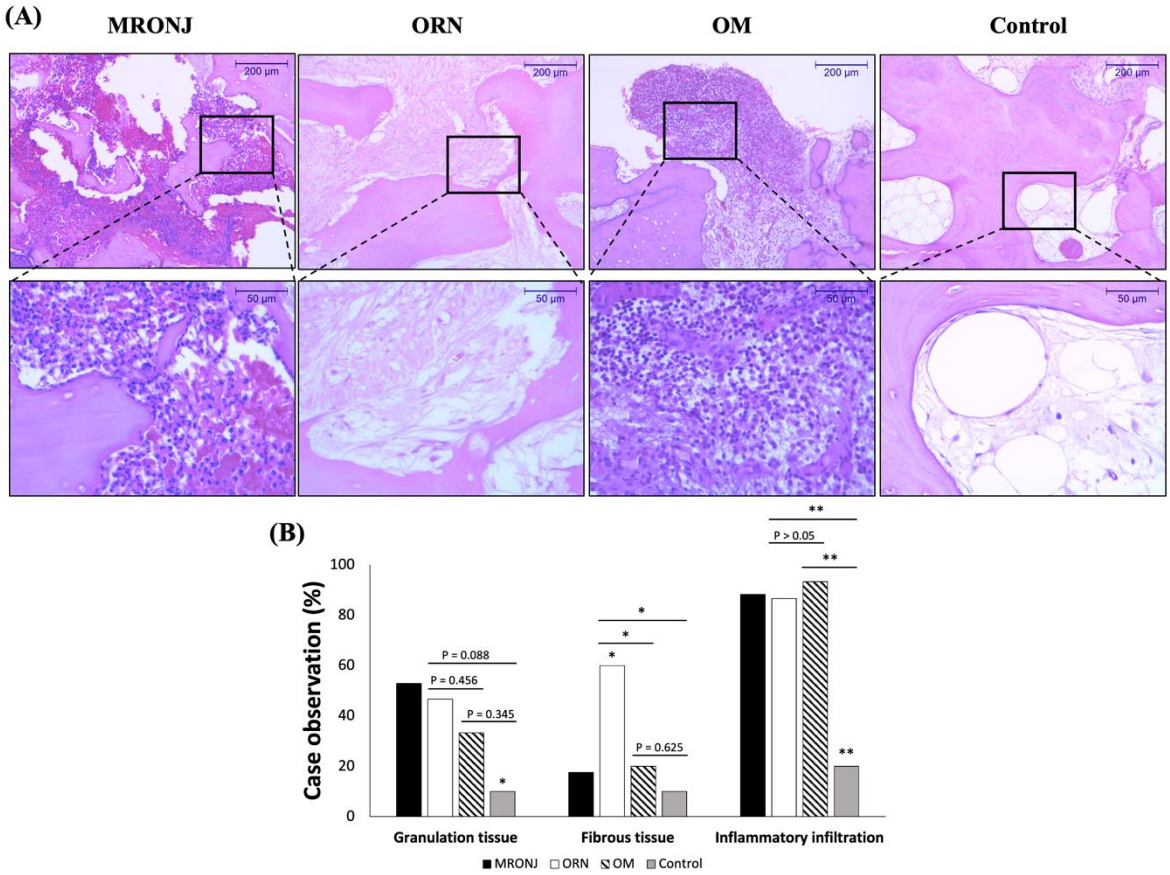


Figure 4.2. Histological feature of soft tissue and inflammation in necrotic bone groups and normal jaw bone. (A) Representative images of soft tissue are showed at 2 magnifications. (B) Histological analysis of soft tissue.

Dense clusters of microorganisms in MRONJ

Histological evaluation of the bone specimens showed the presence of bacterial colonies in 15/17 cases (88%) of MRONJ. Similarly, ORN showed the presence of bacterial colonies in 13/15 cases (86%) and this percentage in OM was 9/15 cases (60%). Normal jaw bone had no sign of infection. The characteristic of bacterial colonies was also quite different among groups. As shown in Figure 4.3A-D, MRONJ and ORN specimens showed a lot of dense bacterial clusters found at the bone periphery, whereas sparse bacterial colonies were observed to locate within the marrow bone tissue of OM specimens. The analyzed result indicated that there was a significant difference in the status of microorganisms between MRONJ and OM ($p<0.05$), but not between MRONJ and ORN or ORN and OM (Figure 4.3E). Intriguingly, the statistical analysis pointed out the significant association between the bacterial density and the presence of osteoblasts ($p<0.01$) in the necrotic specimens. However, a significant association was only found between the status of microorganisms and the presence of osteoblasts in MRONJ ($p=0.026$) and OM ($p=0.007$) when analyzing individual groups. A logistic regression model fitted with other factors as covariates also showed that there was no significant correlation between the bacterial density with the presence of osteoblasts ($p=0.243$) (Table 4.2).

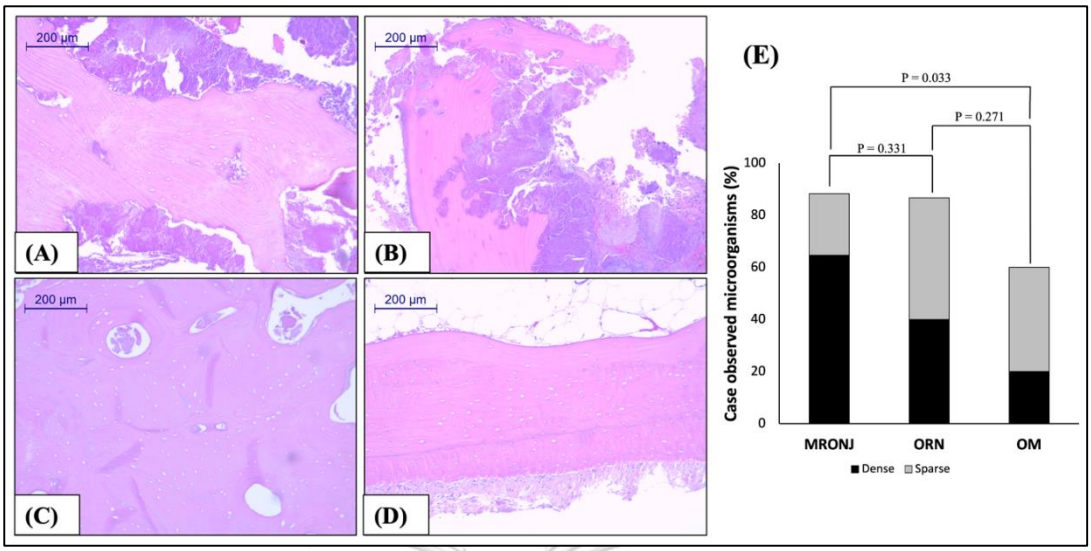


Figure 4.3. The presence of bacterial colonies observed in necrotic bone groups. Massive bacterial colonies were found in bone surface of (A) MRONJ specimens and (B) ORN specimens. (C) Sparse bacterial colonies were observed to locate within the marrow bone space of OM specimens. (D) Normal jaw bone had no sign of infection. (E) Analysis of bacterial colonies.

Table 4.2. Logistic analysis result of bacterial density and other factors

	Odd ratio	95% confidence interval	p-value
Osteoblast	0.267	0.029 – 2.451	0.243
Osteoclast	3.561	0.388 – 32.667	0.261
Gender	8.258	0.851 – 80.119	0.069
Age	0.939	0.870 – 1.013	0.104
Lesion location	2.853	0.391 – 20.831	0.301
Diagnosis	4.725	1.164 – 19.177	0.030*
Inflammation	11.319	0.646 – 198.269	0.097

*P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences

The presence of osteoclasts in MRONJ

An image of dispersed nuclei into the cytoplasm and loss of ruffle border indicative of apoptosis cell was observed in osteoclast of MRONJ as shown in Figure 4.4. However, there was no significant difference in the presence of osteoclasts when compare groups in pairs ($p>0.05$). Interestingly, many osteoclasts of MRONJ were noted with giant shape comparing with small osteoclasts normally found in other groups (Figure 4.5). The analytical result showed that there was a significant relationship between the presence of osteoblast and osteoclast ($p<0.05$) in MRONJ and OM, but not in ORN ($p = 0.103$). Adjusting for patient demographic data, diagnosis group, peripheral resorption, inflammatory cell infiltration, and level of bacterial colonies as covariates, a logistic regression model showed the presence of osteoblast (OR = 64.374, one-sided $p = 0.007$) still be significantly associated with the presence of osteoclast (Table 4.3).

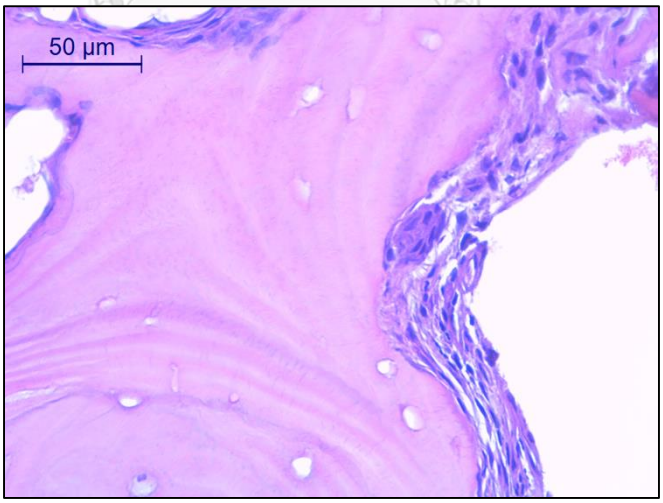


Figure 4.4. Osteoclast in MRONJ showed an image of dispersed nuclei into the cytoplasm and loss of ruffle border (Magnification 400×)

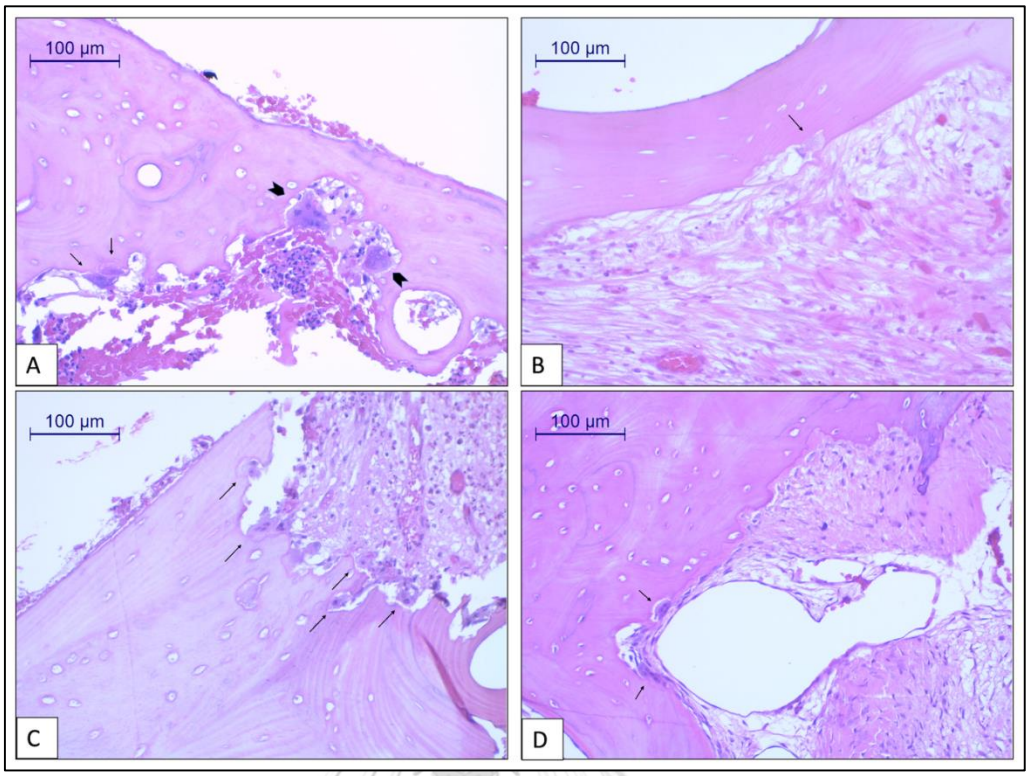


Figure 4.5. Osteoclasts (black arrow) were seen to digest bone. (A) Giant osteoclasts (black chevron) and small osteoclasts of MRONJ were digesting necrotic bone, surrounding area showed blood clot and cellular debris. (B) Necrotic bone with prominent marrow fibrosis was resorbed by the osteoclast. (C) Many small osteoclasts with 2-4 nuclei were digesting necrotic bone in OM specimens, inflammatory cell infiltration was also observed. (D) Bone resorption by osteoclasts in normal jaw bone.

Table 4.3. Association between the presence of osteoclast and osteoblast with other factors

	Odd ratio	**95% confidence interval**	**p-value**
Osteoblast	64.374	3.073 –1348.612	0.007**
Gender	0.821	0.175 – 3.852	0.803
Age	1.058	0.994 – 1.126	0.076
Lesion location	5.874	0.640 – 53.889	0.117
Diagnosis	0.693	0.213 – 2.251	0.541
Peripheral resorption	0.860	0.062 – 11.947	0.911
Inflammation infiltration	1.166	0.100 – 13.618	0.902
Bacterial colony	0.561	0.094 – 3.366	0.527

*P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences

The difference in morphology of osteoclasts

To investigate the morphology of osteoclasts in MRONJ compare with ORN, OM, and control group, a diameter of osteoclasts was measured and nuclei of osteoclasts were counted. Many multinucleated giant osteoclasts with round shapes were often found in MRONJ compare with oval shape osteoclasts in ORN, OM, and control groups (Figure 4.6A). The analyzed result showed that osteoclasts of MRONJ become bigger with more nuclei than those in other groups ($p<0.001$). Particularly, the diameter of osteoclasts in MRONJ was significantly larger than which in ORN, OM, and control on average of 1.25 times, 1.23 times, and 1.34 times, respectively (Table 4.4, Figure 4.6B). There was no significant difference found between the diameter of osteoclasts in ORN, OM, and control groups. Similarly, the number of nuclei of osteoclasts in MRONJ was significantly higher than which in other groups. On average, the number of nuclei of osteoclasts in MRONJ was 1.65 times, 1.58 times, and 1.87 times higher than which in ORN, OM, and control groups, respectively (Table 4.4, Figure 4.6C). There was no significant difference observed in the number of nuclei of osteoclasts among ORN, OM, and control groups. The correlative analysis also indicated a positive relationship between diameter and nuclei of osteoclasts increased cell fusion ($r = 0.579$, $p<0.001$). The regression analysis showed that the number of nuclei ($p<0.001$) was significant associated with the diameter of osteoclasts (Figure 4.6D). However, no significant association was found between the number of nuclei and the diameter of osteoclasts when investigating groups separately ($p>0.05$).

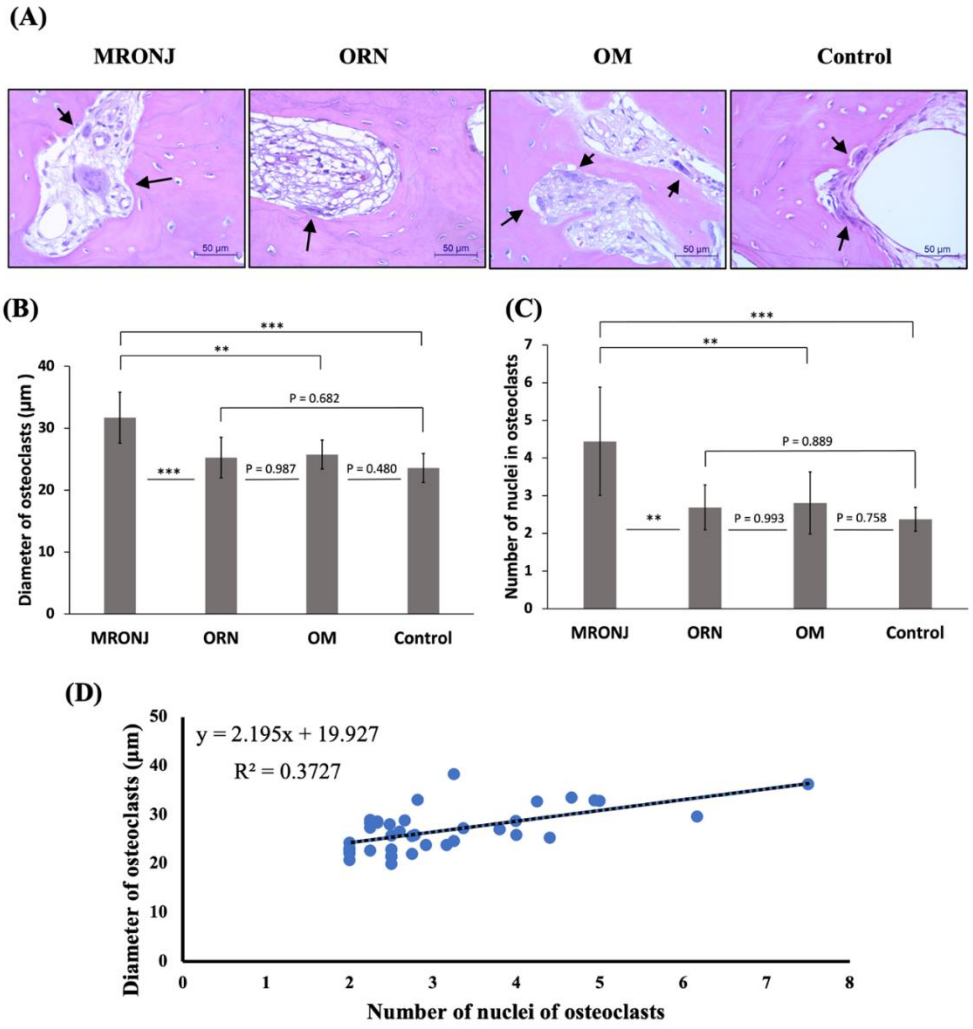


Figure 4.6. Morphology of osteoclasts. (A) Giant osteoclasts with round shape were often found in MRONJ compare with oval shape osteoclasts in ORN, OM, and control group. (B) Compare diameter and (C) the number of nuclei of osteoclasts in MRONJ, ORN, OM, and control group. (D) A positive correlation between diameter and the number of nuclei in osteoclasts. Data are presented as mean $\pm$ SD ; *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences between groups.

Table 4.4. Descriptive data of osteoclasts morphology and quantity

		Mean	SD	Median	IQR	Min	Max
Diameter of osteoclasts (μm)	MRONJ	31.6	4.1	32.8	4.8	23.7	38.3
	ORN	25.2	3.2	25.7	6.1	19.8	28.9
	OM	25.7	2.3	25.6	4.4	22.0	28.2
	Control	23.5	2.3	22.8	4.3	20.7	27.2
Number of nuclei in osteoclasts	MRONJ	4.4	1.4	4.2	1.7	2.8	7.5
	ORN	2.6	0.5	2.5	0.6	2	4
	OM	2.8	0.8	2.4	1.2	2	4.4
	Control	2.3	0.3	2.5	0.6	2	2.75
Osteoclasts per medullary area (osteoclasts/mm^2)	MRONJ	58.5	32.3	63.8	58.7	19.5	119.0
	ORN	27.3	11.9	30.7	19.3	8.3	45.3
	OM	36.8	12.3	36.3	21.3	19.6	56.6
	Control	27.8	9.1	27.2	14.9	16.7	44.3
Osteoclasts per bone length (osteoclasts/mm)	MRONJ	3.4	2.1	3.4	2.8	0.9	8.2
	ORN	1.5	1.0	1.3	1.2	0.5	3.8
	OM	2.2	1.1	2.0	1.4	0.9	4.5
	Control	1.6	0.7	1.6	1.0	0.6	2.8

Number of osteoclasts in MRONJ higher than in ORN and control group

To detect the response of osteoclast shown in quantity, we counted the number of osteoclast in regions of interest. The result of one-way ANOVA showed that there was a significant difference in the number of osteoclasts of MRONJ, ORN, OM, and the control group ($p<0.05$, Table 4.4, Figure 4.7A-C). Post-hoc Tukey's HSD tests showed that osteoclasts per medullary area of MRONJ were significantly higher than this in ORN, and control group ($p<0.05$) but this number was no significant difference comparing with OM ($p>0.05$). Similarly, osteoclast per bone length counted also exhibited a significant increase in the number of osteoclasts in MRONJ compare with

ORN and control group ($p<0.05$), not with OM ($p>0.05$). No significant difference was found in the number of osteoclasts in ORN, OM, and control when compare in pairs ($p>0.05$). The relationship of osteoclasts number and morphology of osteoclasts was also analyzed. There was a positive correlation between diameter ($p<0.05$) and the number of osteoclasts per bone length (Figure 4.7D). However, no significant association was found between the number of osteoclasts and the morphology of osteoclasts when investigating groups separately ($p>0.05$).

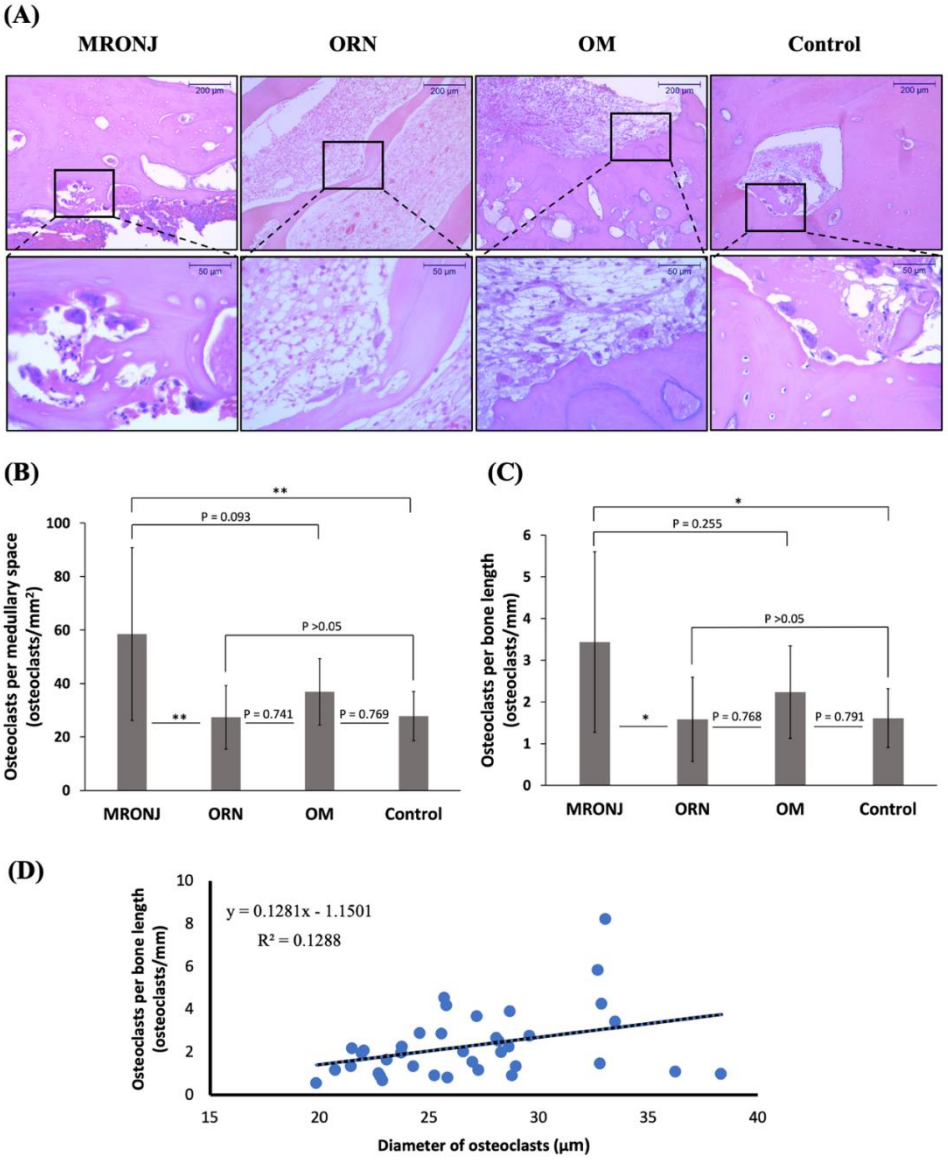


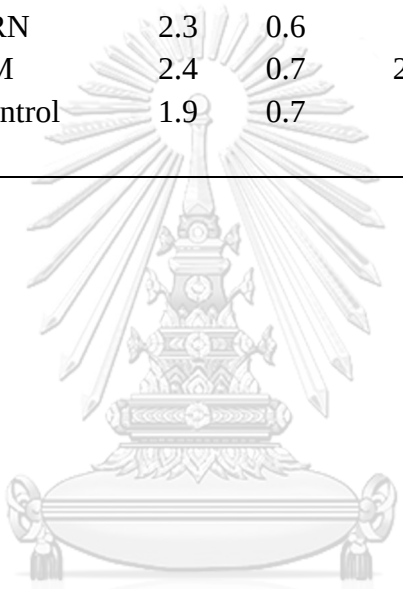
Figure 4.7. Osteoclasts quantity analysis. (A) Osteoclasts were observed and counted in MRONJ, ORN, OM, and control groups. Statistical analysis of (B) number of osteoclasts expressed per medullary area and (C) number of osteoclasts expressed per bone length in MRONJ, ORN, OM, and control group. (D) A positive correlation between diameter and the number of osteoclasts expressed per bone length. Data are presented as mean $\pm$ SD ; *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences between groups.

TRAP expression in osteoclasts

Osteoclasts can be positive with the brown color of TRAP in the cytoplasm. TRAP-positive mainly in osteoclast but some non-osteoclast cells are still detectable scatter with TRAP sometimes. Overall, 4 groups all showed TRAP-positive osteoclasts (Table 4.5, Figure 4.8A). The Kruskal-Wallis test showed a significant difference of TRAP-positive osteoclasts in 4 groups ($p<0.05$). However, when comparing groups in pairs, only OM showed significantly higher TRAP-positive osteoclasts per view than the control group (Figure 4.8B). Furthermore, TRAP-positive osteoclasts per view showed a positive correlation with osteoclast quantity ($p<0.01$) but did not correlate with osteoclast morphology ($p>0.05$). The regression analysis of TRAP-positive and osteoclasts numbers showed that osteoclasts per medullary area ($p<0.001$) and osteoclasts per bone length ($p<0.001$) significantly associated with the number of TRAP-positive osteoclasts (Figure 4.8D-E). On the other hand, OM showed the highest intensity score when evaluating the intensity of TRAP-staining (Table 4.5, Figure 4.8C), however, no significant difference was found in the intensity score of the 4 groups ($p>0.05$).

Table 4.5. Descriptive data of osteoclasts staining with TRAP

		Mean	SD	Median	IQR	Min	Max
TRAP-positive osteoclasts per view	MRONJ	4.5	4.2	2.5	4.5	0.5	14.5
	ORN	2.8	0.9	2.5	1.8	1.5	4
	OM	3.9	1.3	4	2.5	2	6
	Control	2.1	0.7	2	1	1.5	3.5
Intensity score of TRAP-positive cells	MRONJ	2.3	0.7	2	1	1	3
	ORN	2.3	0.6	2	1	1.5	3
	OM	2.4	0.7	2.5	1	1	3
	Control	1.9	0.7	2	1.3	1	3



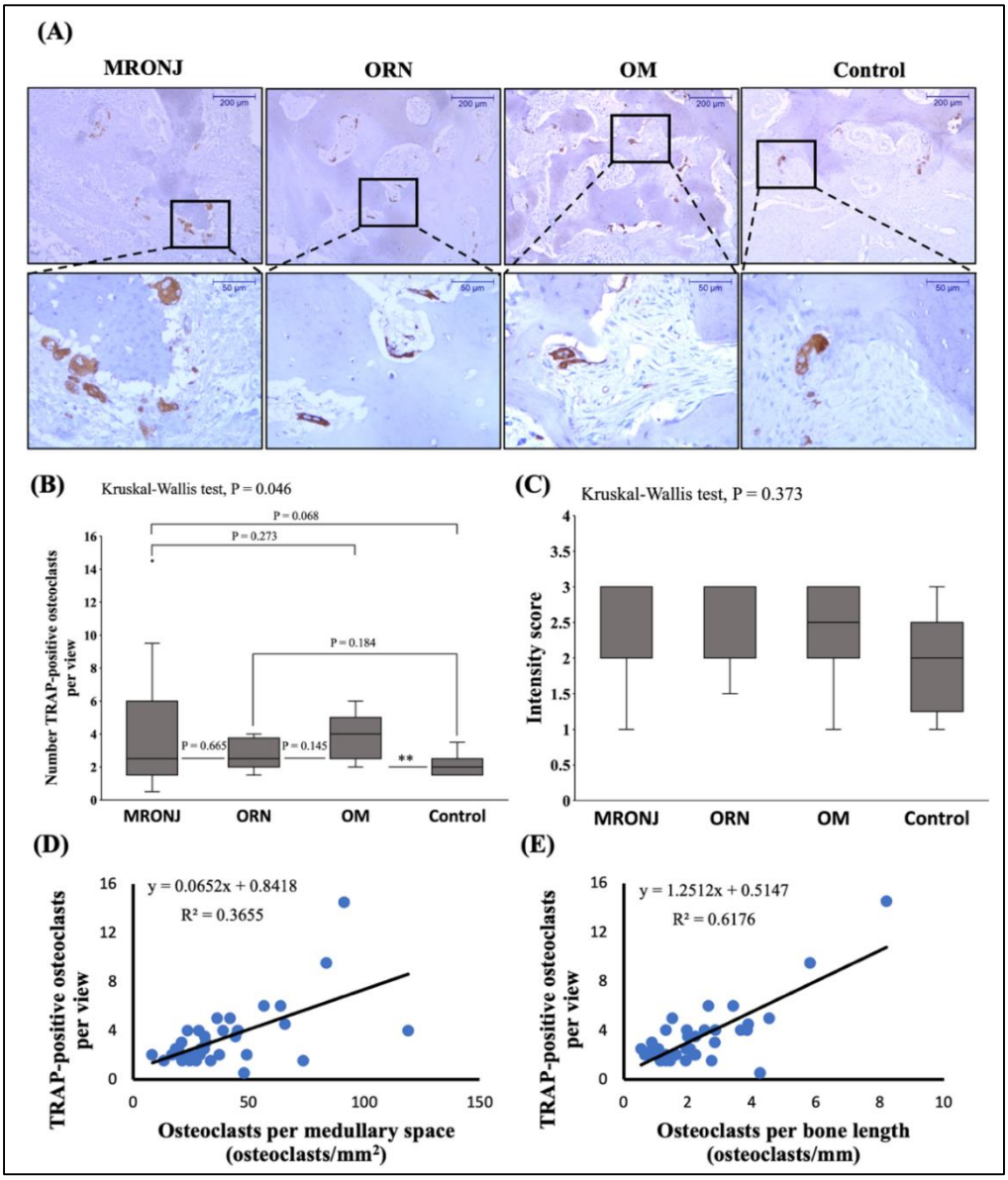


Figure 4.8. TRAP-positive analysis. (A) TRAP-positive osteoclasts in MRONJ, ORN, OM, and control groups. (B) Analyze the number of TRAP-positive osteoclasts per view and (C) intensity score in MRONJ, ORN, OM, and control group. A positive correlation between TRAP-positive osteoclasts per view with (D) osteoclasts per medullary area and (E) osteoclasts per bone length. Data are presented as box plot diagrams; *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences between groups.

Relationship of osteoclasts features with histological characteristics

To evaluate osteoclasts in a complete picture of histopathology, the histological characteristics such as the presence of osteoblasts, the level of a bacterial colony, and the presence of inflammation were assessed in the multiple linear regression model. Regression analysis showed that there was an association between inflammation with the diameter of osteoclasts ($p<0.05$). Furthermore, the level of bacterial colony affects the number of nuclei in osteoclasts ($p<0.05$). With bacteria, the presence of osteoblasts also affects the number of osteoclasts expressed per medullary area. However, no association was found between the number of osteoclasts per bone length and histological features ($p>0.05$). The detail of regression analysis of osteoclasts features was shown in Table 4.6.

Table 4.6: Resulting multiple linear regression models for the osteoclasts features

	Beta	**95% Confidence interval**	**p-value**
Diameter of osteoclasts (R^2=0.166)			
The presence of osteoblasts	0.197	-2.025 – 5.845	0.331
Inflammatory infiltration	0.333	0.050 – 6.821	0.047*
Bacterial colony	0.266	-0.762 – 3.651	0.192
Number of nuclei in osteoclasts (R^2=0.179)			
The presence of osteoblasts	0.147	-0.690 – 1.482	0.464
Inflammatory infiltration	0.161	-0.471 – 1.397	0.321
Bacterial colony	0.436	0.048 – 1.266	0.035*
Number of osteoclasts per medullary area (R^2=0.297)			
The presence of osteoblasts	0.565	9.676 – 47.538	0.004**
Inflammatory infiltration	0.260	-2.221 – 30.350	0.088
Bacterial colony	0.535	4.585 – 25.813	0.006**
Number of osteoclasts per bone length (R^2=0.102)			
The presence of osteoblasts	0.223	-0.683 – 2.215	0.290
Inflammatory infiltration	0.232	-0.398 – 2.095	0.176
Bacterial colony	0.257	-0.319 – 1.306	0.225

*P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences

Negative expression of Bcl-xL in osteoclasts of MRONJ

Bcl-xL is an antiapoptotic protein that modulates the intrinsic pathway of osteoclasts and showed high expression in osteoclasts in an animal study of MRONJ. We hypothesize that osteoclasts express Bcl-xL to survive and resist the apoptotic induce from MRONJ. Thus, we evaluated Bcl-xL expression in the osteoclast of MRONJ. The result showed that no survival signal was found, all osteoclasts cells in MRONJ were negative with antibody Bcl-xL although the positive control using each staining round demonstrated Bcl-xL antibody still work (Figure 4.9).

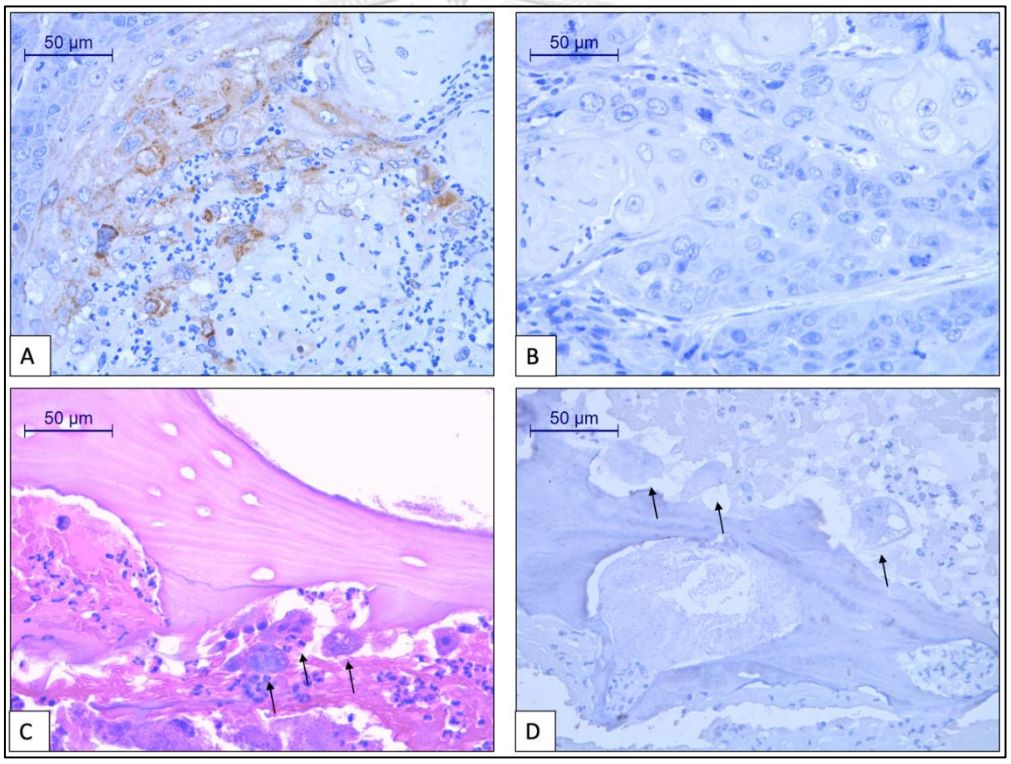


Figure 4.9. Negative expression of antibody Bcl-xL in osteoclasts of MRONJ. (A) Positive control and (B) negative control of antibody Bcl-xL on squamous cell carcinoma of tongue. (C) H&E staining of osteoclasts (black arrow) in MRONJ. (D) Osteoclasts (black arrow) of MRONJ showed negative result with immunohistochemistry.

Chapter 5 Discussion

MRONJ is a skeletal disease with a complex mechanism that remains unexclusive until now. There are many risk factors in which their roles have to be seen in the full picture. In this study, we focus on the histologic analysis first to ensure whether MRONJ showed different histological characteristics with ORN, OM, and control groups. A histological investigation is a powerful tool to offer a broad view and basic knowledge about histopathological characteristics, thereby conducting further research on the underlying mechanism. The demographic feature of sample groups in this study was not quite similar to the previous study [7, 14]. This might lead to different results in evaluating and analyzing histological characteristics among these disease groups.

In general, the histopathologic observation from this study showed the similarities in necrotic bone and inflamed soft tissue characteristics among MRONJ, ORN, and OM. The significant difference noted between MRONJ and ORN was fibrosis which is evidence to support a newly accepted theory about radiation-induced fibrosis damages to normal tissue [46]. However, these differences in finding are not characteristic enough and it is almost impossible to distinguish the disease diagnostic-based solely on the histopathological characteristics of the hard tissue and soft tissue. These observations were consistent with previous findings except that neutrophils were equally found in three necrotic groups, not the same as the significant lack of leukocytes in MRONJ reported in the previous studies [3, 14]. There were no notable histological findings in inflamed tissue of MRONJ among necrotic groups although inflammation is one of the proposed hypotheses of MRONJ pathology [1]. The occurrence of inflammation and the presence of inflammatory cytokines have been shown to play an

important role in the process that drives MRONJ to occur [65]. Thus, further studies focus on inflammation response in MRONJ should be performed using a molecular biological technique to evaluate the changes that happended at the molecular level.

The highlighted point in this study is the status of microorganisms. Analysis result of bacterial colony status between MRONJ and OM showed a significant difference. MRONJ specimens exhibited a lot of dense bacterial clusters found in the bone periphery whereas sparse bacterial colonies were observed to locate within the marrow bone space of OM specimens, in agreement with previous studies [3, 14, 74]. Furthermore, bacterial density on necrotic bone groups was found to be related to the presence of osteoblasts and osteoclasts. However, the actual relationship between them was not clear on further analysis. A bacterial infection is considered to be an important component in the pathogenesis of MRONJ when necrosis occurs only in the jaw where it is easily damaged and penetrates by microorganisms. The high prevalence of bacteria, especially *Actinomyces spp.* in MRONJ, has been reported and is receiving increasing attention [75].

Osteoclasts are worth noting although there was no significant difference in their presence when compare groups in pair. As the cells are directly influenced by the effect of antiresorptive agents, the role of osteoclasts is highlighted. Histopathological evidence of empty Howship's lacunae was observed in most specimens of MRONJ suggests that many osteoclasts have disappeared due to drug effect. Images of dispersed nuclei into the cytoplasm in osteoclasts also confirmed the apoptosis process of these giant bone-eating cells. However, the frequency of osteoclast encounters does not change significantly, suggesting that osteoclast might against drug-induced apoptosis and somehow persisted. The giant hypernuleated osteoclasts and the increase in the

number of osteoclasts were also reported [7, 9]. This conflicting finding showed the complicated response of osteoclast in a complicated oral environment with multi influencing factors. The response of osteoclast in MRONJ remains unclear now. The analytical result showed that there was a significant relationship between the presence of osteoblast and osteoclast in MRONJ and OM, but not in ORN. Therefore osteoblast might play a role in osteoclast's response in MRONJ. These results support findings that osteoblasts, osteoclasts, and osteocytes respond mutually in MRONJ [64].

To go further, the osteoclasts profile of MRONJ was investigate. As the only cells were definitively shown to have the capacity to digest bone, osteoclasts are key mediators of skeletal diseases and thus become a target of drugs prescribed to limit bone resorption. To rebalance bone remodeling, these prescribed drugs as bisphosphonates and denosumab, worked by interfering with the migration, function, and apoptosis of osteoclast to prevent bone resorption [27, 29]. Investigating the response of osteoclasts which was directly subjected to drugs, maybe the key to understand the histopathology of MRONJ. In this study, osteoclasts in MRONJ patients showed unusual responses that showed a morphological change when compared with other necrotic jaw bone diseases and the healthy group. More specifically, osteoclasts in MRONJ became larger with more nuclei compared with normal oval small shape osteoclasts in other groups. The presence of giant cells in MRONJ has also been observed and reported in many previous studies [7-9]. The increase in size and nuclearity in osteoclasts required the process of cell-cell fusion [76]. The positive correlation between the diameter and number of nuclei of osteoclasts in this study strengthens the theory of the increase of cell-cell fusion as an impact step to increase osteoclasts size to become giant cells in

MRONJ. However, cell-cell fusion only is not sufficient to account for giant shape abnormalities of osteoclasts [7].

The regression result between osteoclasts morphology and histologic features indicated the association between the presence of inflammatory cells with the diameter of osteoclasts. The status of the bacterial colony was also found to relate with the number of nuclei counted in osteoclasts in 4 experimental groups. This finding suggests the role of inflammatory cells and bacterial cluster as influence factors in the response of osteoclasts in MRONJ. The enlargement of osteoclasts also proves that osteoclasts' life spans are longer. The response of osteoclasts in MRONJ might be one of the ways to against the drug's action, in another word, resistance the mechanism of the drug-induced osteoclasts apoptosis. MRONJ reproducible in animal studies indicated elevated concentrations of survival signaling proteins such as Bcl-2, Bcl-xL, and Mcl-1 in the MRONJ mice group compared with the control group [43, 58, 73]. Bcl-xL which is one of the main pro-survival members of the Bcl-2 family is found on osteoclasts [71]. This may be the reason why inactivated giant osteoclasts are found almost exclusively on MRONJ. However, the immunohistochemistry in this study showed the negative result of Bcl-xL expression in osteoclasts of MRONJ. The increase of the diameter of osteoclasts in MRONJ may have to be explained by different mechanisms which need further investigations.

The accepted mechanism of action of bisphosphonates and denosumab on osteoclasts is to prevent osteoclasts' development and function [5]. The decreased amounts of osteoclasts were reported in humans and animals as an obvious consequence [3, 77, 78]. However, in our study, not only increasing in the diameter and nuclei, but the number of osteoclasts in MRONJ also increased significantly. The quantity of

osteoclasts is considered as a measurement of bone resorption but must be reviewed in the case of MRONJ because osteoclasts are inactivated [79]. Osteoclasts number increase is consistent with recent studies on human samples of MRONJ, however, our result indicated the number of MRONJs is not more prominent than the number of osteoclasts in OM, unlike the previous study [7]. As defined as the inflammatory condition of the bone, the increase in the number of osteoclasts in OM is the same as MRONJ suggesting a dynamic role of inflammation in increasing the number of osteoclasts in MRONJ. However, the correlation between osteoclasts number and the presence of inflammatory cells was not significant. More specific information on inflammatory cytokine might be required rather than only observing the presence of chronic and acute inflammatory cells.

Although not showing the relationship between the number of osteoclasts and inflammation, the regression analysis pointed out the presence of osteoblasts as a predictor affects osteoclasts' quantity. Osteoblasts are bone-forming cells that play important roles in bone remodeling. Osteoblasts regulate the migration and differentiation of osteoclasts through Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) secretion which is an important element that triggers osteoclasts activity [80]. The activity of osteoblasts is affected by bisphosphonates in many studies [81, 82]. Besides, the effect of bisphosphonates was also observed on another type of bone cell, osteocytes, which are differentiated from osteoblasts and embedded in the bone matrix [64]. Therefore, it seems like, osteoblasts and osteocytes have increased secretion of RANKL thereby enhancing osteoclastogenesis under the action of bisphosphonates.

Bacteria can also stimulate secrete RANKL, which indirectly enhances osteoclastogenesis and bone resorption [83]. Commonly pathogenic bacteria induce periodontitis *Actinomyces spp.* and *Porphyromonas gingivalis* have been shown to induce increased bone resorption in animal models [84]. Moreover, 84% of patient with MRONJ was reported with periodontitis and many studies showed the presence of bacterial cluster on the sequestered bone [85]. The scalloped bone borders observed in MRONJ showed that bone resorption activity still happened. These scalloped bone borders were clustered with dense bacterial colony support the proposed theory that bone resorption can be caused directly by bacteria, inflammatory cytokines independent with osteoclasts which were inhibited in MRONJ [86]. Therefore, the presence of border resorption in MRONJ may not prove the disappearance of the osteoclasts as the previous study suggested [3]. However, a previous study showed that bacterial infection in the presence of bisphosphonates treatment converts osteoclasts progenitors to macrophages but not mature osteoclasts [65]. There may be another factor involved and influencing osteoclastogenesis when investigating 2 different groups of research subjects, namely humans and animals.

TRAP is considered a biomarker of osteoclasts [87]. TRAP-positive osteoclasts showed no significant difference between MRONJ and other groups although a high number of osteoclasts was observed on H&E staining of MRONJ. This might due to osteoclasts in MRONJ was less inactivated led to reducing TRAP-positive osteoclasts finding in MRONJ. Instead, the number of TRAP-positive osteoclasts in OM increased significantly when compared with the control group. The intensity score of OM was also highest suggest the activity of osteoclasts, while the lower intensity score of MRONJ suggest the inhibition of osteoclasts. However, no significant difference was

found between those groups. Increased TRAP-positive osteoclasts finding in OM showed an increased osteoclasts activity in OM, which is caused by inflammation and microorganisms. However, the bacterial density on MRONJ is much denser. It is not consistent with other studies, which may be due to different methods of measurement and TRAP is not as accurate in assessing the function of osteoclasts [7, 10]. This difference also may be due to the sample selection combine of bisphosphonate and denosumab which has a different principle of action in the study. A previous study showed a significant difference of TRAP-positive osteoclasts between the bisphosphonate group and the denosumab group [10]. TRAP-positive osteoclasts increase in the bisphosphonate treatment group but not the denosumab treatment group and TRAP mean the number of osteoclasts rises even higher in patients with the combination of bisphosphonate and denosumab in treatment. Drug synergies may trigger another pathway that needs further research.

The response of osteoclasts observed in this study suggests mechanisms related to drug resistance leading to an increase in the number and be enlargement of osteoclasts. Different results were finding in this study compare with previous studies that might come from different risk factors, sample selection, technique, and statistical method. Moreover, the different results might come from the genetic differences which was reported to play a role in MRONJ [88-90]. Therefore, this study performed on Thai people samples and showed different results compare with previous studies which reflect genetic differences may leading to different responses of osteoclasts regardless of dosage and duration of administration. On the other hand, the limitation of this study is the small sample size and the combination of drugs in sample selection that might affect the analysis results. Missing data of dose and stage of MRONJ is also a drawback

of this study when it is not possible to evaluate the severity of the disease. However, this study still provided a general profile of osteoclasts in MRONJ and indicated a close relation of bacteria and inflammatory factors with osteoclasts' response. When the oral environment can be in direct contact with bacteria and frequent microtrauma lead to inflammation and high bone turnover, all these factors including microtrauma, a bacterial toxin, and inflammation lead to bone death, but osteoclasts cannot remove these necrotic bone, thus lead to osteonecrosis in MRONJ. This may be the reason why MRONJ is almost exclusively out in the maxillofacial area.

In conclusion, highlighted points in MRONJ histology are peripheral resorption showing irregular shape, high prevalence of dense bacterial clusters on bone surfaces, and giant osteoclasts. Osteoclasts in MRONJ showed an enhanced response which might relate to inflammation and bacteria. This finding also again supports the idea osteoclasts might be the main key to investigate MRONJ but have to check many risk factors in which their roles have to be seen in the full picture with bone remodeling, as well as the pathway signaling, is triggered by inflammatory factors and the presence of bacteria.

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