博 士 学 位 論 文

EXPERIMENTAL STUDY ON

BONE HEALING OF RABBIT FEMUR UNDER

IMPLANT PLATE FIXATION USING A

NEWLY DEVELOPED TITANIUM ALLOY

近畿大学大学院

総合理工学研究科　メカニックス系工学専攻

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2023 年 1 月 25 日

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SUMMARY

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NORAIN BINTI ABDULLAH

Titanium and its alloys have been used as biomaterials generally. As an example, implant plate for bone fracture treatment has been widely made using titanium alloys such as Ti-6Al-4V ELI (Ti-64). Although stainless steel and cobalt-chromium alloy were being as biomaterials, it is known that stress shielding phenomenon occurs due to their high elastic modulus of 200 [GPa] and 220 [GPa], respectively. As Ti-64 alloy possesses elastic modulus of 110 [GPa], it is expected to be useful as biomaterials to be used in long term implantation. However, there is vanadium element in the Ti-64 alloy that is being said to be a toxic element. It has been rising concern among the researchers.

Hence, our laboratory developed a new titanium alloy, Ti-29Nb-13Ta-4.6Zr (TNTZ) which has elastic modulus of 60 [GPa] and expected can be used safely in a long-term implantation. As general, it is being said that low elastic modulus of biomaterials which are near to that of bone (30 [GPa]) can prevent stress shielding phenomenon. Stress shielding is a phenomenon when a certain stress is removed from the bone due to the elastic modulus difference between the bone and biomaterials (i.e., Implant plate). As a result, bone formation and bone resorption will be unbalance, bone mass loss happened, and eventually led to bone malfunction. This phenomenon is known to happen on the later stage of healing.

On the other hand, stiffness is being said as one of the important factors to be considered when designing the implant plate especially during the early stage of healing. This is because the mechanical stability of fixation system must be gained for bone to heal. If appropriate stiffness of implant plate is used, required mechanical stability can be achieved, a certain degree of interfragmentary movement of bone (IFM) can be obtained to ensure a balance cell differentiation, thus helps in the process of bone healing. This topic has been discussed intensely until now, but the study is merely conducted through computational analysis. Present study proposed an experimental study on bone healing, discussing in regards of elastic modulus along with stiffness and aimed to describe the bone formation pattern under the fixation when using Ti-64 and TNTZ implant plates focusing on early stage of healing.

This dissertation is divided into five chapters; Chapter 1: Introduction, Chapter 2: Literature reviews, Chapter 3: Research Methodology, Chapter 4: Results and discussion, and Chapter 5: Conclusion. Chapter 4 is discussed about the findings from manuscript published in Journal of Materials Research and Mechanical Engineering Journal, respectively.

Chapter 1 outlined the background of present study, research motivation including the current problems and idea, objectives, and the structure of dissertation.

Chapter 2 focused on the literature reviews regarding the bone formation, facture healing process, metallic biomaterials in terms of its suitability and problems as implant plate, factors that are needed to be considered in designing the implant plate, and method or approach that were applied to aid in the animal study.

Chapter 3 described the framework of present study, mainly focused on the research methodology and approach strategized to investigate the bone healing of rabbit femur under implant plate fixation. Mainly, it is divided into three parts which are sample preparation: method of implant plate preparation which produced Ti-64 implant plate and TNTZ implant plate with elastic modulus of 110 GPa and 60 GPa, and thickness of 1.5 [mm] and 0.5 [mm], respectively, surgical procedures, and bone evaluation including the bone sample preparation method for mechanical properties test and staining evaluation. On top of that, the stiffness is calculated and the order from low stiffness to high stiffness is as follows: TNTZ implant plate with thickness of 0.5 [mm], Ti-64 implant plate with thickness of 0.5 [mm], TNTZ implant plate with thickness of 1.5 [mm], Ti-64 implant plate with thickness of 1.5 [mm]. Noted that, it is mentioned as TNTZ 0.5, Ti-64 0.5, TNTZ 1.5 and Ti-64 1.5.

Chapter 4 elaborated the findings of present study through two publications; First objective: regarding elastic modulus difference, Second objective: based on implant plate stiffness. The results shows that the bone formation on anterior region (defect) has similar area. However, posterior region (opposite of defect) resulted in asymmetric callus formation under fixation when using Ti-64 1.5 implant, while other fixation types show regular bone structure. The medial (above of defect) and lateral (below of defect) both shows the callus formation on the outermost layer of cortex. This is quantitatively described by image analysis using ImageJ software. It is known that the defect (anterior region) has almost similar average of total area of callus, while the non-defect region (posterior, medial, lateral) is opposite. The callus formation is the largest under the fixation when using Ti-64 1.5 implant plate, followed by TNTZ 0.5 implant plate, and TNTZ and Ti-64 implant plates. Furthermore, histological observation by H&E staining allows proper investigation on healing progression. It is found that under the fixation when using Ti-64 implant plates have slower healing progression that TNTZ implant plates, noticed by the high percentage of soft tissue in anterior region. Moreover, Vickers hardness of bone callus were measured. As a result, the bone hardness is almost the same under all fixation types. However, under Ti-64 1.5 implant plate results in the lowest hardness compared with other fixations.

Chapter 5 concluded the summary, limitations, and future proposed works. A few limitations were considered such as the actual stiffness and IFM measurements. A few proposed works that are being considered is also described in this chapter. The conclusion of present study is as follows:

- ✓ Implant plate with high stiffness resulted in the asymmetric callus formation due to high mechanical stability of implant plate that suppressed the IFM on anterior region, resulted in non-uniform IFM throughout the bone (Ti-64 1.5 implant plate).
- ✓ Excessive callus formation under fixation when using low stiffness implant plate due to poor mechanical stability that led to intense IFM throughout the bone (TNTZ 0.5 implant plate).
- ✓ Ideal healing can be achieved when implant plate with suitable stiffness is used because

required mechanical stability can be obtained and a certain degree of IFM is transferred (TNTZ 1.5 implant plate may be near to the ideal stiffness).

List of Publications

No.	Manuscripts	Authors	Journal (year, volume, pages)	Dissertation Chapter
1	Effect of low modulus titanium plate fixation on rabbit femur bone healing	N. B. Abdullah D. Miyazaki E. Yamamoto K. Ueki M. Nakai	Journal of Materials Research, 2022, 37, 2536-2545.	Chapter 2 Chapter 3 Chapter 4 (4.2)
2	Effect of titanium plate stiffness on bone healing in rabbit femur	N. B. Abdullah D. Miyazaki E. Yamamoto K. Ueki M. Nakai	Mechanical Engineering Journal, 2022, 9, 22-00282.	Chapter 2 Chapter 3 Chapter 4 (4.3)

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BONE HEALING OF RABBIT FEMUR UNDER
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NEWLY DEVELOPED TITANIUM ALLOY

25 January 2023

Major in Mechanical Engineering

Graduate School of Science and Engineering

Kindai University

Norain Binti Abdullah

Declaration

I hereby declare that this dissertation with titled “Experimental study on bone healing of rabbit femur under implant plate fixation using a newly developed titanium alloy” has been composed solely by myself and that it has not been submitted, in whole or in part, in any university for any degree previously.

I am aware and understand the university’s policy on plagiarism and I certify that this dissertation is my own work, except where indicated by referencing.

Norain Binti Abdullah
25 January 2023

Preface

This dissertation is an original work by me during my Ph.D. study in Mechanical Engineering Department of Kindai University, Osaka, Japan with some parts of it is a research collaboration with Biomedical Engineering Department of Kindai University. It was a tough challenge for me since the first year I started this study is in a midst of Corona pandemic. In the journey to complete this dissertation, I went through many ups and downs to execute the experiment that sometimes turns out a failure.

An experimental approach is carried out to find the properties of implant plate that is beneficial in aid to fracture healing. As generally known, titanium alloys are being used nowadays thanks to its properties such as good corrosion resistance, good biocompatibility, and low elastic modulus. The material with low elastic modulus has been gaining interests from the researchers all around the world. Here, the material originates from my laboratory, Ti-29Nb-13Ta-4.6Zr (TNTZ) is being focused and expected to be a suitable implant plate for a better bone healing outcome.

The framework of this research is focused on the investigation of bone healing under titanium implant plate during the early stage of healing. Mainly, it is divided into three main part which are implant plate preparation, animal experiment, bone evaluation. Based on the knowledge through mechanical field and biological field, discussion is made. The findings of this study can serve as a reference to design a better implant plate in future. Besides, it has been proven that by using an implant plate that have suitable flexibility, better bone healing outcomes can be achieved.

This dissertation marks the end of my study in Kindai University. I am very grateful for all the experiences and memories in Kindai university all this while.

Acknowledgement

In the name of Allah, the Most Gracious, and the Most Merciful

First and foremost, Alhamdulillah, all praises to Allah, for His guidance and showers of blessings throughout my research work to complete this dissertation. The completion of this dissertation is also thanks to the great number of people who have supported and helped me throughout this journey.

I would like to express my sincere gratitude to my supervisor, Professor Masaaki Nakai for his advice, support and encouragement that helped me from the beginning of this journey. He provided me with assistance and remarkable comments since my undergraduate study. I am thankful for his guidance and assisting me in all possible way in developing myself and my research. And thanks to the members of my laboratory for the assistances and discussions during my research's life.

I would also like to sincerely thanks Professor Ei Yamamoto that helped and guided me in experiment and gives relevance advice. My dissertation would not be progressed without his kind help from few years back. Also, thanks for his willingness to be the second examiner of this dissertation.

I would like to acknowledge Professor Kazunori Asano as the main examiner of this dissertation. I am grateful for his comments and feedbacks for this dissertation to be completed.

I want to take a chance to thanks JEES Toyota Tsusho International Student Scholarship and Iwatani Naoji Foundation for the scholarship offered to me. It really helped me a lot both physically and mentally. With the scholarships, I can afford to pay my tuition fees, and allowed me to focus on my study.

Finally, I want to thank my mother for her love, prayers, caring and sacrifices for educating me. Also, thanks to my family and friends for continuing support during my studies. To others that directly or indirectly contributed to this research, I appreciate all the kindness and thanks a lot for believing in me.

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CHAPTER 1

Introduction

1.1 Background

Human body consists of 206 bones helps to protect organ in the body and act as storage of minerals such as calcium. Bone is composed of mainly cortical bone, trabecular bone, and marrow bone. Organic and inorganic component consist of hydroxyapatite in major responsible for hardness, rigidity, and compressive strength of bone. Although bone is stiff, it is also elastic to withstand impact. Fracture of bone are normally happened when force exerted to the bone is higher than the load that it can bear. This is one of the examples of bone trauma and it needs to be healed. Although bone can heal by itself without any medication, it takes a very long times and in the worst case, irregular bone healing might happen.

Due to this, biomaterials are introduced. Biomaterials are artificial or natural materials that are used to restore or replace the loss or failure of a biological structure to recovers its form and function to improve the quality and longevity of human life. It can intervene with regeneration process of the bone in human body. Material implanted into the bone will cause local and systemic biological responses even if they are known to be inert. Materials and body responses will initiate an adaptive and reactive process. Biomaterials are used in different parts of human body as implants. Most of the implants that are being used is for spinal, hip and knee replacements. This is because human joints suffer from degenerative diseases and trauma leading to pain or loss in functions. To meet the demands of longer human life and implantation in younger patients, the development of novel metallic alloys for medical applications is aiming at providing structural materials for replacing hard human tissue.

There are many types of biomaterials that are being used as implant such as internal fixation which are pins, nails, screws and plates or external fixation by using external fixator. In terms of materials, implants are made from variety things such as plastic, ceramics and metals. Nowadays, metals are widely being used. It is due to its strength that is believed can support the body as a replacement of fractured bone. Many researchers have been investigating the materials and properties that needs to be possessed by implants to help bone heal without adverse effect [1-5]. Moreover, not only materials and its properties, but also shape, design and size are very crucial and it needs to be considered depending on the fracture site. One of the most important factors that needs to be considered first and foremost is biocompatibility. Biocompatibility means that the implant does not interact adversely with the physiological environment or vice versa. Biomaterials that are being used include stainless steel, cobalt-chromium alloys, and titanium alloys [4].

From biological point of view, for bone to heal, it requires sufficient blood supply and balance cell differentiation. By insertion of implant, regeneration of bone might be disturbed, thus changing the tissue strain level in the bone. This is directly relating to the change of bone fragments environment. On the hand, mechanical stability, weight-bearing, stress and strain, and loading conditions are the main factors that are being focused from mechanical point of view. These two concurrence factors are important and related to one another. That is why in designing a safe and suitable implant, not only mechanical but biological factors are needs to be considered severely.

1.2 Research motivation

Conventionally, stainless steel and cobalt-chromium alloys were being used as biomaterials. However, nowadays titanium alloys are generally being used. That is mostly because of the needs for long term implantation with no allergic reactions. Stainless steel is not suitable for a permanent implant because of its poor strength and liability undergo plastic deformation, poor corrosion resistance and commonly used for non-permanent implant. On the other hand, cobalt-chromium alloys have better corrosion resistance, but ion release during in vivo (chromium, nickel) are carcinogens while cobalt is a suspected carcinogen.

As that being said, present study chooses titanium alloys as implant plate because it has good biocompatibility, good corrosive resistance, and possesses low elastic modulus are said to be a good implant for long-term implantation. Many researchers stated that implant with low elastic modulus can prevent stress shielding phenomenon which happen after long-term implantation [6-9]. Stress shielding refers to the reduction in bone density because of removal of typical stress from the bone by an implant. This phenomenon happened when there is elastic modulus difference between implant and bone in the body. Due to this, imbalance osteoclast and osteoblast production that leads to the disruption of the balance between bone resorption and bone formation, and thus causes the bone to loss its mass and eventually become malfunction.

Present study selected experimental approach by animal study in rabbit using implant plate. The materials chosen for implant plates are generally used Ti-6Al-4V ELI (Ti-64) and newly developed Ti-29Nb-13Ta-4.6Zr (TNTZ). Ti-64 alloy is indeed has low elastic modulus when compared to conventional biomaterials, but The TNTZ alloy is originally developed in our laboratory with further low elastic modulus than Ti-64 and expected to be suitable biomaterial for long-term implantation. Although stress shielding after long-term implantation needs to be avoided, the fundamental of bone healing especially on the early stage of healing is beneficial to be studied. Other factors such as stiffness, mechanical stability and so on also needs to be considered. This is because, previous studies stated that early stage of healing is a crucial stage which can be affect the bone healing entirely a in healing time and bone properties. Hence, present study focusses mainly on the early stage of healing to better understanding the healing and factors that is required to achieve successful bone healing. In fact, previous studies discussed through computational analysis and described about the cell differentiation during bone healing which happened on the early stage of healing [10-14].

Although many studies mentioned stress shielding, the early stage of healing has not been investigated properly. To the best of our knowledge, most of the findings are based on computational analysis or using cadaver experiment. Therefore, an experimental investigation about early stage of healing under implant plate fixation are required for better understanding to design an implant plate with suitable characteristic, and thus achieve an ideal bone healing.

1.3 Objective

As per discussed in previous section, for bone to heal, implant plate fixation properties are important to be focused on. This means that the fixation must be ideal to aid the bone healing process. However, during the early stage of bone healing, the beneficial properties of implant plate, the required factor of mechanical system, and implant plate fixation effect are not properly discussed until now. Therefore, the main objectives of present study are as follows:

1. To investigate the effects of different titanium alloys fixations on bone formation [15]

2. To investigate the implant plate stiffness influence on the mechanical stability of the fixation system at the fracture site and new bone formation [16]

1.4 Structure of the dissertation

Chapter 1: Introduction
In this chapter, the overview of this study which described the study background and motivation of this research. Here, the problems and focus regarding implant plate fixation along with bone healing nowadays are being presented. Through this, the objective of the study is explained.

Chapter 2: Literature Review
This chapter provides the knowledge on bone, fracture healing, the implant plate fixation characterization, bone healing outcomes, analysis of problems and factors that can be linked to ensure the successful bone healing. Here, a few factors are being discussed to help in better understanding of this study.

Chapter 3: Research Methodology
This chapter contained the approaches that are being used in this study to achieve the aim provided. It is mainly divided into three parts which are sample preparation, animal experiment and bone evaluation. Each part will be described precisely in understandable manner.

Chapter 4: Results and Discussion

In this chapter, the findings of this study are explained following each objective in orderly manner. The effect of using implant plate with different elastic modulus is discussed, followed by further less thickness of each implant plate in order of stiffness are focused.

Chapter 5: Conclusion

This chapter described the summary about the outcomes of this study. The limitations and proposed future works are also shown in the end of this chapter.

Appendix

The appendix included the machines that were being used, list of reagents and staining protocol, list of figures and list of tables. The list of publications and research activities also recorded afterwards.

CHAPTER 2

Literature reviews

2.1 Bone formation

Ossification is a process which new bone is form. Bone continues to grow with ongoing process of resorption and formation throughout the lifetime (Fig. 1). In a normal process, bone resorption and bone formation occur in a balance cycle and continue. Bone cells consists of osteocytes, osteoclasts, and osteoblasts. Osteocytes are inactive osteoblasts that have become trapped in the bone that have been created. Osteoblasts are responsible to make a new bone and repairing the older ones. Osteoclasts are large cells with more than one nucleus, breakdown bone, release enzymes and acids to dissolve minerals in bone through a process called resorption and help to remodel bone. Bone in the resting state consists of many osteocytes. The bone is then resorbed by osteoclasts. Then, osteocytes will be triggered to produce osteoblasts to from a new bone. There are two types of ossifications which are endochondral ossification and intermembranous ossification. All flat bone are forms through intramembranous ossifications, while others are forms through endochondral ossifications.

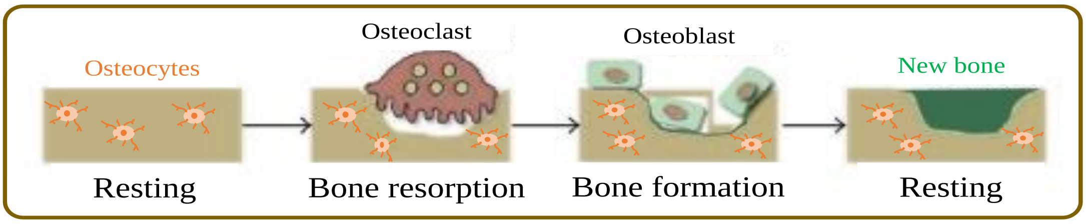


Fig. 1 Bone growth.

Endochondral ossification is the development of bone from hyaline cartilage. As an examples, in long bones, chondrocytes from a template of hyaline cartilage diaphysis. Responding to complex development signals, the matrix begins to calcify. This calcification prevents diffusion of nutrients into the matrix, resulted in chondrocytes dying and the opening of cavities in the diaphysis cartilage. Blood vessels invade the cavities, and osteoclast and osteoblast modify the calcifies cartilage into spongy bone. Osteoclasts then breakdown some of the spongy bone to create marrow or medullary cavity in the center of the bone. The first site of the ossification is on the middle of the diaphysis, while secondary ossification from in the epiphyses that forms spongy bone.

Intramembranous ossification is the development of bone through fibrous membranes. Ossifications begins as mesenchymal cells from a template of the future bone. They then differentiate into osteoblasts at the ossification center. Osteoblasts secretes the extracellular matrix and deposit calcium, which hardens the matrix. The osteoid continues to form around blood vessels, forming a spongy bone. It will then be remodeled into a thin layer of compact bone on the surface of the spongy bone. Lastly, compact bone develops to the trabecular bone, and crowded blood vessels condense into red marrow.

2.2 Fracture healing

Bone is known as a tissue that heals without scars. Fracture healing is a complex biological process that involves bone development and tissue regeneration. Generally, fracture healing is divided into primary healing and secondary healing (Fig. 2) [14, 17-24].

Primary or direct healing happens where there is rigid fixation which offers mechanically stable conditions. This type of healing is does not commonly occur in the natural process. This is possible when the bone fragments are in direct contact. Primary healing happens without the formation of callus. During primary healing, it often relates to intramembranous ossification which osteoblasts form through mesenchymal stem cell, and then becomes bone tissue. In a small fracture gap, or strain level of below 2%, primary healing occurs in aid of rigid fixation [14]. It is a slow process of fracture healing.

On the other hand, secondary or indirect healing is a process which consists of four overlapping stages and relates to the formation of callus [23-26]. It occurs when there is a movement at the fracture site. This is natural healing process and the most common fracture healing process. Secondary healing includes the process of intramembranous and endochondral ossifications depends on strain and vascularity level at different area of fracture sites [14-27]. The cartilage is formed, calcified, and then replaced by bone. It does not require a rigid fixation and enhanced by micromotion and weight bearing activities. Here, mechanical stimuli play an important role. If a good mechanical environment is achieved, fracture healing can be promoted. Otherwise, delayed healing or non-union may happen. To further described the secondary healing, four overlapping stages are explained in the next sections.

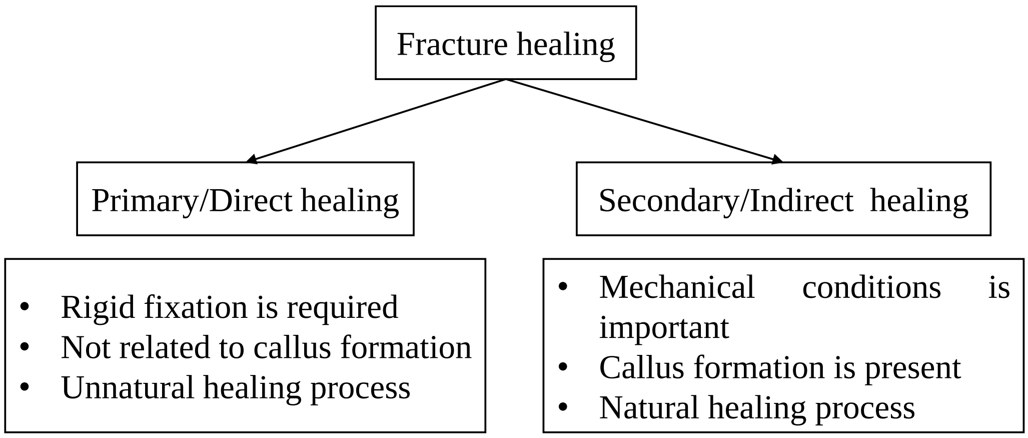


Fig. 2 Fracture healing through primary and secondary healing.

2.2.1 Secondary healing

As per mentioned in previous section, secondary healing consists of four overlapping stages which are inflammatory phase, reparative phase that divided into soft callus formation and hard callus formation, and remodeling phase (Fig. 3) [21, 23, 26]. First, when fracture happened, bone, blood vessels and soft tissues are destructed, thus starts the inflammatory phase. This begins with the formation of hematoma. The inflammatory cells, macrophages, and endothelial cells are migrated to the fracture region. This will then form a blood clot that turns into granulation tissue.

Second, reparative phase. It has been said in other publication that this phase is crucial in determining the successfulness of fracture healing. Here, mechanical stimuli play an important role. On this phase, the granulation tissue will either turns into chondrocytes or fibroblasts. In general, fracture often induces certain level of mechanical instability and heal through endochondral ossification. Through this, chondroblasts will form cartilaginous matrix. However, if the cartilage is not enough, fibroblasts will replace those regions with fibrous tissue. These cartilaginous matrixes will develop into soft callus.

Third, as high osteoblasts activity occurs, soft callus is gradually replaced by hard callus. This hard callus is more mineralized which changed of cartilage to woven bone happened in this stage. Mineralization of soft callus is said to begin from fragment ends towards the center of fracture site. Above all, reparative phase which includes soft callus formation and hard callus formation are depending on the mechanical stability. In some cases, both intramembranous and endochondral ossification happen simultaneously in different region of fracture depends on the strain level, oxygen level and so on [25, 28].

Forth, remodeling phase consists of the resorption and formation of bone. Bone resorption by osteoclasts happens on the bone that is poorly arranged, while lamellar bone is formed by osteoblasts. Indeed, remodeling takes the longest times to gain the original shape and strength of bone. This also differs between species, size, and age of animals [29]. The remodeling process will result in a successful fracture healing if there is sufficient mechanical environment around the fracture site, but if the environment is not favorable, eventually bone healing can resulted in non-union.

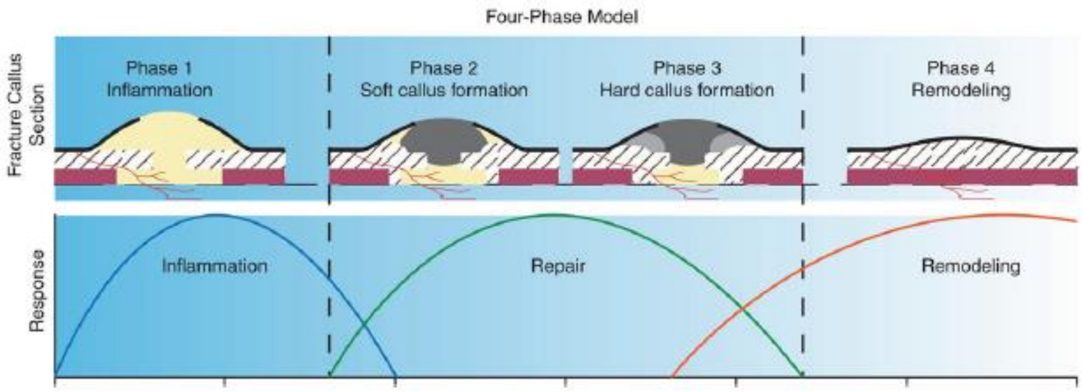


Fig. 3 Four overlapping stages of secondary healing [21].

2.3 Metallic biomaterials

Metallic biomaterials have been used for a long time. However, there is still ongoing research to obtain a metal that is ideal to be used in accelerating the fracture healing. As internal fixation, stainless steel and cobalt-chromium alloy have been used conventionally. It possesses elastic modulus of 200 [GPa] and 220 [GPa], respectively [3, 4]. Due to its high elastic modulus, it has been said that in long term implantation, it triggers the stress shielding phenomenon. Stress shielding phenomenon is a removal of certain stress from bone due to the insertion of implant plate in the body (Fig. 4). This is because of the elastic modulus difference between metallics biomaterial and the bone (10-30 [GPa]) [3,4]. Upon the insertion of implant plate, the load transfer to implant plate will be greater than bone due to the elastic modulus difference. Then, the balance between osteoclast and osteoblast is disrupted for bone resorption and bone formation respectively, which lead to the reduction of bone mass. This is because in this case, bone resorption will be higher than bone formation. Eventually, bone will lose its function. To avoid this, a metallic biomaterial with much lower elastic modulus is introduced.

Generally, titanium alloys are being used nowadays. It mainly because of its strength, high corrosive resistance, good biocompatibility, and for sure low elastic modulus. Nowadays, Ti-6Al-4V ELI (Ti-64) alloy with elastic modulus of 110 [GPa] is being used as materials for implant plate [3, 4]. Since it has elastic modulus of about half than conventional alloys, it is expected than it can be used for long term implantation without stress shielding phenomenon. However, the used of vanadium element in the alloy has been raising concern [30, 31]. Vanadium element is a potential toxic element. Although it is not toxic in the alloy, due to implant plate wear, the possibility of ion dissolvement in the body is not favorable [30]. This can cause an adverse effect. So, the research regarding low elastic modulus alloys have been studied actively these days.

In relation to that, our laboratory introduced Ti-29Nb-13Ta-4.6Zr (TNTZ) alloy which much lower elastic modulus which nearer to that of bone (60 GPa) [3, 4]. It is expected to be used as implant plate for long term implantation without any complication as it possesses of non-toxic element and may be able to avoid stress shielding phenomenon. However, there is not much study that can support this hypothesis. It is believed that a proper investigation about the effect on bone healing, along with the bone properties during healing and so on is beneficial to be evaluated. This not only help researchers to develop a new biomaterial, but also designing a safe implant plate to be used in internal fixation, and further enhance the knowledge about the bone healing outcomes. All the metallic biomaterials mentioned above are grouped in the bar chart in Fig. 5 to show the difference in elastic modulus with respect to bone.

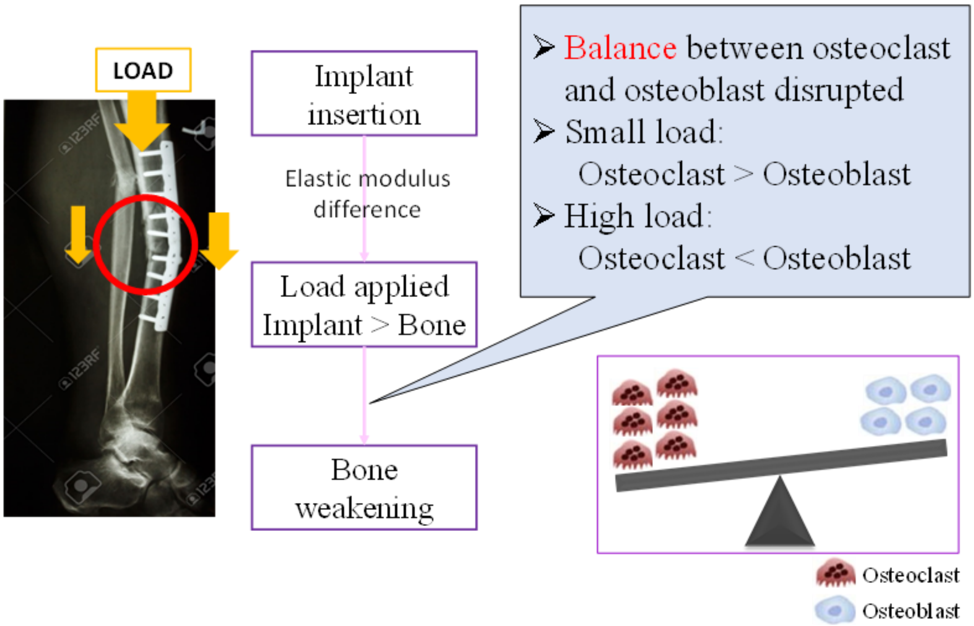


Fig. 4 Stress shielding phenomenon.

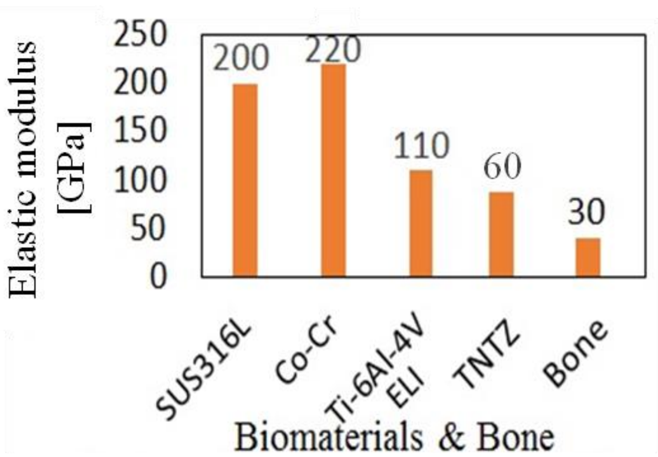


Fig. 5 Elastic modulus of bone and metallic biomaterials.

2.4 Mechanical conditions

Fracture healing through secondary or indirect healing are dependable to the mechanical conditions of the fracture site. Mechanical conditions can help the fracture to heal faster or even worst, non-union will happen. Due to this, mechanical conditions are very important to consider in designing fracture healing studies. In general, mechanical stability are often being considered. This is because mechanical stability is the basic that can trigger the interfragmentary movement of bone (IFM) distribution, mechanical stimuli, cell differentiation and so on. Many of previous studies have discussed about these factors either through computational analysis or testing in laboratory by using cadaver or artificial bone in experiment [10-14]. This is summarized in Fig. 6.

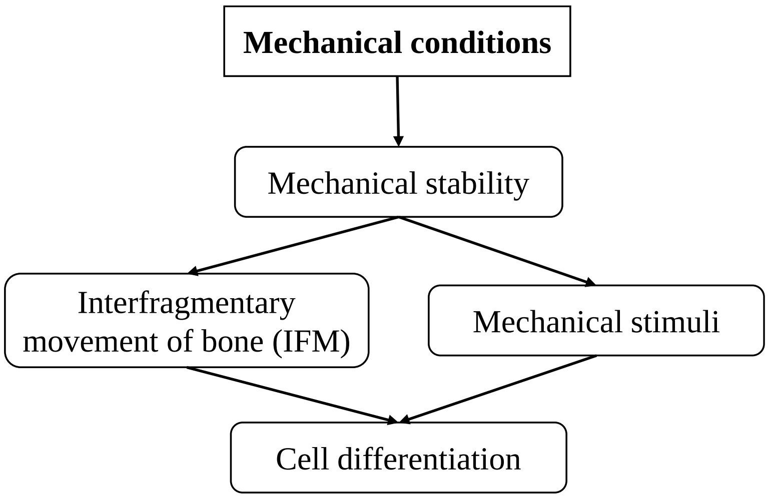


Fig. 6 Mechanical conditions related to fracture healing in summary.

First, mechanical stability is gain through a suitable fixation configuration. The fixation system should provide a suitable flexibility to ensure that mechanical stability is achieved. Based on the mechanical stability, IFM is differ and mechanical stimuli will decide the cell differentiation that is suitable. Besides, loading conditions and strain levels are also being discussed. This factor relates to the anatomy of bone itself that is either undergo endochondral or intramembranous ossification. Strain level are said to be important as it can also result is tissue distortion in a worse case situation. In general, endochondral ossification is favorable since the formation of cartilaginous callus is faster which lead to faster bone healing. However, in some cases, the combination of intramembranous and endochondral ossification is needed because there are differences in the tissue strain level. Here, the fracture site will undergo endochondral ossification, while the callus away from fracture site will undergo intramembranous ossification [21].

2.4.1 Mechanical stimuli

Previous study suggests that mechanical stimuli are affected by mechanical stability of the fixation system [32-35]. These studies tried a few combinations of fixation configuration, fracture angle, fracture gap size to describe the mechanical stimuli and its effect on the fracture healing through computational analysis. In most of the studies, the large fracture gap size has been said to be more significant to be investigated rather than small fracture gap size as it is applicable in a real situation. Hence, present study intends to consider this as a fracture model to be investigated. The mechanical stimuli are calculated using formula which is described by the relation of octahedral shear strain, γ and interstitial fluid flow, v in equation 2.1 [10-14].

$$S = \frac{\gamma}{a} + \frac{v}{b} \tag{2.1}$$

Mechanoregulation is a study of mechanical and physical conditions relationship to regulate biological responses. The study includes investigating at the organ level which is healing outcomes and tissue level which is healing progresses. From the formula in eq. 2.1, mechanical stimuli are calculated and a theory was made in relation to the cell differentiation and healing outcomes predicted (Fig. 7).

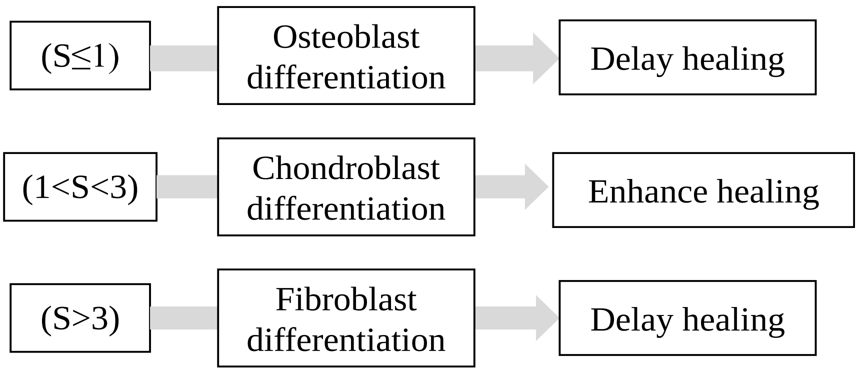


Fig. 7 Prediction of bone healing outcomes through computational analysis from previous studies [10, 13].

There are three common level of mechanical stimuli that is stated in previous studies. From Fig. 7, when the mechanical stimuli are low, osteoblast differentiation happened. During early stage of healing, osteoblast differentiation led to intramembranous ossification. Because of this, fracture healing might not be able to heal through indirect healing because to be able to heal, IFM must be kept as low as possible, or rigid fixation system need to be used. If none of this is achievable, it will result in delay healing. Previous study stated that the IFM $< 2\%$ can lead to the bone formation through intramembranous ossification, while endochondral ossification can happen when the IFM is as follow; 2% < IFM < 10% [10]. By contrast, when mechanical stimuli are high, fibroblast differentiation is likely happened. The fibroblast differentiation can form cartilaginous callus, and stabilize the fixation system, but too much fibroblast formation due to excessive strain and interstitial fluid can result in delay healing or non-union. On top of that, when chondroblast differentiation is favorable during the early stage of healing. by chondroblast differentiation, cartilaginous callus will form and follow through indirect healing. This can enhance the healing outcomes with better bone quality.

As mechanical stimuli and cell differentiation being discussed, other factors such as IFM and fixation configuration are also important to be investigated. This is because all these factors are not independent, and related to each other, especially on the early stage of fracture healing.

2.4.2 Implant plate configuration

Internal fixation such as implant plate is being used to ensure indirect healing of bone is pursued. To design a safe implant plate to be used, stiffness is one of the factors that needs to be considered. In general, too rigid fixation will suppress IFM, thus delay healing may happen. On the other hand, too flexible fixation can lead to excessive IFM which eventually resulted in overly formed callus. Therefore, a relative flexible fixation is needed to induce certain degree of IFM, thus enhanced bone healing. Previous publications often discussed IFM in relation to cell differentiation has been discussed in previous section [35, 36]. Since the implant plate is fixed on one side of bone, the IFM might not be uniform across the bone cross section. This will be a problem as non-uniform IFM can lead to non-uniform cell differentiation due to the different in mechanical stimuli between the side with implant plate and the opposite of it. Previous study refers implant plate side and opposite of implant plate side as near cortex and far cortex, respectively (Fig. 8) [12-14]. Most of studies described fracture model while cutting the bone in both near cortex and far cortex. However, present study decided to create a model with defect only on the near cortex. This is to distinguish existed bone and newly formed bone in a proper manner and to explore the fixation influence on the untouched far cortex.

Implant plate stiffness can be adjusted with many methods. As an example, using different material with the same implant plate size, i.e., stainless steel and titanium alloy. Other than that, changing the bone-plate distance, working length or even the shape or size of the implant plate with the same material. By adding or lowering the number of screws to fix the implant plate can also adjust the stiffness of implant plate. Fig. 8 shows the example of bone-plate distance and working length definition. The bone-plate distance is the distance in between the implant plate and outermost layer of cortex (near cortex), while working length is the distance between the innermost of screw on above and below the fracture gap. The fixation system will be flexible when the working length is longer and when bone-plate distance is farther.

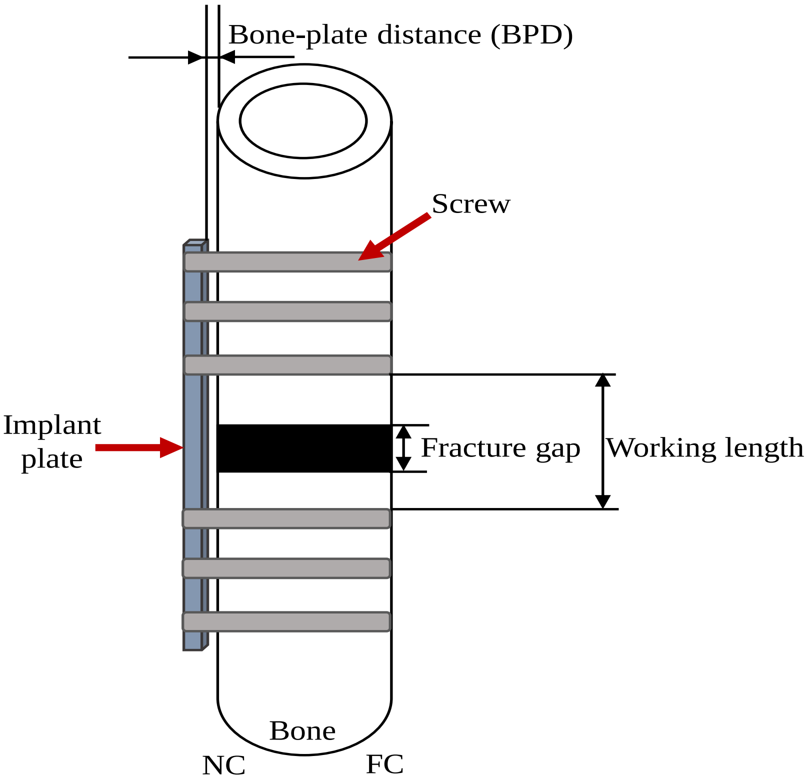


Fig. 8 A sketch diagram describing the bone-plate distance and working length.
(NC: Near cortex, FC: Far cortex)

When suitable implant plate stiffness is used, IFM in the near cortex and far cortex will be uniform. Due to this, a balance cell differentiation can be achieved. Indeed, the implant plate stiffness needed is also depending on the fracture gap size. If the fracture gap size is small, relative flexible fixation led to excessive strain at the fracture site but using the same implant plate configuration can benefit a callus formation if the fracture gap size is large [12-14]. Noted that the angle of fracture gap is also affecting the cell differentiation. Other than that, uniform IFM by relative flexible fixation can avoid the formation of asymmetric callus [37]. A uniform IFM on the bone cross section also enhances healing with better callus properties and may take shorter time to heal. On top of that, a uniform IFM guaranteed a balance cell differentiation across the bone cross section following a good mechanical stimuli to aid in an ideal bone healing through indirect healing that is preferable.

Despite, stiffness is categorized as tensile (compressive), bending and torsional. In bone healing, there is no such thing as pure stiffness. All the three stiffness are affected when loading, movement and weight bearing [38, 39]. On of top that, in long bone, tensile (compressive) and bending stiffness are said to be widely affected the bone healing. The ability of the implant plate to replace the specific amount of stiffness that is removed from the bone after fracture is important. Indeed, it is a difficult task to solve, but by adjusting the stiffness of implant plate to be near to that of fractured bone, can ensure that bone deformation when load is applied and IFM is equal to avoid bone healing failure.

Although a computational analysis has been discussed in many previous studies, it is believed that animal study is needed to be carried out. The cell differentiation might be able to be observed by staining or fluorescent coloring during animal study. However, the limitations are expected to pursue in animal study, even though it might be a better finding that can be used as basic knowledge in order to design the most suitable implant plate to be used in the future.

2.5 Bone staining protocol

Fracture healing progresses can be found on tissue level investigation such as bone staining method. There are many types of bone staining protocol that can be used which serve its purpose independently. As an example, the commonly used protocol is hematoxylin and eosin (H&E) staining, Masson-Goldner trichrome, modified Masson-Goldner trichrome, Movat's pentachrome and alcian blue staining. These staining protocols is quite different between each other in terms of observation objective, types of cells or tissue that is needed to be observed and so on. Other than that, the staining protocol is also chosen by deciding the type of cells etc., that needs to be in distinct color for easier observation to be done.

In general, to determine the bone healing progress, osteocytes, osteoblasts, and osteoclasts are being observed. Besides, the different is ossification can also be investigated using different protocol, for example, by using Movat's penachrome staining, bone healing progresses can be evaluated, also endochondral and intramembranous ossification can be distinguished. This is done by definite coloration between cartilage, mature bone, connective tissue, and nuclei [40-42]. As quantitative analysis, Masson's trichrome protocol can be used to distinguish the connective tissue and bone matrix, and then imaging software can be used to calculate the area that is needed. However, a detailed investigation of cell is quite difficult using this protocol. Overall, H&E staining protocol is widely used in cell investigation as it is the most basic staining protocol in understanding the morphology of bone tissue.

2.6 Animal selection for in vivo

In vivo is a study which is perform in the living organism. An animal with variety of size and species is being used in regards the objective of the study itself. A small animal like mice is widely being used as it is easier to handle, easy to reproduce and so on. However, in fracture healing study, larger animal is more favorable. As an example, rabbit or sheep is commonly used in tibial or femoral study, while in dental implant, dog is also being used. As an investigation regarding bone healing, either on loading conditions or fixation configurations, small or large animal can be used, but it has been stated in previous study that rabbit is the most favorable animal to be utilized because the forces that acts on the bone is more similar to the forces in human [43, 44]. Hence, animal model for fracture healing can be discussed with simple fixation configuration an easy to handle throughout the experiment.

CHAPTER 3

Research Methodology

3.1 Sample preparation

3.1.1 Materials

Present study chooses Ti-6Al-4V ELI (Ti-64) which is a generally used titanium alloy nowadays, and Ti-29Nb-13Ta-4.6Zr (TNTZ) which is a newly developed titanium alloy. The chemical compositions of both materials are shown in Table 1. Both alloys possess of different elastic modulus; Ti-64: 110 [GPa], TNTZ: 60 [GPa]. The properties of both alloys are evaluated and shown in Appendix 5. Noted that the elastic modulus is in the range of the commonly cited values. In addition to that, two types of implant thickness (1.5 [mm] and 0.5 [mm]) are also being tested. From these two factors, we calculated the stiffness of implant plate which are tensile (compression) stiffness and bending stiffness following the formula in eq. 3.1 where E is elastic modulus, A is cross section area, and L is length and eq. 3.2 where E is elastic modulus and I is moment of inertia. Then, the results were tabulated in Table 1. Since present study is carried out in long bone (femur bone), only tensile (compressive) and bending stiffness are taken into consideration. From the calculation, it is suggested that the bending stiffness is highly influenced by the change of elastic modulus and thickness of implant plate.

Table 1 The chemical compositions of Ti-6Al-4V ELI (Ti-64), and Ti-29Nb-13Ta-4.6Zr (TNTZ) [mass%].

Ti-64	Ti	Al	V	O	Fe	C	N	H
	Bal	6.155	4.070	0.106	0.175	0.022	0.003	0.003
TNTZ	Ti	Nb	Ta	Zr	O	C	N	
	Bal	29.4	13.0	4.64	0.064	0.013	0.008	

$$\text{Tensile (Compressive) stiffness} = \frac{EA}{L} \ \text{[N/m]} \tag{3.1}$$

$$\text{Bending stiffness} = EI \ \ [\text{N/m}^2] \tag{3.2}$$

Table 2 Tensile (Compressive) and bending stiffness of implant plate.

Implant plate (thickness)	Elastic modulus [GPa]	Tensile (Compressive) stiffness [N/m]	Bending stiffness [Nm^2]
TNTZ (0.5 mm)	60	$11.53x10^9$	$3.125x10^{-3}$
Ti-64 (0.5 mm)	110	$21.15x10^9$	$5.729x10^{-3}$
TNTZ (1.5 mm)	60	$34.62x10^9$	$84.38x10^{-3}$
Ti-64 (1.5 mm)	110	$63.46x10^9$	$154.7x10^{-3}$

Annealed Ti-64 alloy sheets with thickness of 2.0 mm and as-forged TNTZ cylindrical rods with diameter of 10.0 mm were received. The sample preparation steps are shown in Fig. 9.

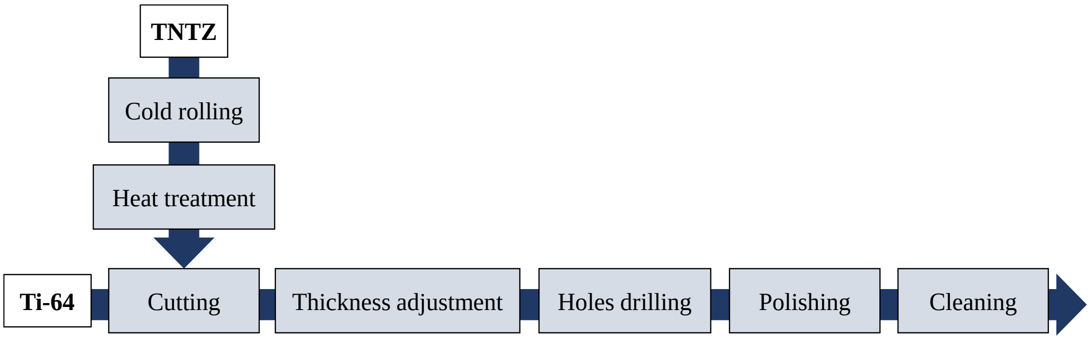


Fig. 9 Sample preparation process of Ti-64 and TNTZ implant plate.

3.1.2 Cold rolling

TNTZ alloy was received as cylindrical rod, so cold rolling was needed. Cold rolling is a process which passes metal through rollers at temperatures below its recrystallization temperature. The TNTZ rod was cut into 10.0 mm height and subjected to cold rolling. Cold rolling was carried out using rolling mills by Yoshida Kinen Co. Ltd. During overall experiment, the speed of roller and direction of plate during insertion into the roller are maintained to ensure there is no breakage or failure. The thickness of the rod was reduced by 0.1 mm per one time rolled. This action was repeated until 2.0 mm thickness of plate is ready. Fig. 10 shows the TNTZ alloy before and after cold rolling.

(a) (b)

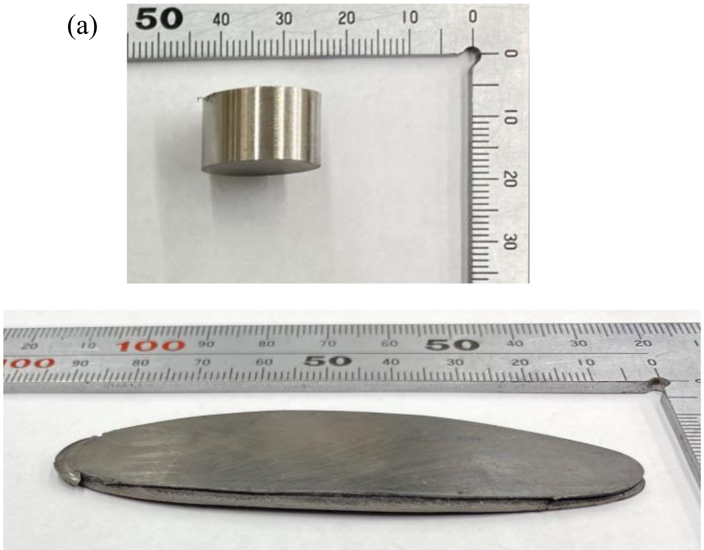


Fig. 10 The TNTZ alloy (a) before, and (b) after cold rolling.

3.1.3 Heat treatment

After cold rolling, TNTZ plate was subjected to heat treatment. Here, TNTZ plate was solution-treated to gain an initial state of crystal which is single β phase [45, 46]. This is because, deformation happens during cold rolling, which then broaden the XRD peaks. By solution treatment, recrystallization occurred along with sharpening of XRD peaks due to the relief of residual stress and strain removal after deformation. By this, the properties of TNTZ plate are believed to gain the initial properties (Refer Appendix 5.). In present study, TNTZ plate was solution-treated at temperature of 790 °C and 1 hour process in vacuum. The cooling process chosen was rapid cooling.

3.1.4 Cutting

Ti-64 plate and TNTZ plate needs to be cut down into 25.0 [mm] x 5.0 [mm] by using electric discharge machine (EDM), Fanuc Robocut α-iE series. The plate was fixed on the jig one by one. The program that is being used is shown in Fig. 11. The plate positioning was checked first to ensure no error happened during the cutting process.

Fig. 11 The program that is being used to cut both of Ti-64 and TNTZ plate.

3.1.5 Thickness adjustment

Since the plate was only cold rolled until 2.0 mm, further thickness adjustment was needed. This action can be done by using milling machine, Prospec tools PSF400. The plate was milled layer by layer with constant speed until its thickness achieved both 0.5 [mm] and 1.5 [mm]. This experiment was done one piece per time. Fig. 12 shows the setting of the plate.

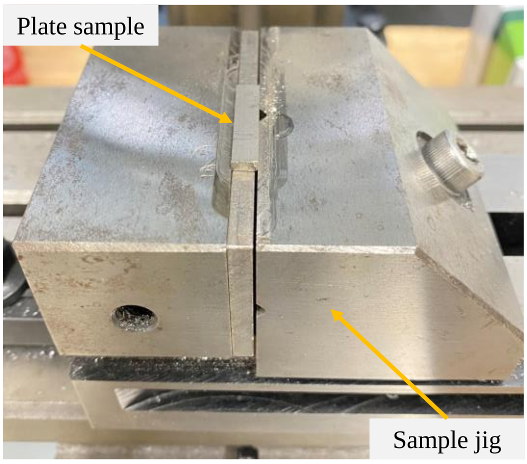


Fig. 12 The setup of plate for thickness adjustment.

3.1.6 Drilling

All the plates were prepared for drilling. The machine that was used is super high speed small hole discharge perforator from Japan Electric Discharge Machine Co. Ltd., JEM-25A. The plate was fixed on the jig and ready to drill. Each plate was drill with four holes which two holes on both end of the plate. The distance between the holes from the end of the plate is 3.0 [mm], and the holes size is 1.0 [mm]. At this point, the plates are ready as implant plate but surface finishing is needed (Fig. 13).

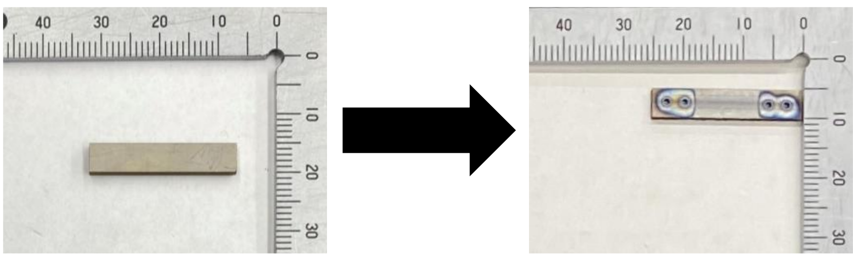


Fig. 13 Ti-64 implant plate before and after drilling.
(Noted that same method was done for TNTZ plate)

3.1.7 Polishing

The Ti-64 and TNTZ implant plates were polished by using waterproof abrasive paper with #80 grit until #800 grit. The finishing surface were made not so smooth but not too rough, with surface finishing of 0.170-0.171 [µm] for Ti-64 implant plate, and 0.220-0.294 [µm] for TNTZ implant plate. All the procedures were done manually by hand and observation was made to ensure that the surface was polished evenly. As a last step, the implant plates were immersed in acetone and place in the ultrasonic cleaning machine, which then transferred into ethanol for final cleansing. The implant plates were placed nicely in sample containers to avoid scratch and impurities. The finished products are shown in Fig. 14.

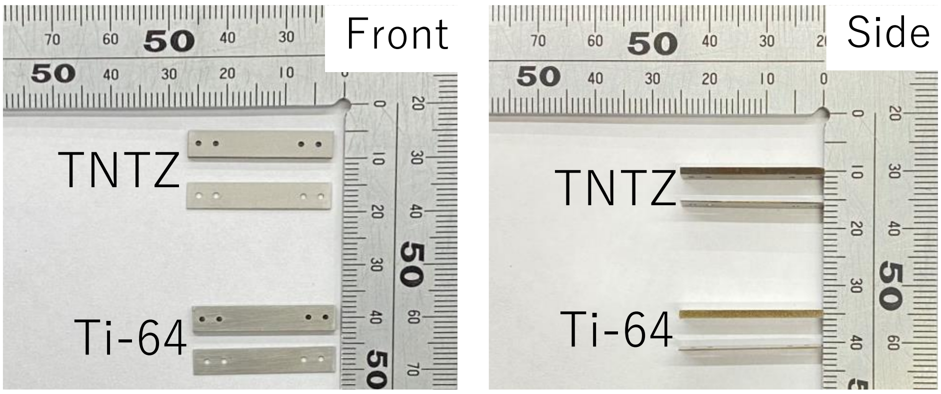


Fig. 14 Final product of Ti-64 and TNTZ implant plates for thickness of 1.5 [mm] and 0.5 [mm].

3.2 Surgical procedures

Four adult female white rabbits weighing 3.0-4.0 kg were kept in room for two weeks before surgical procedure. The rabbits were given free access to food and water throughout this period but kept away from it a day before the surgery. On the day of surgery, the rabbits were anesthetized through intravenous injection. The injection consists of three types of mixed anesthetic which are medetomidine, midazolam, and butorphanol. These anesthetics were diluted with physiological saline beforehand before administered to the rabbits. Then, the fur on the rabbit's legs were shaved until the skin was seen, followed by applying iodine on the knee joint region. Skin incision was made until femur bone was seen. Although the fracture model in most of the previous studies were carried out on near cortex and far cortex, in present study the bone defect was made by half-circular rasp with size of 5.0 [mm] x 2.0 [mm] on the near cortex only. This was done for proper comparison between new bone and existed bone, since present study not only focused on defect, but also its surrounding. The bone was drilled following the holes on implant plate, and then the screws were fixed. Refer Fig. 15

to see the fixation state. Noted that the control, sham surgery was carried out for comparison. The surgical region was rinsed with penicillin solution that is diluted with physiological saline to avoid infection before clamping the skin. After that, the skin was again applied with iodine. On the same time, nutrient was injected into the rabbit gradually to avoid sudden rise in heartbeat that might danger the rabbit. All the rabbits were then transferred to the cages again with access to food and water starting from the next day of surgery. The observation was made every day to ensure the rabbit's conditions are good and get enough food and water, until three weeks.

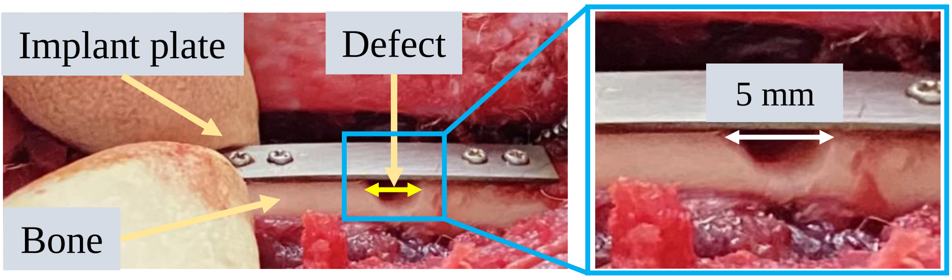


Fig. 15 Fixation configuration of rabbit femur bone using TNTZ implant plate.
(Noted that the fixation configuration is the same in Ti-64 implant plate)

3.3 Bone evaluation

3.3.1 Bone sample preparation

After three weeks of surgical procedure, femur bone was harvested (Fig. 16). Femur bone was cleansed from flesh, leaving only the bone itself. It will then immerse in either physiological saline solution or 10% formalin solution. This is because two types of bone samples need to be prepared. First, bone samples for observation and mechanical properties test. Before cutting down the bone, implant plate is removed first.

Fig. 16 Harvested bone after removal of implant plate.

Harvested femur bone which was immersed in physiological saline solution is cut down from the center of the defect with size of 4.5 [mm]. The bone region is shown in Fig. 17(a). Anterior represent defect region, posterior represent opposite of defect region, and medial and lateral represents above and below the defect region, respectively. Then, the bone sample was embedded in resin. The embedded bone sample was then polished with waterproof abrasive paper with grit of #80 until #800 for two minutes in each stage. As surface finishing, the bone sample was polished on the red felt with diamond spray of 1.0 μm particles size for about 10 to 15 minutes.

Second, bone samples for histological observation. The femur bone was cut down in the same manner and left to immersed in 10% formalin solution. After that, procedure for bone staining is being proceed and will be discussed in the next section. The prepared bone samples are shown in Fig. 17(b).

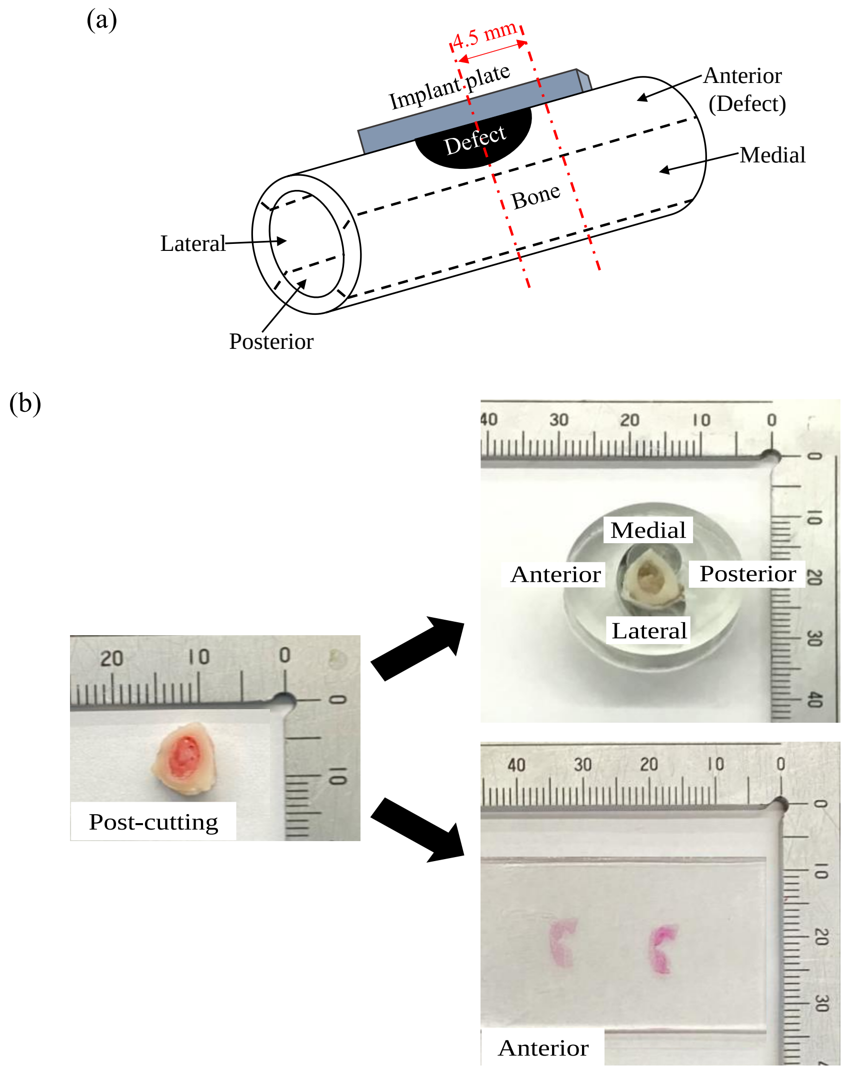


Fig. 17 (a) Sketch diagram of bone region, and
(b) bone samples for mechanical properties test and H&E staining.

3.3.2 Scanning electron microscopy (SEM)

The bone sample which is embedded in the resin is being used for bone cross-sections observation by scanning electron microscopy (SEM) using Hitachi Miniscope TM3030. Since the bone sample is embedded in the resin, carbon coating was performed post-polishing, before beginning the observation. The machine that is being used is CADE carbon coating machine by Meiwafosis Co. Ltd. The observation was performed using x30 magnification but since the bone cross-sections cannot be captured entirely at one time, this step is repeated for a few times. Present study used SEM to observe callus and bone, thus distinguish the newly formed bone and existed bone.

3.3.3 Hematoxylin & Eosin staining (H&E)

The procedure H&E staining was described in Fig. 18(a). The bone sample was immersed in small container (Fig. 18(b)). For sampling, there are two main parts. First, preparation which the protocol that is to prepare the bone sample before decalcification. The bone sample which was immersed in 10% formalin solution is being used in this experiment. The immersion was continuous for a week. After that, phosphate buffered saline (PBS) solution for 1 hours. The bone sample was then immersed in 70% ethanol for 30 minutes and followed by immersion in 100% ethanol for about one week. All immersions were performed in room temperature except for 100% ethanol which done in 4 ℃.

Second, decalcification. Before started, the bone sample was cleansed with PBS solution, which then immersed in decalcifying solution (G-Chelate Mild) for about a month in 4°C. The decalcifying solution is changed every day in the first week and 2 to 3 times a week in following weeks. After bone is decalcified, the bone sample will then be cleansed using PBS solution for 1.5 hours in room temperature. To prepare the bone sample before going to paraffin embedding, the bone sample was first immersed in 70% ethanol in 4°C for a few days, then immersed in 100% ethanol for one hours, followed by 3 hours in room temperature and lastly overnight in 4°C.

For paraffin embedding, the bone sample is first placed in the automated machine for resin embedding sample preparation. After that, liquid paraffin was poured into a small case contained bone sample and left until harden. The bone sample was then sliced for about 5 μm in size and attach to the glass slide. After it dry, the glass slide with sliced bone sample is ready to be stained. The bone sample is stack in the small rack, then immersed following the H&E staining protocol (Fig. 19, Fig. 20). Each procedure was carried out with independent time frame.

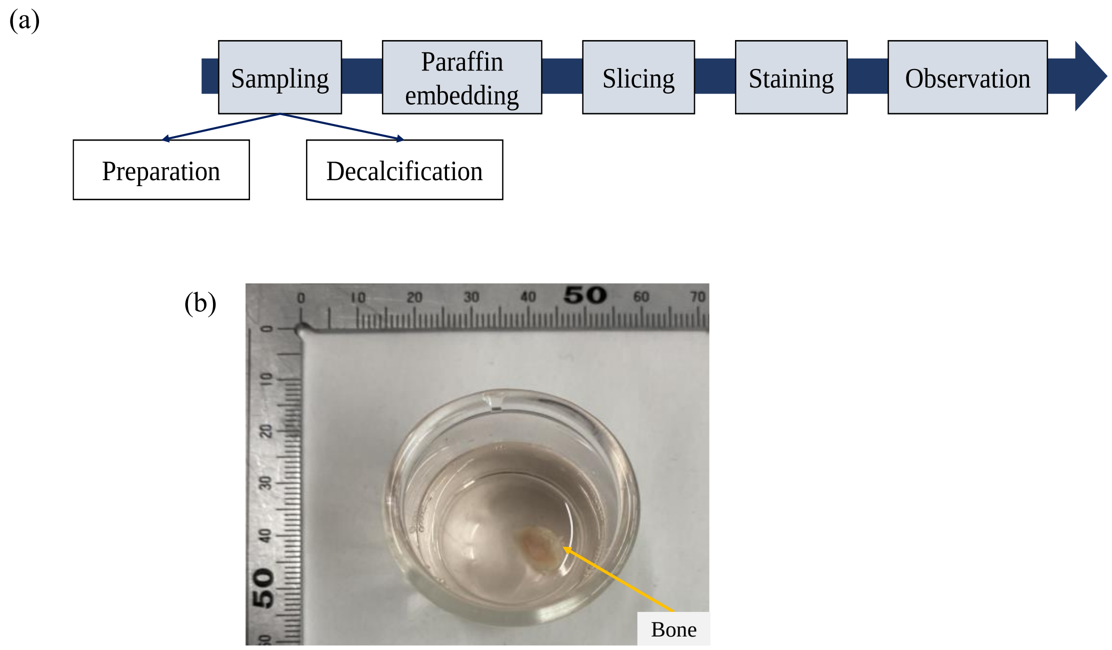


Fig. 18 (a) The staining procedure for bone sample, and (b) Bone sample immersed in 10% formalin.

Fig. 19 Glass slide stacked in the rack for staining protocol.

Fig. 20 H&E staining procedure.

H&E-stained sample are observed by using all-in-one Fluorescent Microscope, Keyence BZ-X series. Firstly, full image of bone sample is captured with magnification x10. After that, the observation was done with much detailed view especially on the anterior or defect region. This method is being used to observe not only the bone, but also soft tissue presents during healing. Compared to SEM micrograph, bone progression can be seen through staining method due to the existence of different types of bone during maturation.

3.3.4 Image analysis

Image analysis was carried out by using ImageJ software. There are two parts of image analysis which is the SEM micrographs is being used to calculate the area of callus and bone, while the H&E-stained micrograph is being used to calculate the area of soft tissue presents.

First, the SEM micrograph will be trimmed into callus and bone separately as shown in Fig. 21(a). Then, the scale was set to avoid miscalculation. The area of callus on anterior or defect region, and non-defect region (posterior, medial, lateral) are calculated separately. All the results are accumulated, then plotted in the bar chart. The SEM micrograph is chosen as it is easier to distinguish the callus and bone for proper measurement.

Second, by using the H&E-stained micrograph, the soft tissue, cancellous-like bone, and callus were trimmed into three images (Fig. 21(b)). Then, the scale was set, the threshold of images is adjusted, and the area was measured independently. The data was used to plot a bar chart. This area is used to discuss the bone progression of healing. It will be further described in 4.4.1.

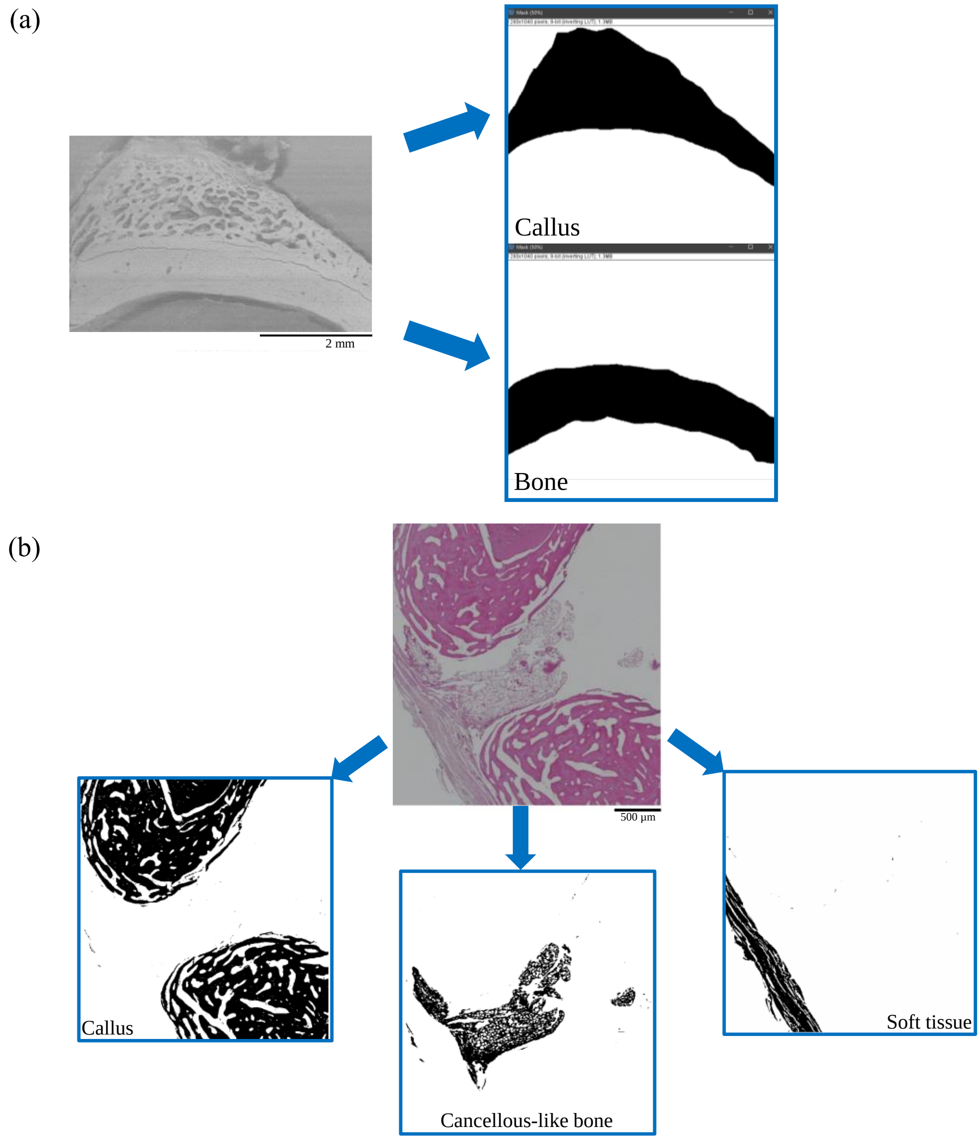
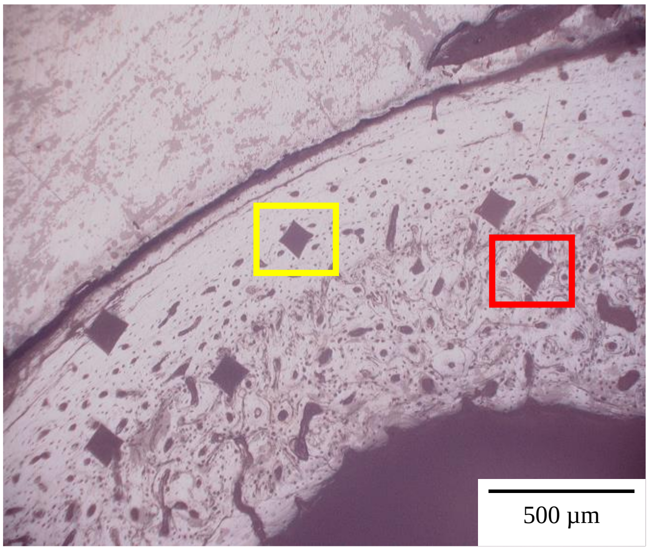


Fig. 21 Example on trimming of (a) SEM micrograph into separate part of callus and bone, and (b) H&E-stained micrograph into three types of bone: soft tissue, cancellous-like bone, callus.

3.3.5 Vickers hardness

By using the same sample as in SEM observation, Vickers hardness was performed with Mitutoyo Hardness Testing machine. The bone cross-section was divided into four parts. On the bone structure, a total of 40 indentations are performed, while a minimum of 10 indentations were carried out on callus. The indentations were shown in Fig. 22(a) and (b). The hardness of both bone and callus are recorded, then the average was calculated and plotted in bar chart.

(a)



(b)

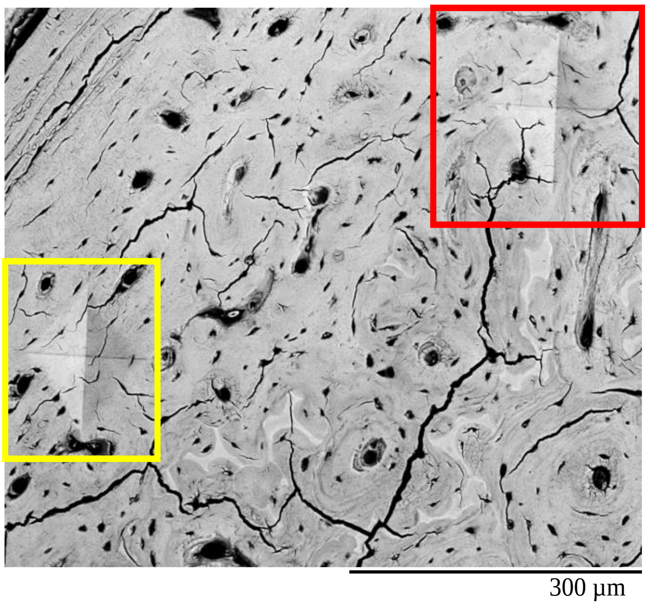


Fig. 22 The indentations performed on bone observed by (a) light microscope, and (b) SEM.

CHAPTER 4

Results and Discussion

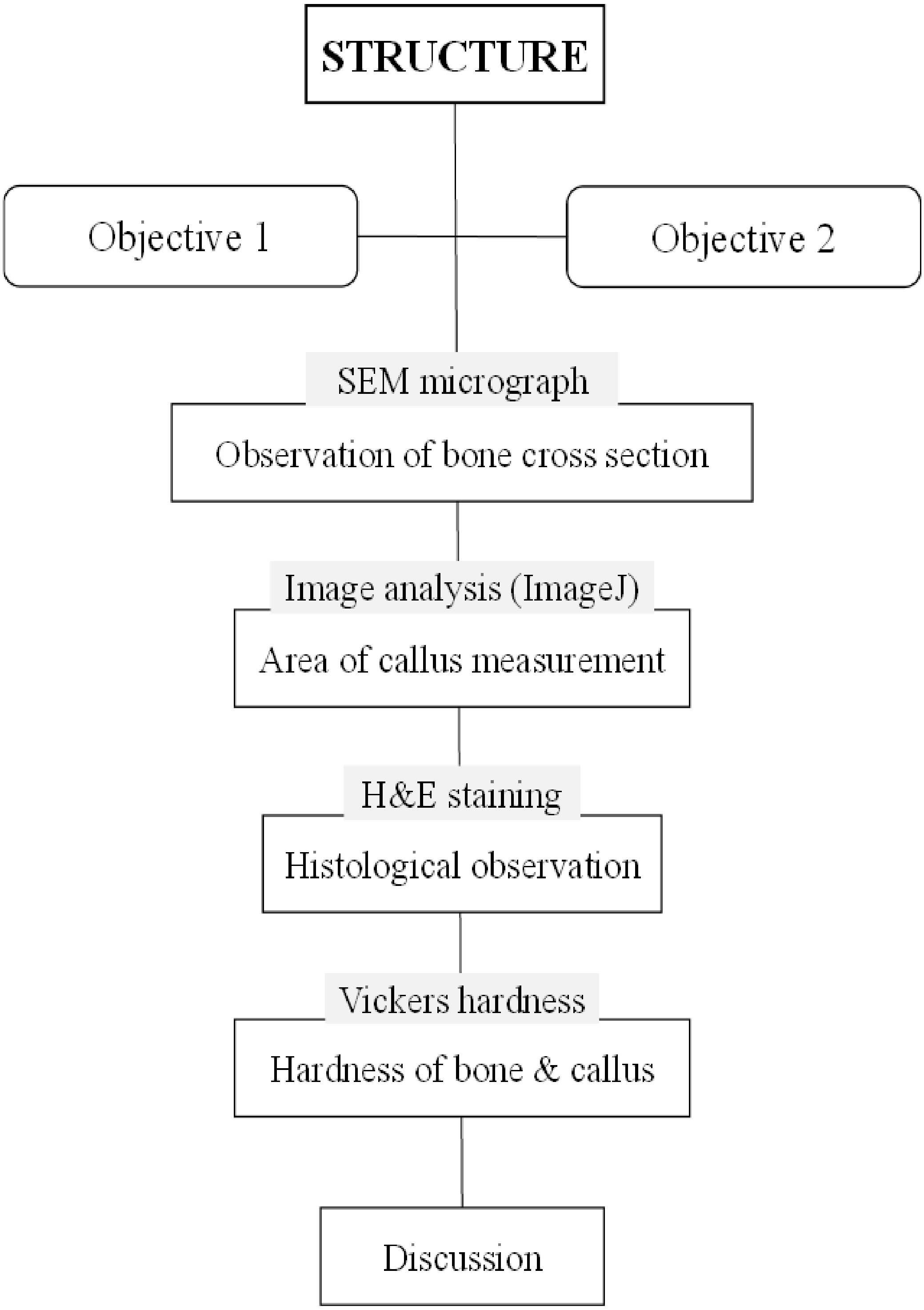


Fig. 23 The chart showing the structure of this chapter.

4.1 Overview

Present study is conducted on rabbit femur bone by using implant plate with different elastic modulus such as Ti-64 and TNTZ. The findings will be first discussed following Objective 1 which focusing on elastic modulus difference. Further, by using the same types of implant plate, the thickness of each implant plate is changed into a thinner implant plate to reduce the stiffness, and then compared with the thicker implant plate following Objective 2. As summary, results and discussion will be explained as summarize in the previous page. The results will be divided in two parts following Objective 1 and Objective 2 that refers to the first and second publications. Then, discussion will be made, altogether.

4.2 Elastic modulus difference (Objective 1)

In these sections, bone evaluations after the fixation of Ti-64 and TNTZ implant plates with thickness of 1.5 [mm] will be discussed. The discussion is based on elastic modulus difference between these two implant plates. The elastic modulus of TNTZ is double from Ti-64 alloy. When compared to bone, elastic modulus of TNTZ is double, while Ti-64 is about four times higher. Through this, the new bone formation is observed and its properties was evaluated.

4.2.1 Bone cross section observation

Bone cross section are divided into anterior, posterior, medial and lateral when SEM micrographs were taken. The bone cross section after fixation when using Ti-64 implant plate is shown in Fig. 24, Fig. 26. Fig. 28, and Fig. 30 and TNTZ implant plate is shown in Fig. 25, Fig. 27, Fig. 29, and Fig. 31. When looking at anterior or defect region, although there are four samples that are being observed, it can be said that both fixations show almost similar new bone formation (Fig. 24 and Fig. 25). The new bone that is formed on defect region has a porous-like structure referred as callus. Since there is some defect that are left yet, it can be said that the bone formation is still in progress. This may be due to the short period of healing, which is only three weeks, can be said as early stage of healing. Here, the bone is still in regeneration stage.

Besides, as being observed in posterior or opposite of defect region, the bone that is fixed with Ti-64 implant plate shows callus appearance in most of the bone samples (Fig. 26). The callus is formed in the outermost layer of the cortex. By contrast, when using TNTZ implant plate, it can be said that there is no callus appearance, except in one sample (Fig. 27). Overall, as per observed, callus appearance in the bone when using Ti-64 implant plate is larger, and it only occurs in the outermost layer of cortex. On the other hand, in the inner layer of the bone cross -section, there are hollow-like structure that can be seen when fixation using Ti-64 implant plate. Indeed, this structure also can be seen in some of the bone cross section when fixation using TNTZ implant plate.

Furthermore, when looking at the SEM micrographs of the medial bone cross section, callus is formed in both fixations (Fig. 28 and Fig. 29). On the other hand, distal of bone cross section also shows the callus appearance on the outermost layer of cortex for both fixations (Fig. 30 and Fig. 31). When compared to posterior, it can be said that the callus formation is larger. From these micrographs, it is suggested that the callus formation is larger when fixation using Ti-64 implant plate, when compared to TNTZ implant plate. These micrographs were then being used for image analysis to measure the area quantitatively.

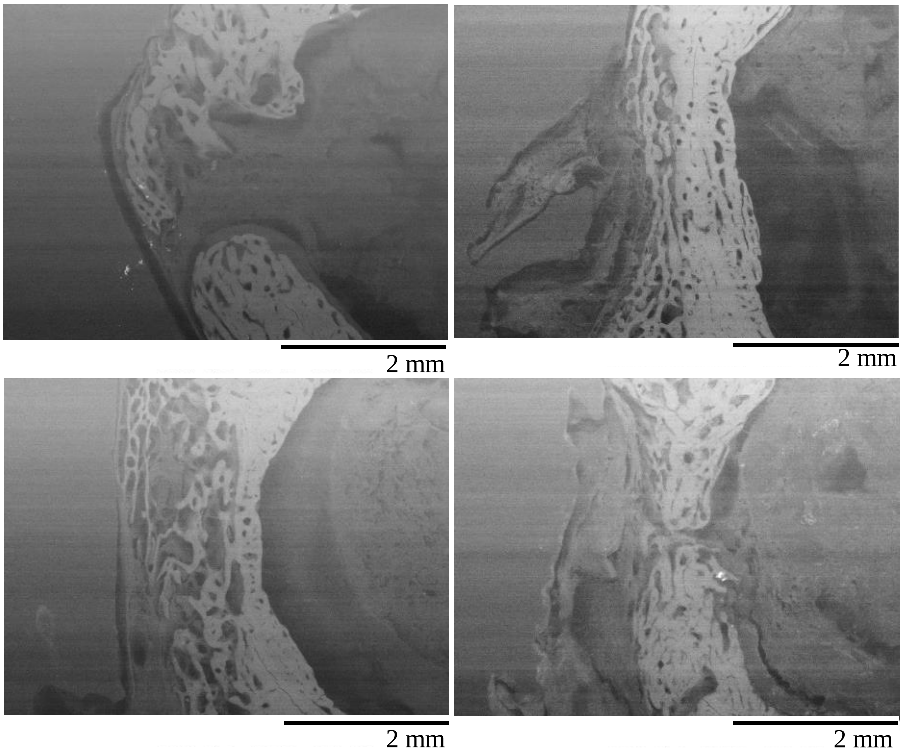


Fig. 24 SEM micrographs of anterior or defect region under the fixation when using Ti-64 with thickness of 1.5 [mm]. (n=1,2,3,4)

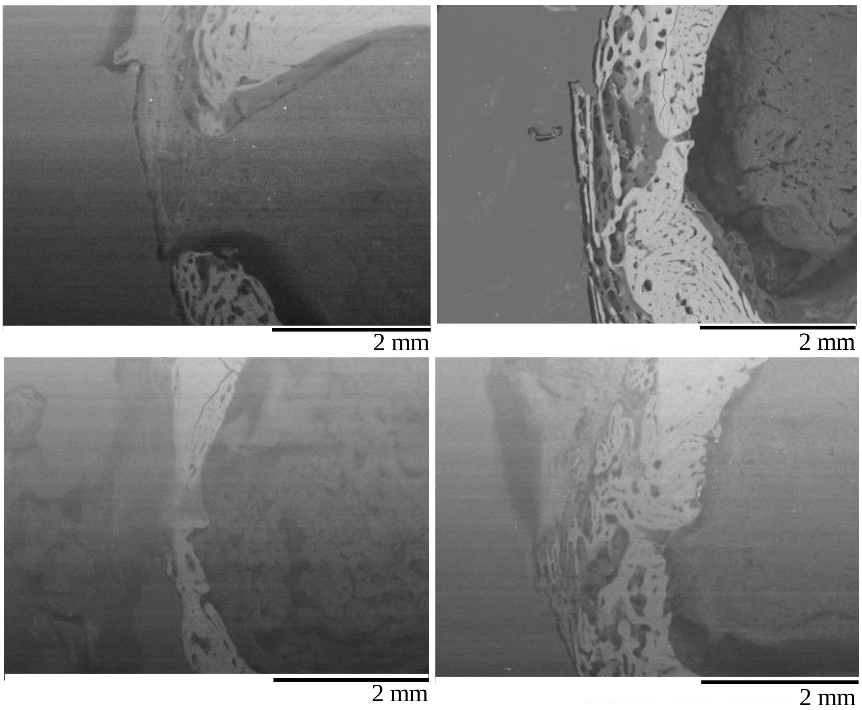


Fig. 25 SEM micrographs of anterior or defect region under the fixation when using TNTZ with thickness of 1.5 [mm]. (n=1,2,3,4)

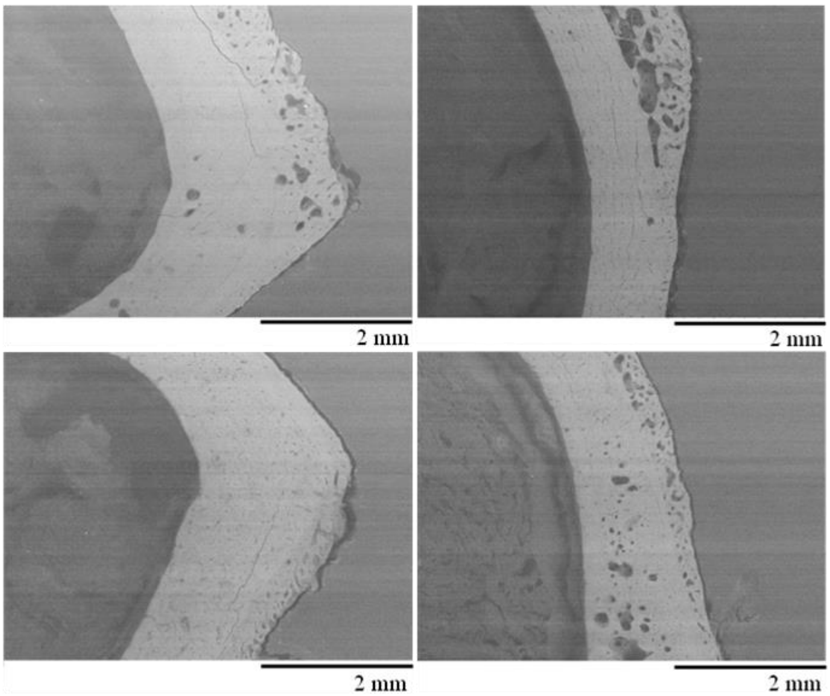


Fig. 26 SEM micrographs of posterior or opposite of defect region under the fixation when using Ti-64 with thickness of 1.5 [mm]. (n=1,2,3,4)

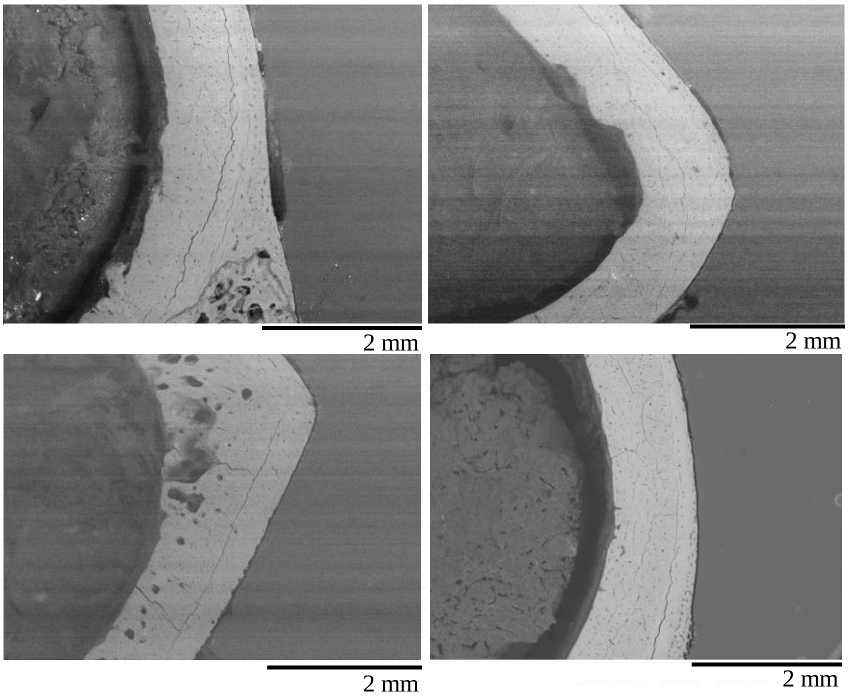


Fig. 27 SEM micrographs of posterior or opposite of defect region under the fixation when using TNTZ with thickness of 1.5 [mm]. (n=1,2,3,4)

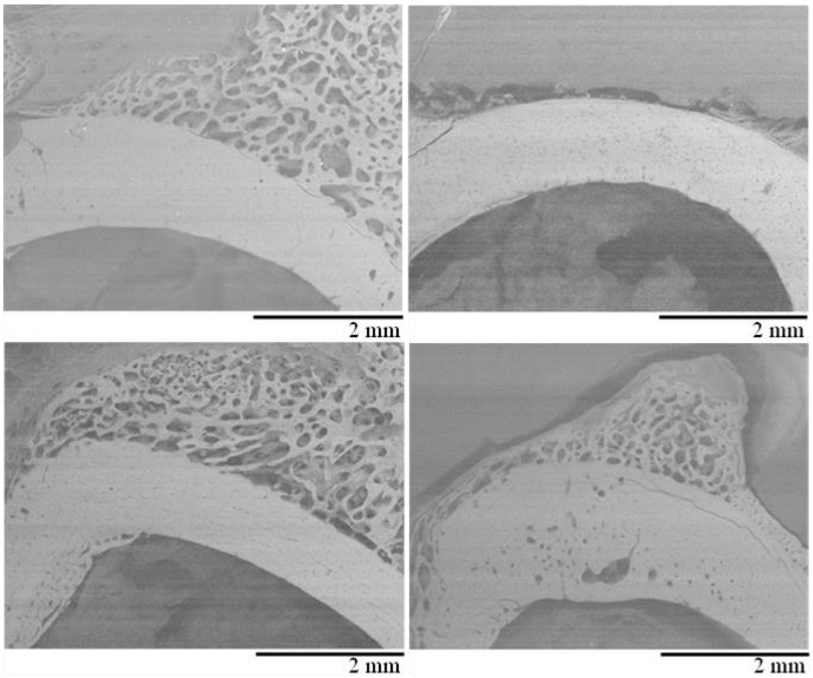


Fig. 28 SEM micrographs of medial or above the defect region under the fixation when using Ti-64 with thickness of 1.5 [mm]. (n=1,2,3,4)

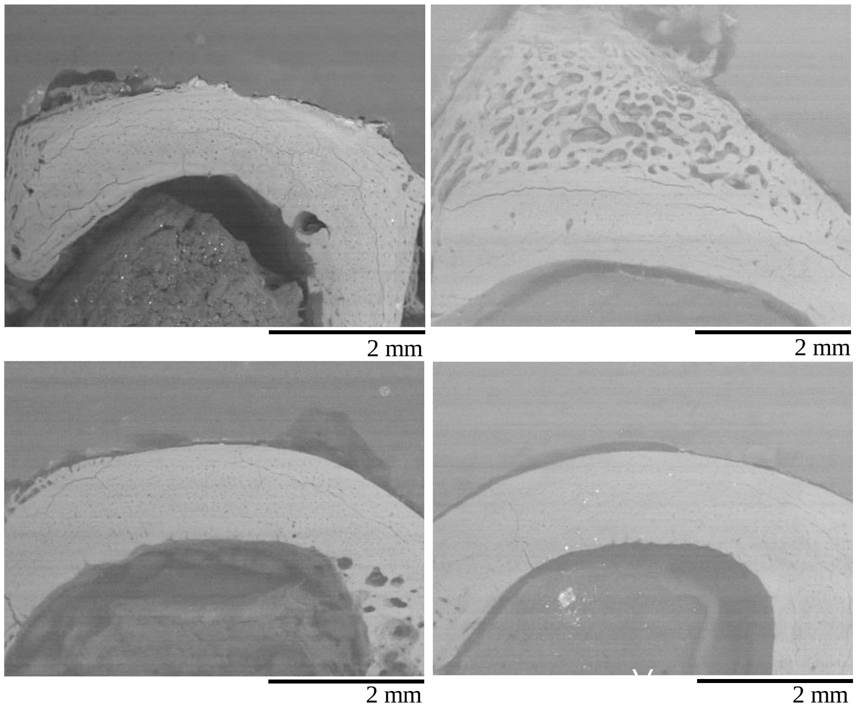


Fig. 29 SEM micrographs of medial or above the defect region under the fixation when using TNTZ with thickness of 1.5 [mm]. (n=1,2,3,4)

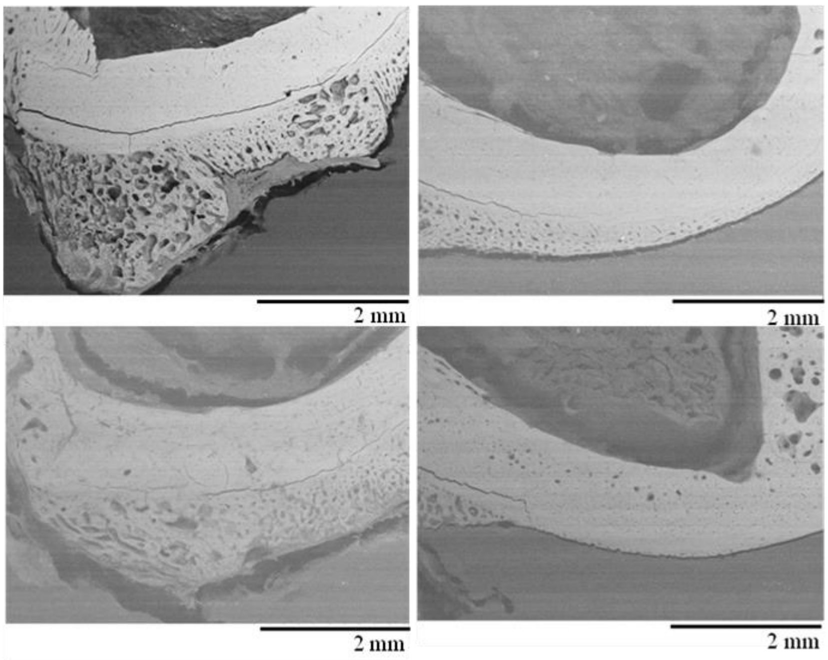


Fig. 30 SEM micrographs of lateral or below the defect region under the fixation when using Ti-64 with thickness of 1.5 [mm]. (n=1,2,3,4)

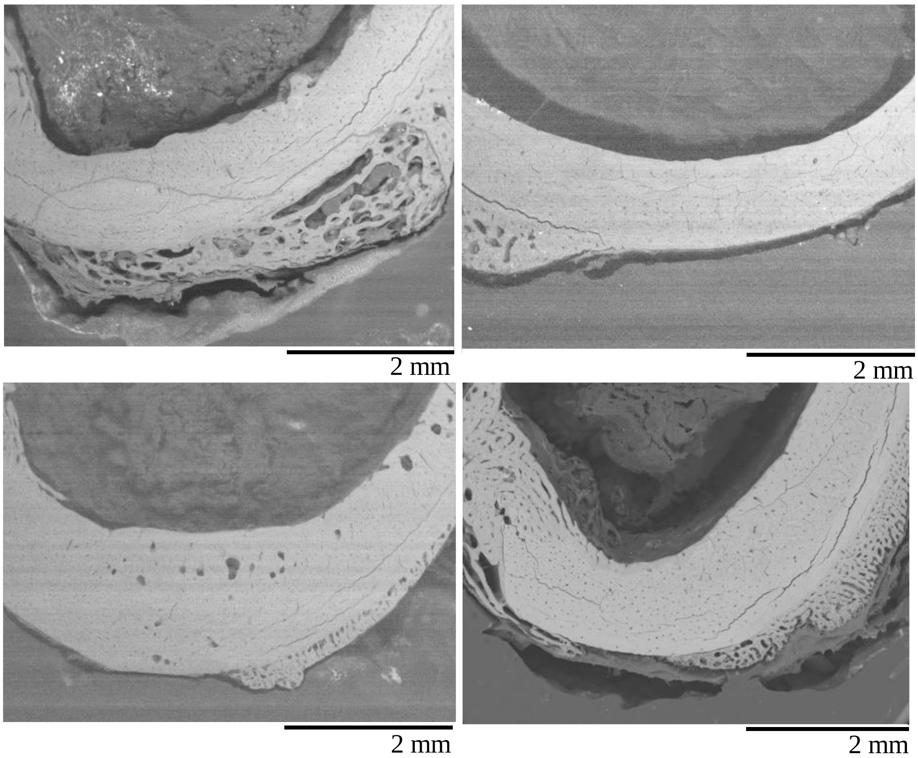


Fig. 31 SEM micrographs of lateral or below the defect region under the fixation when using TNTZ with thickness of 1.5 [mm]. (n=1,2,3,4)

4.2.2 Image analysis

SEM micrographs are being used in image analysis by using ImageJ software. Fig. 32 shows the average of total area of callus and bone for both fixations. Here, the callus on the anterior or defect region is almost similar in both fixations. However, the callus on the non-defect regions which are posterior, medial, and lateral were larger in the fixation when using Ti-64 implant plate than when fixation using TNTZ implant plate. Although the callus formed on the outermost layer of cortex is larger in fixation when using Ti-64 implant plate, new bone formation in defect region is almost similar under both fixations. Hence, it is suggested that larger callus formation does not promote the new bone formation in defect region. This is because TNTZ implant plate led to a similar new bone formation in the defect region, but it can suppress the callus formation in other regions. On top of that, the average of total callus area under fixation when using Ti-64 implant plate was nearly threefold larger than fixation when using TNTZ implant plate. As note, total bone area that was calculated in both implant plates are practically identical.

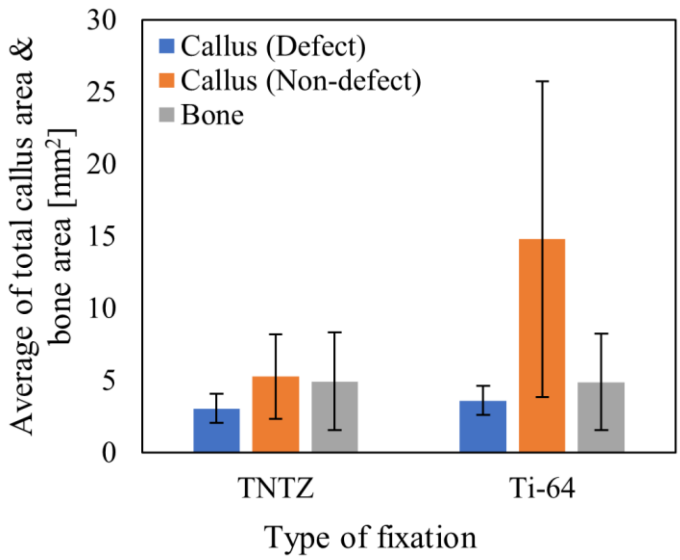


Fig. 3 The average of the total callus area and bone area under the fixation when using Ti-64 and TNTZ implant plate with thickness of 1.5 [mm].

4.2.3 Histological observation

Through histological observations, bone maturation was investigated. Here, hematoxylin and eosin (H&E) staining procedure was carried out on the bone cross section. In this experiment, bone types on the anterior or defect region are being focused. A shown in Fig. 33, different implant plate led to different types of bone structure that filled the defect region. When fixation using Ti-64 was performed, the defect was mostly filled with soft tissues and porous structure bone (Fig. 32(a)). On the other hand, fixation when using TNTZ implant plate led to the formation of mostly cancellous-like bone and callus (Fig. 33(b)). On top of that, the bone nearest to the defect also has porous structure which suggests that bone density is lower than under the fixation when using TNTZ implant plate. Although the new bone formation has similar pattern as suggested in bone cross section observations by SEM, bone is more mature under fixation when using TNTZ implant plate than Ti-64 implant plate. Along with that, it can be said that the bone healing progress is faster under fixation when using TNTZ implant plate. In general, fracture healing begins with soft tissue formation. It will then transform into soft callus and promotes into hard callus or cancellous-like bone. After that, the hard callus will mineralize and turns into bone as time progresses [19-21, 23, 24]. As a comparison for callus and bone structure, posterior of bone under fixation when using TNTZ implant plate is shown in Fig. 33(c). From here, it is suggested that bone structure is highly similar in all regions of bone cross section for both fixations. Refer to the magnified micrograph in Fig. 33(c), even though bone and callus can be identified, somehow their boundaries cannot be discriminated.

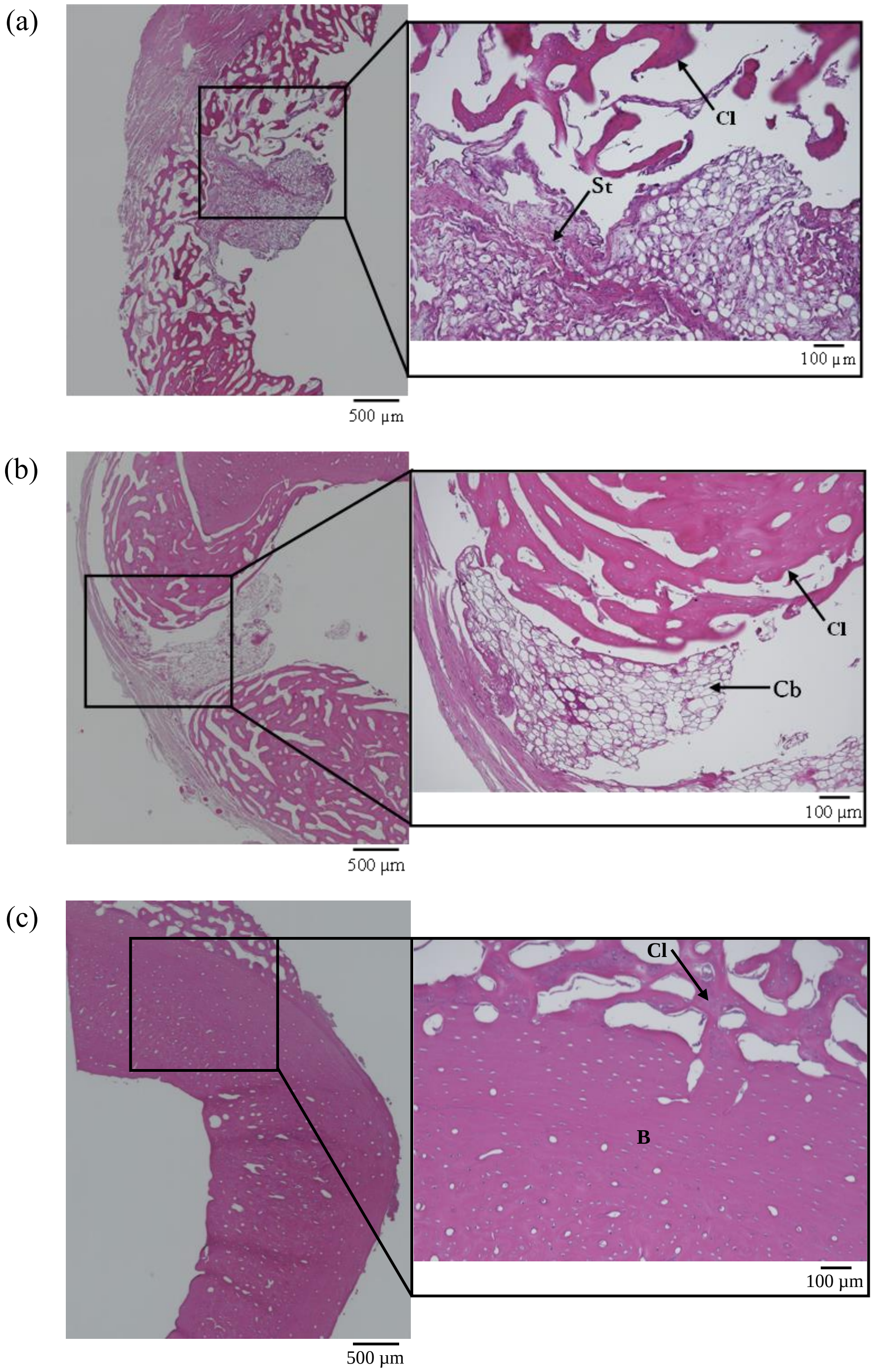


Fig. 33 H&E staining micrographs of bone on the anterior or defect region under the fixation when using (a) Ti-64 and (b) TNTZ implant plates with thickness of 1.5 [mm], and posterior or opposite of defect region under the fixation when using (c) TNTZ implant plate with thickness of 1.5 [mm].

4.2.4 Hardness of bone and callus

The mechanical properties of bones under different types of fixations were evaluated. As shown in Fig. 34, bone hardness is almost similar between both fixations. However, the callus hardness is slightly higher in fixation when using TNTZ implant plate than Ti-64 implant plate. The fixation when using Ti-64 implant plate resulted in larger callus but softer than the fixation when using TNTZ implant plate. Due to this, it is suggested that the hardness is not influenced by area of callus, but it is influenced by porosity and density of the structure. So, larger callus does not mean that its mechanical properties is better. Indeed, as healing progress, hardness is expected to increase. This is because bone will be mineralized and become matured [47-50].

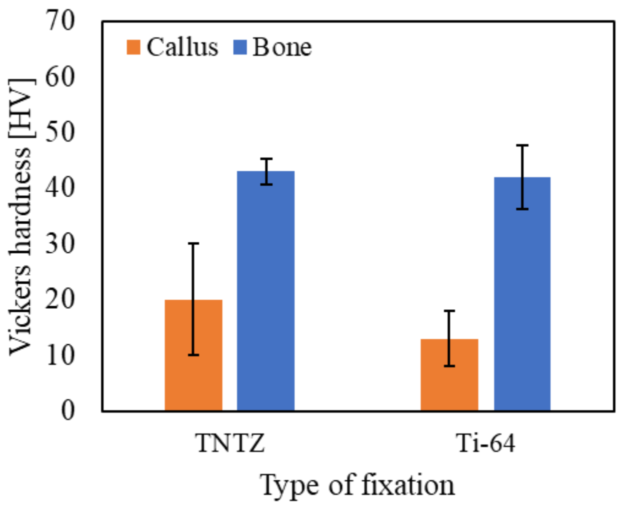


Fig. 34 The Vickers hardness of callus and bone under the fixation when using Ti-64 and TNTZ implant plates with thickness of 1.5 [mm].

4.3 Implant plate stiffness (Objective 2)

4.3.1 Bone cross section observation

As stated in previous section, the bone cross section is observed using the same method. But, in this section, both Ti-64 and TNTZ implant plates that were used are implant plate with thickness of 0.5 [mm]. The results for both fixations are discussed. This follows the Objective 2 which focused on the stiffness of implant plate. In regards of stiffness, TNTZ implant plate possesses lower stiffness than Ti-64 implant plate. First, the anterior or defect region under fixation when using Ti-64 implant plate is filled with new bone, but in some sample, defect region is still clearly seen (Fig. 35), and the fixation when using TNTZ implant plate shows identical pattern (Fig. 36). However, under TNTZ implant plate, the result is intense between all the samples. Some sample shows good new bone formation and no defect was seen, but most of the samples shows poor new bone formation and the defect is clearly seen and large. Overall, when comparing the anterior of these two implant plates, it can be said that the new bone formation is almost identical. It can also be seen that the new bone formation shows porous structure (callus).

Besides, posterior, or opposite of defect region shows only bone structure, with no callus formation in both fixations unlike in the previous section which used thicker implant plate (Fig. 37 and Fig. 38). When looking at medial, there is callus formation on the outermost layer of cortex in both fixations. As being observed in Fig. 39 and Fig. 40, the fixation when using Ti-64 implant plate led to smaller callus formation, when compared to the fixation when using TNTZ implant plate, respectively. The callus is also formed in lateral for both fixations, while the fixation when using Ti-64 implant plate have smaller callus (Fig. 41). Altogether, the fixation when using TNTZ implant plate led to a slightly good new bone formation, but the callus is larger in all regions, when compared to Ti-64 implant plate (Fig. 42).

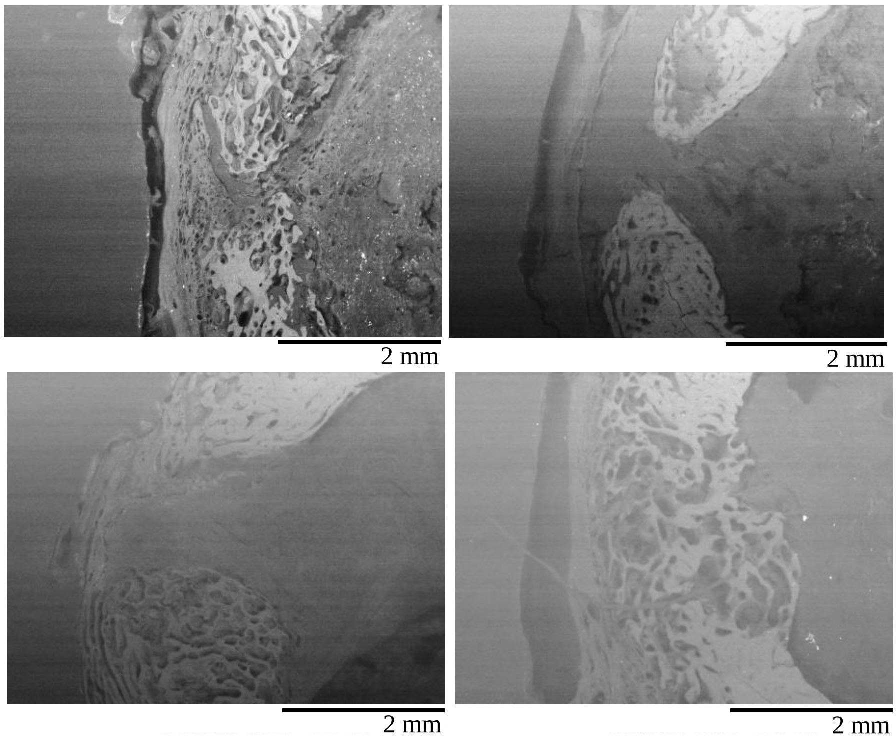


Fig. 35 SEM micrographs of anterior or defect region under the fixation when using Ti-64 with thickness of 0.5 [mm]. (n=1,2,3,4)

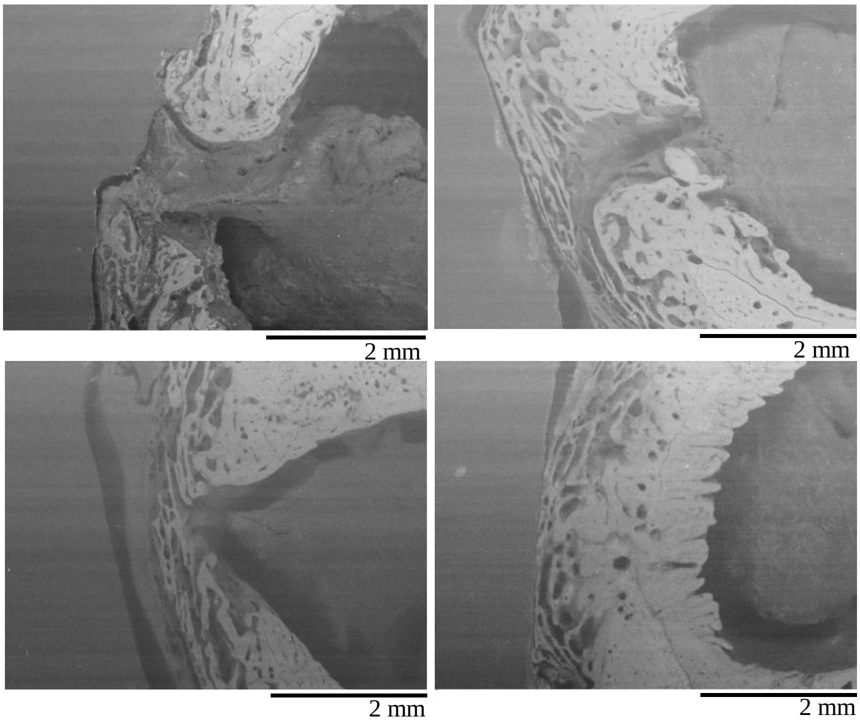


Fig. 36 SEM micrographs of anterior or defect region under the fixation when using TNTZ with thickness of 0.5 [mm]. (n=1,2,3,4)

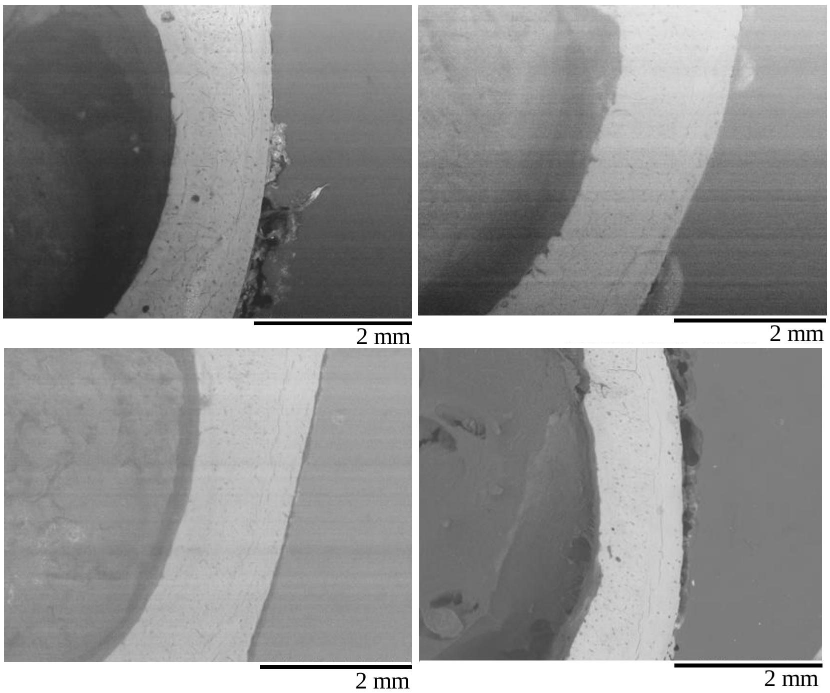


Fig. 37 SEM micrographs of posterior or opposite of defect region under the fixation when using Ti-64 with thickness of 0.5 [mm]. (n=1,2,3,4)

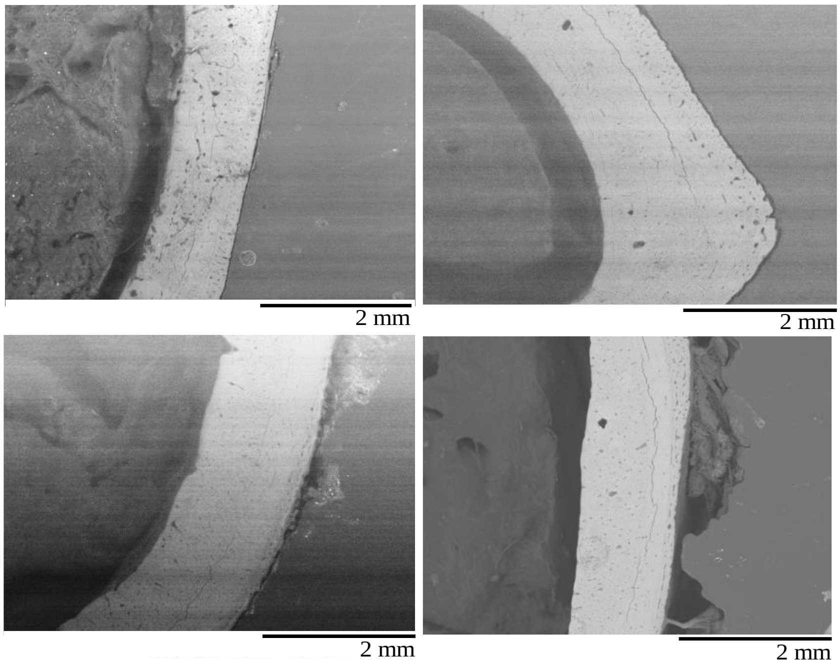


Fig. 38 SEM micrographs of posterior or opposite of defect region under the fixation when using TNTZ with thickness of 0.5 [mm]. (n=1,2,3,4)

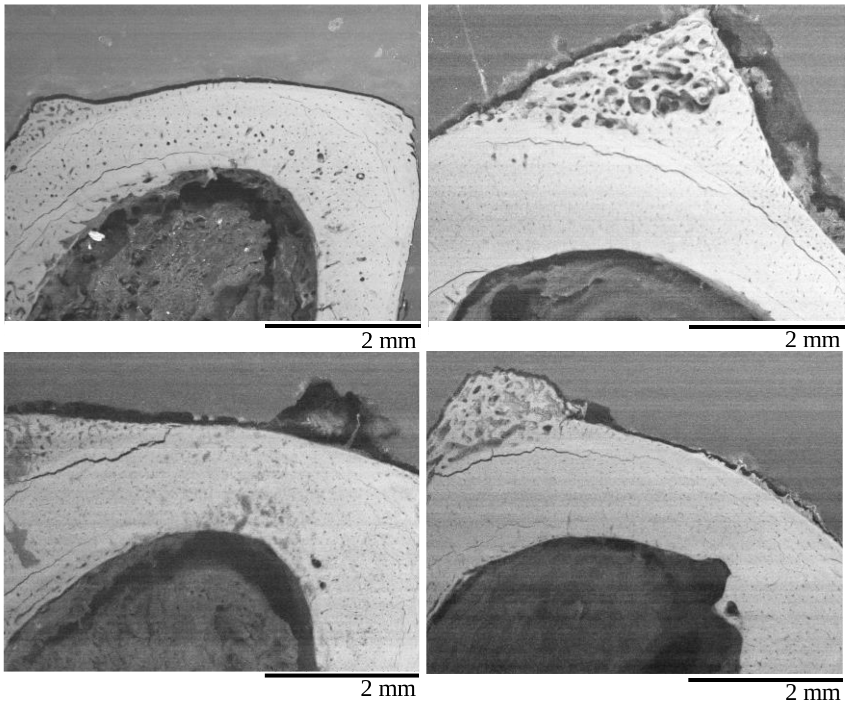


Fig. 39 SEM micrographs of medial or above of defect region under the fixation when using Ti-64 with thickness of 0.5 [mm]. (n=1,2,3,4)

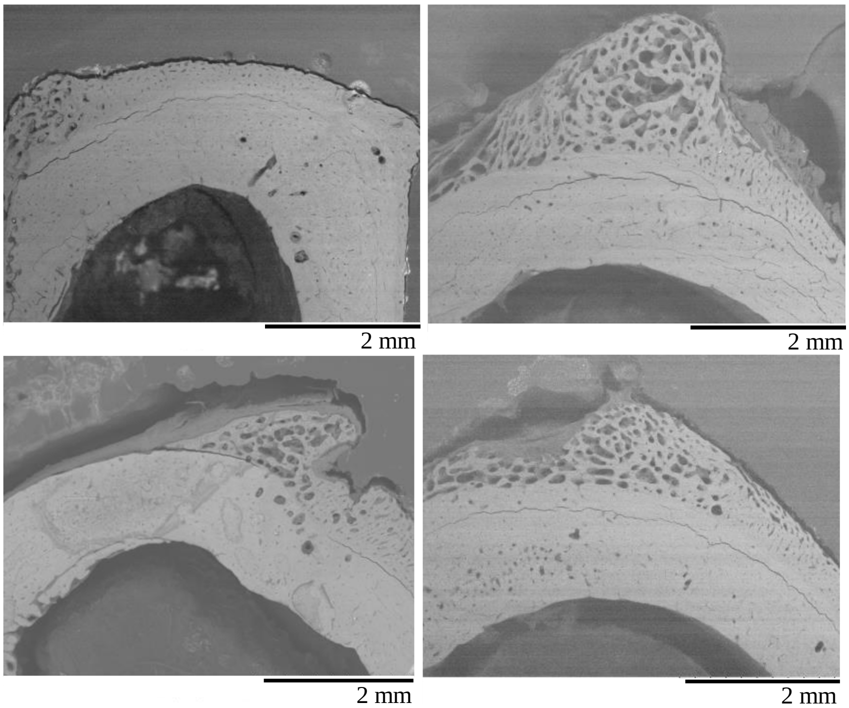


Fig. 40 SEM micrographs of medial or above of defect region under the fixation when using TNTZ with thickness of 0.5 [mm]. (n=1,2,3,4)

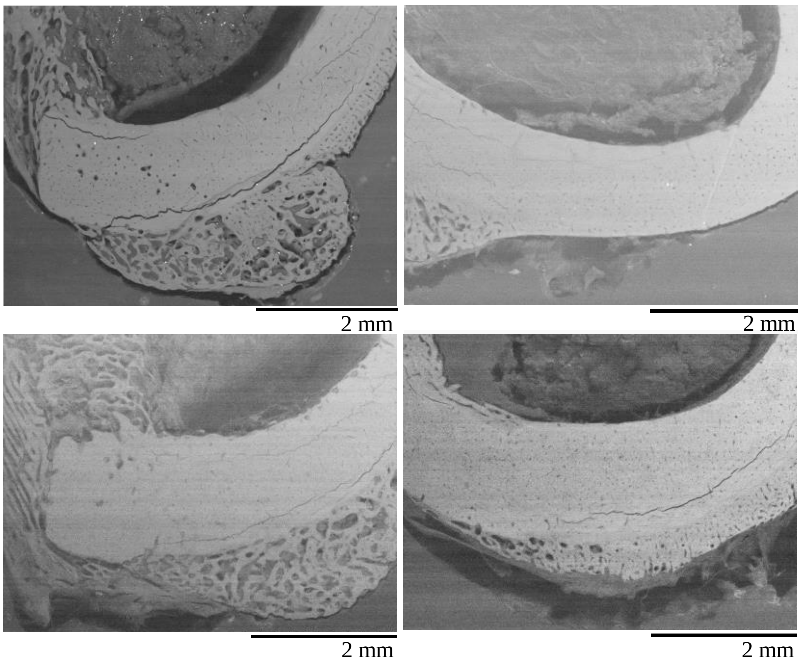


Fig. 41 SEM micrographs of lateral or below of defect region under the fixation when using Ti-64 with thickness of 0.5 [mm]. (n=1,2,3,4)

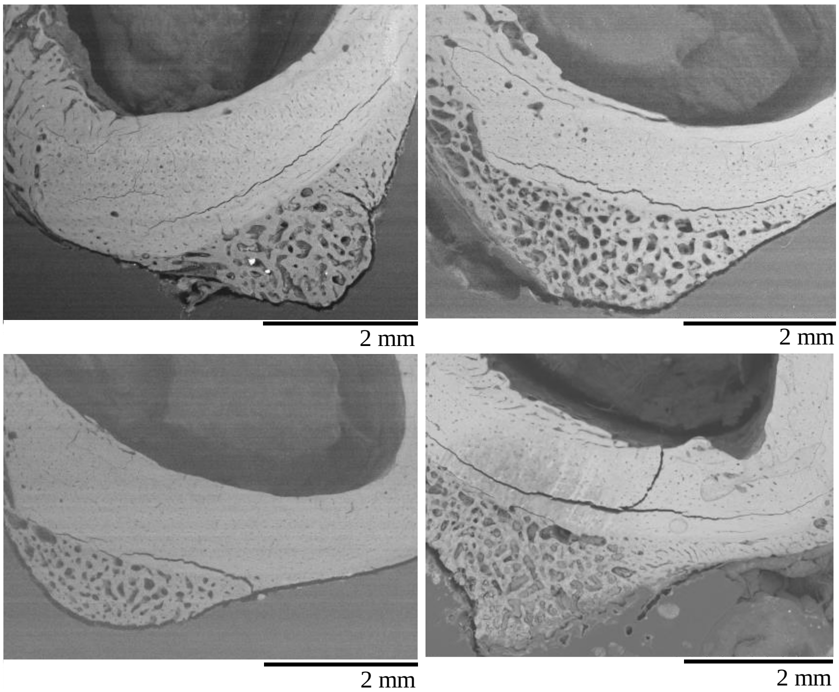


Fig. 42 SEM micrographs of lateral or below of defect region under the fixation when using TNTZ with thickness of 0.5 [mm]. (n=1,2,3,4)

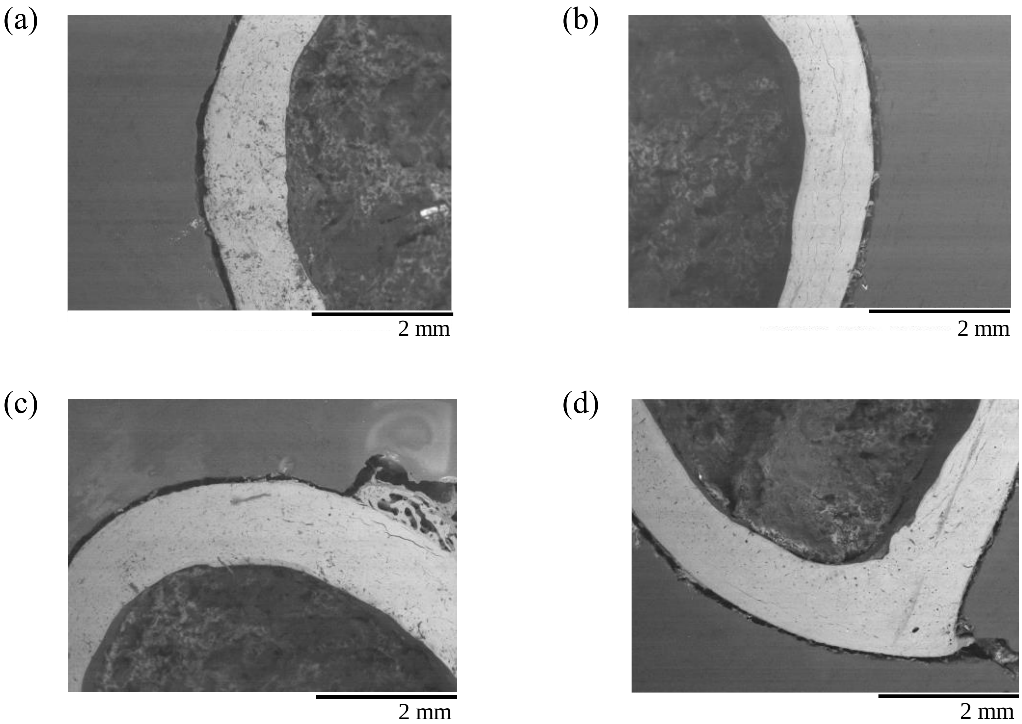


Fig. 43 SEM micrographs of bone for control (sham surgery) on (a) anterior, (b) posterior, (c) medial, and (d) lateral.

Further, SEM micrographs of control; sham surgery in shown in Fig. 43. The sham surgery indicates the normal bone condition with no extra formatted callus. This was carried out for comparison. So, it can be said that the insertion of implant plate regardless of its properties induced the formation of callus.

4.3.2 Image analysis

SEM micrographs are being used for image analysis by ImageJ, and then compared with the previous thicker implant plates as shown in bar chart (Fig. 44). In this section, the average of total callus area on defect (anterior), and non-defect (posterior, medial, lateral) are being calculated. From the results of using thinner plate of 0.5 [mm] thickness, the callus formation is larger under the fixation when using TNTZ implant plate than Ti-64 implant plate. The callus area under the fixation when using TNTZ implant plate is as follows: Defect region: 4.706 [mm^2], non-defect region: 7.180 [mm^2]. On the other hand, the callus area under the fixation when using Ti-64 implant plate is as follows: Defect region: 3.823 [mm^2], non-defect region: 6.092 [mm^2]. The callus in the defect region is considered as aiding to the bone healing, while callus in non-defect region is said to be unrelated to the bone healing. So, it can be said that callus on non-defect region is unnecessary and might be a disturbance towards bone healing which should be focused only on defect region. This might relate to mechanical instability of the fixation system. In the previous section, when thicker implant plates were used, both fixations show almost similar callus area in the defect region. However, the fixation when using Ti-64 implant plate with thicker plate induced more callus formation in non-defect region when compared with the fixation when using TNTZ implant plate of the same thickness. In total, the callus area is the largest under the fixation when using Ti-64 implant plate with thickness of 1.5 [mm] (18.394 [mm^2]), then followed by TNTZ implant plate with thickness of 0.5 [mm] (11.886 [mm^2]), Ti-64 implant plate with thickness of 0.5 [mm] (9.924 [mm^2]), and TNTZ implant plate with thickness of 1.5 [mm] (8.316 [mm^2]) [15, 16]. Here, the highest stiffness of implant plate is said to induce more callus especially on non-defect region. On the other hand, the lowest stiffness of implant plate also induced quite high stiffness may be due to the mechanical instability of the fixation system.

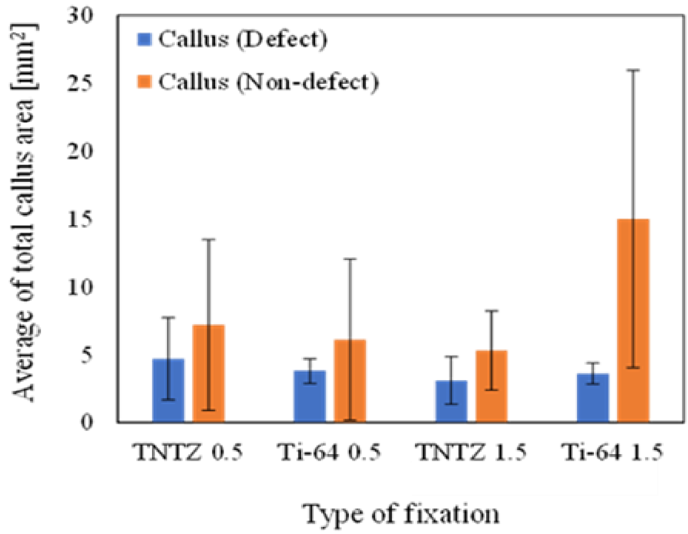


Fig. 44 The average of total callus area under the fixation when using Ti-64 and TNTZ implant plates with thickness of 0.5 [mm] which are compared with both Ti-64 and TNTZ implant plates with thickness of 1.5 [mm].

4.3.3 Histological observation

H&E staining performed on the bone cross sections after the fixation when using Ti-64 implant plate and TNTZ implant plate with thickness of 0.5 [mm] are shown in Fig. 45. As stated in previous section, bone healing started from the formation of soft tissue (St), followed by cancellous-like bone (Cb), callus (Cl), and then mineralized to become bone (B). The fixation when using Ti-64 implant plate shows the appearance of callus (Cl) and many soft tissues (St) (Fig. 44(a)). By contrast, the fixation when using TNTZ implant plate resulted mostly as Cl, some St with cancellous-like bone (Cb) in between (Fig. 45(b)). Although there is Cl in the fixation when using Ti-64 implant plate, there is still many soft tissues that are present. On the other hand, the structure of the bone under the fixation when using TNTZ implant plate consists of three types of bone structure, shows that the healing is progressing. Compared these two fixations, as the defect region is mostly filled with callus under the fixation when using Ti-64 implant plate, it is suggested that the healing is more progressive here.

(a) (b)

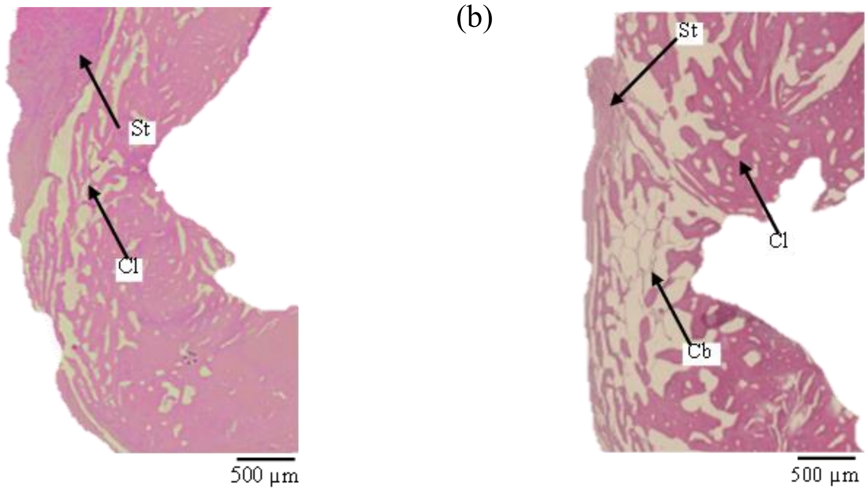


Fig. 45 H&E staining micrographs of bone on the anterior or defect region under the fixation when using (a) Ti-64 and (b) TNTZ implant plates with thickness of 0.5 [mm].

4.3.4 Hardness of callus

The bar chart of bone and callus hardness are plotted (Fig. 46). Here, the result from previous section is also grouped. The bone hardness is nearly similar in all fixations, which ranging from 42-44 [HV]. The fixation when using Ti-64 implant plate and TNTZ implant plate led to an almost similar callus hardness which are 20 [HV] and 18 [HV], respectively. This is because both fixations resulted in almost similar structure that consist of many calluses. As explained in previous section, callus hardness is expected to increase as healing progresses as it will become more mature and mineralizes over time. On the other hand, the fixation when using Ti-64 implant plate with thickness of 1.5 [mm] resulted in the lowest callus hardness, while TNTZ implant plate with thickness of 1.5 [mm] led to a similar hardness to both fixations with thickness of 0.5 [mm].

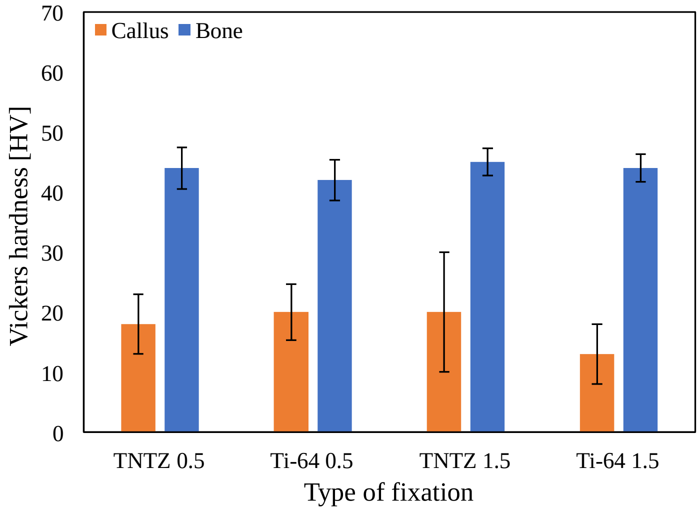


Fig. 46 The Vickers hardness of callus and bone under the fixation when using Ti-64 and TNTZ implant plates with thickness of 0.5 [mm] which are compared with Ti-64 and TNTZ implant plates with thickness of 1.5 [mm].

4.4 Discussion

Briefly, this research investigates the bone healing under implant plate fixation by using Ti-64 and TNTZ alloys with two variables which are elastic modulus and thickness differences. The elastic modulus difference is being said that it can prevent stress shielding phenomenon after long-term implantation [5, 6, 9]. So, it is expected that the nearer the elastic modulus of implant plate to that of bone, the higher the possibility to avoid stress shielding phenomenon.

On the other hand, thickness can be used to adjust the stiffness of the same implant plate. It has been discussed that flexible materials can ensure a good fracture healing especially providing a suitable mechanical stability and ensure a balance cell differentiation in the healing bone [10-14]. Hence, these two variables can be directly link to the stiffness of the implant plate. Here, when using different type of implant plate with the same thickness, the ratio of TNTZ implant plate to Ti-64 implant plate is 1 : 2. On the other hand, when using the same implant plate with different thickness, the ratio of 0.5 [mm] thickness to 1.5 [mm] thickness is 1 : 3. The stiffness is calculated, and the order from the lowest stiffness is TNTZ with thickness of 0.5 [mm] → Ti-64 with thickness of 0.5 [mm] → TNTZ with thickness of 1.5 [mm] → Ti-64 with thickness of 1.5 [mm]. Hereafter, the implant plate types will be mentioned as TNTZ 0.5, Ti-64 0.5, TNTZ 1.5 and Ti-64 1.5. Although previous publications have stated that the elastic modulus along with stiffness are crucial in gaining successful fracture healing, there is no solid proof to support this statement especially on the pattern of callus formation which happen on the early stage of healing. Moreover, the experimental study has not been done as much as computational analysis [10-14]. For that reason, present study focused on describing the callus formation pattern and its properties under the fixation of implant plate made of Ti-64 and TNTZ alloys.

4.4.1 Bone formation pattern

Firstly, comparing the fixation when using Ti-64 and TNTZ implant plates with the same thickness, either 0.5 [mm] or 1.5 [mm], the new bone formation on the anterior or defect region is almost similar, and in some regions, the defect is still seen. This suggests that the bone formation is still in progress as the healing period is three weeks, which is in the early stage of healing. In general, fracture healing of rabbit is six weeks [29]. So, as time passes the defect region is expected to fully heal and gain its initial structure and properties again.

Second, the posterior or opposite of defect region shows non-identical pattern. The fixation when using Ti-64 1.5 implant plate induced the formation of callus in the outermost layer of cortex, while Ti-64 0.5 implant plate didn't show any callus formation in the same region. On the other hand, the fixation when using TNTZ implant plate does not show any callus formation too.

Despite, medial and lateral of bone cross section shows that Ti-64 1.5 implant plate has larger callus than Ti-64 0.5 implant plate, while the pattern is opposite under the fixation when using TNTZ implant plate (TNTZ 0.5 > TNTZ 1.5). When the same thickness of implant plate is being used, Ti-64 1.5 implant plate shows larger callus than TNTZ 1.5 implant plate, but TNTZ 0.5 implant plate shows larger callus formation than Ti-64 0.5 implant plate. This shows that when different elastic modulus of implant plate is being used, either high or low elastic modulus does not really relate to any specific pattern. So, stiffness of implant plate is suggested to be crucial factor that influencing the new bone formation.

Quantitatively, defect region (anterior) indeed shows almost similar callus area in all fixations. However, non-defect region (posterior, medial, lateral) has highest callus formation in the fixation when using Ti-64 1.5 implant plate followed by TNTZ 0.5 implant plate, Ti-64 0.5 implant plate and TNTZ 1.5 implant plate. In total, the highest stiffness of Ti-64 1.5 implant plate led to the largest callus formation, and the lowest is the fixation when using TNTZ 1.5 implant plate. Previous study stated that larger callus formation enhances the fracture healing, but in present study we noticed that it doesn't necessarily true [51, 52]. This is because although the fixation when using Ti-64 1.5 has the largest callus formation, the callus on defect is similar in all fixations, and thus means that the healing progress is just the same.

In early stage of healing, mechanical stimuli will affect the cell differentiation because of mechanical stability of the fixation system. The previous computational analysis studies stated that mechanical stimuli can alter the period and route of bone healing through cell differentiation [10-14, 53, 54]. If good mechanical stimuli are achieved, bone maturation will be faster, thus bone healing is enhanced. The preferable mechanical stimuli and its cell differentiation is described in Fig. 7 [10, 13].

Bone maturation is being evaluated through H&E staining that is focused on the anterior or defect region. Although SEM micrographs found that the new bone formation is almost similar, H&E staining reveals the bone types presents on the bone cross section. Here, bone is suggested to heal through indirect healing which include the formation of callus [19, 25, 26]. The bone healing start with hematoma formation. After that, the soft callus will form, then followed by hard callus. It will then mineralize and become bone. In this stage, the bone will regain its structure and properties. As results, there are soft tissue (St), cancellous-like bone (Cb), callus (Cl), and bone deposited in the bone cross section. In case of the fixation when using Ti-64 implant plate, both of thickness (1.5 [mm] & 0.5 [mm]) led to the deposition of St and Cl. The only difference is that under thicker implant plate, the surrounding bone is also affected, as in it seems more porous and lose density. On the other hand, the fixation when using TNTZ implant plate shows that under a thicker implant plate, only Cb and Cl are present, but under thinner implant plate, there is some region that St still present.

In addition, further investigation on H&E-stained bone sample was carried out to describe bone progression quantitatively. The H&E micrograph was enlarged, bone types were observed, and image analysis to measure the area of each type of bone was carried out. Even though in first publication stated that there is no sign of St in bone cross-section under fixation when using TNTZ 1.5, the enlarged micrograph shows some St in the outermost layer of cortex. However, the presence is insignificant. All measured area is shown in Fig. 47. By comparing the fixation type, it is known that no Cb is deposited under the fixation when using Ti-64 implant plate, while all three types of bone were present under the fixation when using TNTZ implant plate. This shows that under the fixation when using Ti-64 implant plate, some region has progressive healing while some are not. On the other hand, under the fixation when using TNTZ implant, all three types of bone were present shows that the progression of healing is dispersed. On top of that, under fixation when using TNTZ 1.5 implant plate, healing is more progressive than the fixation when using TNTZ 0.5 implant plate which are shown by the larger area of St and Cb. Here, in bone maturation, elastic modulus is said to be important. It is suggested that when bone is healing, if elastic modulus of the implant plate is nearer to the bone, the anterior region (defect) can heal faster with more mature bone as the load act on micro level of bone is uniform. However, there is no realistic proof to this event yet.

Indeed, under the fixation when using TNTZ implant plates resulted in more progressive bone healing with a small percentage of St formation left on the anterior (defect) than Ti-64 implant plates. Hence, rather than stiffness, it is believed that elastic modulus difference plays an important role in bone maturation during healing.

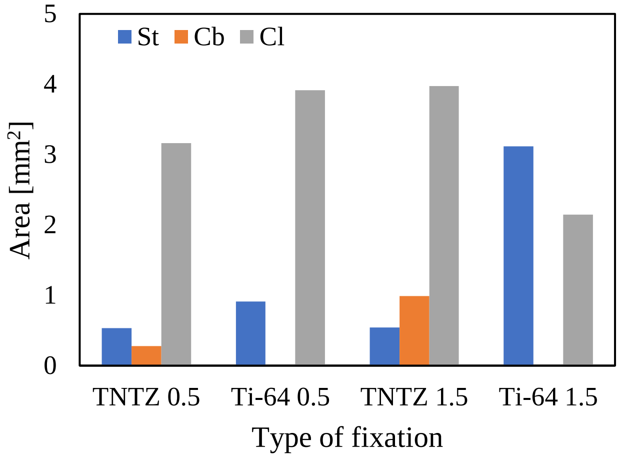


Fig. 47 Area of soft tissue (St), cancellous-like bone (Cb), and callus (Cl) on the anterior region (defect).

4.4.2 Stress shielding

Elastic modulus is often referred as one of the factors that can be linked to stress shielding phenomenon [5-9]. In general, stress shielding is expected after long-term implantation. However, in present study, since different elastic modulus of implant plates are being used, its appearance also expected. Overall, the findings through H&E staining can be used to discuss the stress shielding phenomenon (Fig. 33 and Fig. 45). Compare all type of fixations, it can be said that the fixation when using Ti-64 1.5 implant plate shows large area of affected bone surrounding the defect region. This bone seems to have porous structure and looks like the density is losing. Although there is no significant evidence, this structure may have related to stress shielding, thus suggest that even in early stage of healing, sign of stress shielding was seen. However, further investigation is needed.

4.4.3 Mechanical stability

Post-fracture, bone loss its stability, and the interruption of functioning regularly happened. By the insertion of the implant plate, support is given to bone, until it healed. This support should be adequate in the fixation system near to the stability of bone before fracture. Hence, the act of inserting implant plate can be beneficial or worst thing as it may turns out to be insufficient support to help bone healed. In this context, it is believed that mechanical stability of the fixation system is needed to support the bone healing. The mechanical stability is often related to the interfragmentary movement (IFM) of bone fragment [55-62]. IFM is the bone movement in acts of certain force added to the bone. It is proven that the IFM is affected by locking plate configurations [63-65]. By adjusting the stiffness of implant plate, the mechanical stability of the fixation system and IFM of bone fragment are changed, and thus resulting in the different healing outcomes. Present study considered a theory where the stiffness of the implant plate should be able to replace the stiffness of the defect that was removed from the bone. As that being said, the stiffness of the defect was calculated in respect of the ideal shape of half-circular (eq. 4.1). Here, the bending stiffness of the implant plate was calculated again using parallel axis theorem. As explained in 3.1.1, the bending stiffness is considered. The result was then compared with the bending stiffness of the implant plate (Table 3, 4). Noted that the elastic modulus of bone is assumed as 30 [GPa] even though there is a possibility that the elastic modulus is varied when there is slight difference interspecies, age, gender, and so on. As comparison, it can be seen that the bending stiffness of TNTZ 0.5 implant plate is very low, but Ti-64 1.5 implant plate has higher stiffness, while the bending stiffness of defect is in between Ti-64 0.5 and TNTZ 1.5 implant plates. As an ideal theory, the stiffness of implant plate is desirable in between the Ti-64 0.5 and TNTZ 1.5 implant plates. In fact, the findings of present study did describe that the healing under fixation when using TNTZ 1.5 implant plate are the most favorable.

$$E \, x \, I = E \, x \, \frac{r^4}{8}(\theta - \sin\theta + 2\sin\theta \; \sin^2\frac{\theta}{2}) \tag{4.1}$$

Table 3 Bending stiffness of defect.

Elastic modulus of bone [GPa]	Bending stiffness [Nm^2]
30	12.28

Table 4 Bending stiffness of implant plate.

Implant plate (thickness)	Bending stiffness [Nm^2]
TNTZ (0.5 mm)	5.403
Ti-64 (0.5 mm)	9.906
TNTZ (1.5 mm)	16.28
Ti-64 (1.5 mm)	29.85

Previous studies often stated that IFM of bone will affect the cell differentiation in defect region [12, 14, 53]. This will either succeed, delay or failed the bone healing. Present study found that the bone formation in the anterior and posterior under the fixation when using Ti-64 1.5 implant plate is asymmetric; the posterior of bone induced callus formation in the outermost layer of cortex. This suggests that the IFM of bone fragments is not uniform as the fixation is too rigid that limits the IFM on the posterior, and then triggers the formation of callus that is not needed. This is supported by previous studies that mentioned the flexibility of the fixation plays an important role in assisting bone healing through a balance IFM on the fracture site to achieve successful bone healing [66, 67]. The term flexible here refers to the flexibility in metal specifically such as titanium alloys is more flexible than stainless steel. This is proven in previous studies that suggested endochondral ossification which is preferable in aid to bone healing can possibly be achieved better by using titanium alloys rather than stainless steel [14]. Through this, it can be said that using the same implant plate with different thickness, which in this case thinner implant plate might be beneficial. As a result, the fixation when using Ti-64 0.5 implant plate shows more regular callus formation on the posterior (Fig. 37).

Furthermore, in terms of the flexibility of implant plate, it is known that the TNTZ implant plate is more flexible than Ti-64 implant plate in the same thickness. So, the IFM is expected to be more uniform in aid to bone healing. Indeed, the fixation when using TNTZ 1.5 implant plates resulted in good bone formation with no asymmetric callus (Fig. 27). Although the fixation when using TNTZ 0.5 implant plate got no asymmetric callus formation, the callus formation in non-defect region, in total is quite large (Fig. 43). It is suggested that this can be called as excessive callus formation. Further, intense IFM on the fracture site, as a result of mechanical instability of fixation system leads to more callus formation which is clearly not needed. In this case, TNTZ 0.5 implant plate is categorized as too flexible.

In general, uniform IFM can ensure a balance cell differentiation for a better healing outcome and avoid excessive strain on the fracture site to prevent delay union. The IFM of 0.2 [mm] to 1.0 [mm] is stated as suitable to stimulate good callus formation [36]. Though, it is dependent on the fracture gap size. The fracture gap also influences the healing outcomes using different configurations of implant plate, in terms of flexibility. Previous study stated that using a flexible fixation, a small fracture gap could experience excessive strain that will result in delay union while enhance cartilaginous callus is formed and bone healing is improved in large fracture gap with flexible fixation which are described through computational model [10-14]. Hence, the defect region in present study can be considered as large fracture gap.

Above all, Ti-64 and TNTZ implant plates are expected to provide a good mechanical stability as both of alloys are more flexible than stainless steel. However, between these alloys itself, the stiffness is adjusted by changing the thickness of implant plate. Due to this, the highest stiffness of Ti-64 1.5 implant plate is categorized as too rigid, thus high mechanical stability is held. Due to this, the IFM is suppress and non-uniformly transfer to the bone led to formation of asymmetric callus on posterior. On the other hand, the lowest stiffness of TNTZ 0.5 implant plate induced excessive callus formation especially on non-defect region (posterior, medial, lateral), as influenced by intense IFM that eventuate mechanical instability of the fixation system. To the best of our knowledge, TNTZ 1.5 implant plates may be nearest to the ideal stiffness that is required by bone to heal successfully.

In whole, the relationship between IFM and mechanical stability are simulated in Fig. 48. When the fixation is too flexible, mechanical stability is poor, IFM will be high, thus resulted in excessive callus formation in non-defect region. By contrast, when the fixation is too rigid, mechanical stability will be high, IFM is suppressed and non-uniform, thus lead to the formation of asymmetric callus. Therefore, the usage of suitable flexibility of fixation can lead to sufficient mechanical stability which provide a certain degree of IFM that is mechanically consistent with elastic deformation of bone, especially of the posterior or opposite region of defect, to aid in successful bone healing without any consequences.

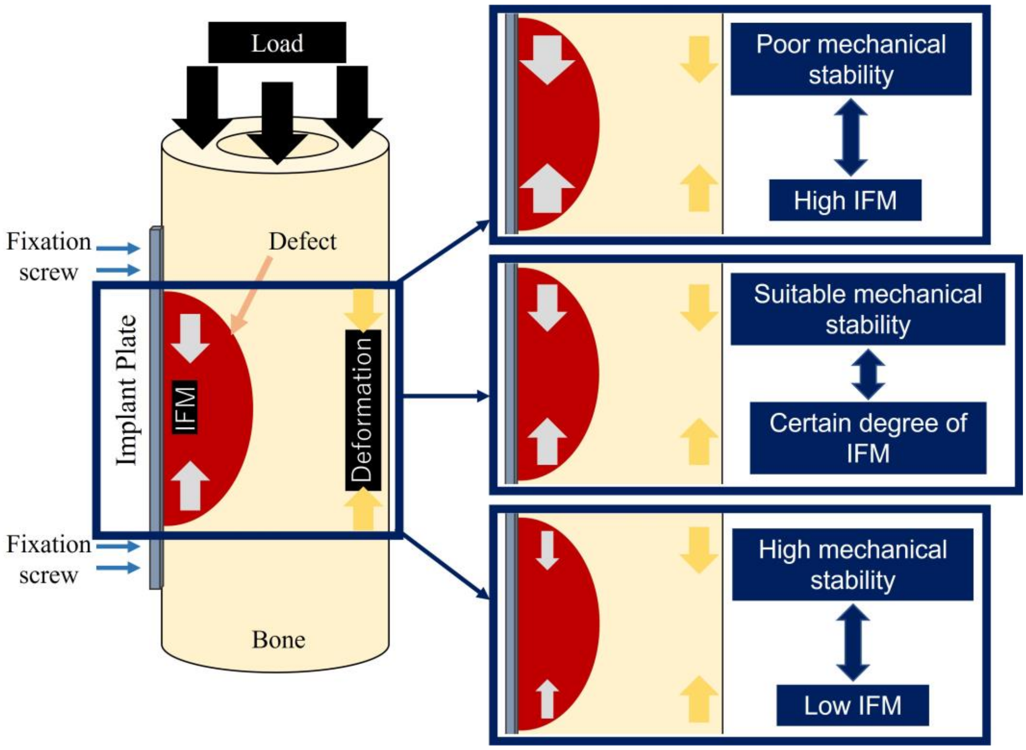


Fig. 48 Diagram of the relationship between IFM and mechanical stability of the fixation system.

CHAPTER 5

Conclusion

5.1 Summary

In this work, the experimental study on bone healing under the implant plate fixation is being discussed. The framework of present study started with sample preparation of implant plate as follow: Ti-64 with thickness of 1.5, 0.5 [mm], and TNTZ with thickness of 1.5, 0.5 [mm]. These two types of titanium alloys have different elastic modulus of 110 [GPa] and 60 [GPa], respectively. Further, by modifying the thickness of both implant plates, the stiffness is adjusted. The stiffness from the lowest is as follow: TNTZ with thickness of 0.5 [mm] < Ti-64 with thickness of 1.5 [mm] < TNTZ with thickness of 1.5 [mm] < Ti-64 with thickness of 1.5 [mm]. This will be referred as TNTZ 0.5, Ti64 0.5, TNTZ 1.5, and Ti-64 1.5 hereafter.

As a result, the newly formed bone on interior or defect region for all fixations are almost similar and show porous structure of new bone which refer as callus. It is noticed that some defect region is still present, suggests that the bone healing is still in progress. This may be because of the healing period of three weeks only, which is still in the early stage of healing. On the other hand, posterior or opposite of defect region shows asymmetric callus formation under the fixation when using Ti-64 1.5. It is suggested that rigid fixation cause high mechanical stability of fixation system, thus IFM is suppress and cannot be delivered uniformly across the bone cross section. By contrast, excessive callus formation on non-defect region (posterior, medial, lateral) is shown under the fixation when using TNTZ 0.5. Fixation system with low mechanical stability that resulted from over-flexible implant plate trigger intense IFM, thus resulted in large callus formation which is not needed for bone healing.

Moreover, although the healing period in this study is only three weeks, bone is more matured under the fixation when using TNTZ 1.5. This is observed through the existence of only cancellous-like bone and callus on the bone cross section, after H&E staining. Bone types in other fixations still have soft tissue presence which is the earliest tissue to form during bone healing. The mechanical properties of bone and callus is also evaluated by using Vickers hardness test. From the result, it is known that bone has higher hardness from the callus. But it is believed that the callus hardness will become higher as healing progress.

Overall, it can be concluded that when the fixation is over flexible, low mechanical stability induced intense IFM, thus resulted in excessive callus formation especially in the non-defect region, while if the fixation is too rigid, high mechanical stability suppress IFM, thus resulted in asymmetric callus formation. Due to this, a suitable flexibility of fixation is needed, thus resulted in medium mechanical stability that will induce a certain degree of IFM to aid in bone healing.

5.2 Limitations

The present study has some limitations. For example, the implant plate stiffness is calculated but the actual defect stiffness cannot be quantified. Due to this, an ideal shape of defect is taken into consideration to calculate the defect stiffness. Other than that, the IFM is not measured due to experimental in sufficient, so only theoretical explanation is discussed. Then, it is believed that number of animals should be increased to gain more precise results.

5.3 Future proposed works

The early stage of healing is described in present study. The findings stated that mechanical stability by suitable stiffness of implant plate is crucial. However, in present study, a few more adjustments to the stiffness are needed. Although the decision to use 0.5 [mm] and 1.5 [mm] seems to hold a good variation in stiffness, from the findings thickness of 1.0 [mm] might be beneficial to be investigated, since the ideal stiffness is still not clearly known.

As per mentioned, early stage which is three weeks was held, thus longer healing period might be needed to be tested. This is because when the healing period is prolonged, the properties of implant plate that is needed might be different. Again, the entire fracture healing process need to be understood well to design an ideal implant plate. Other than that, the add-on in histological observation should be considered. In future, more intense staining should be performed to explain the cell type in defect and other region. This will be advantageous if osteoclast or osteoblast can be identified, especially as a proven fact if there is stress shielding phenomenon.

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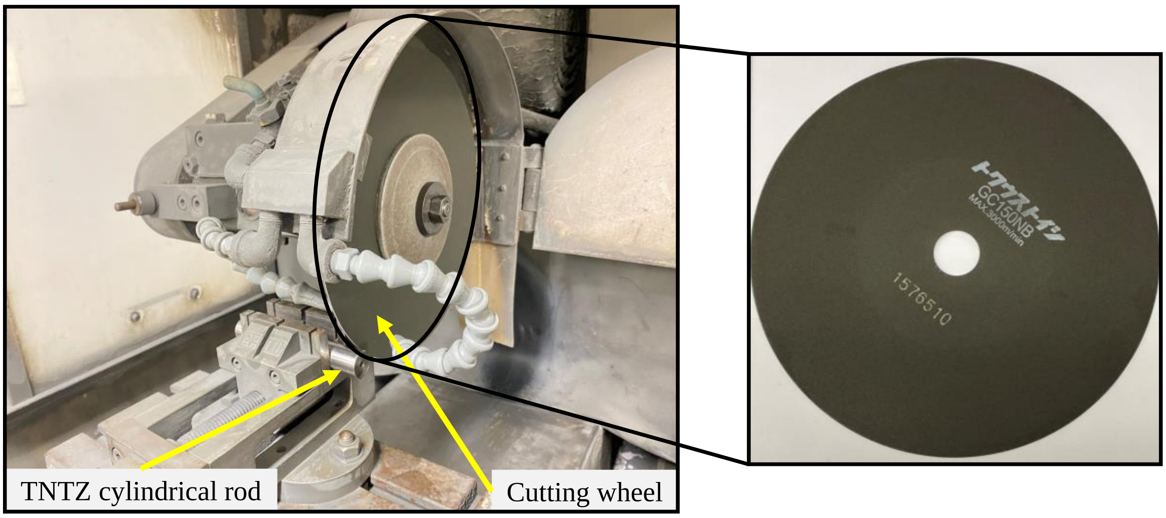
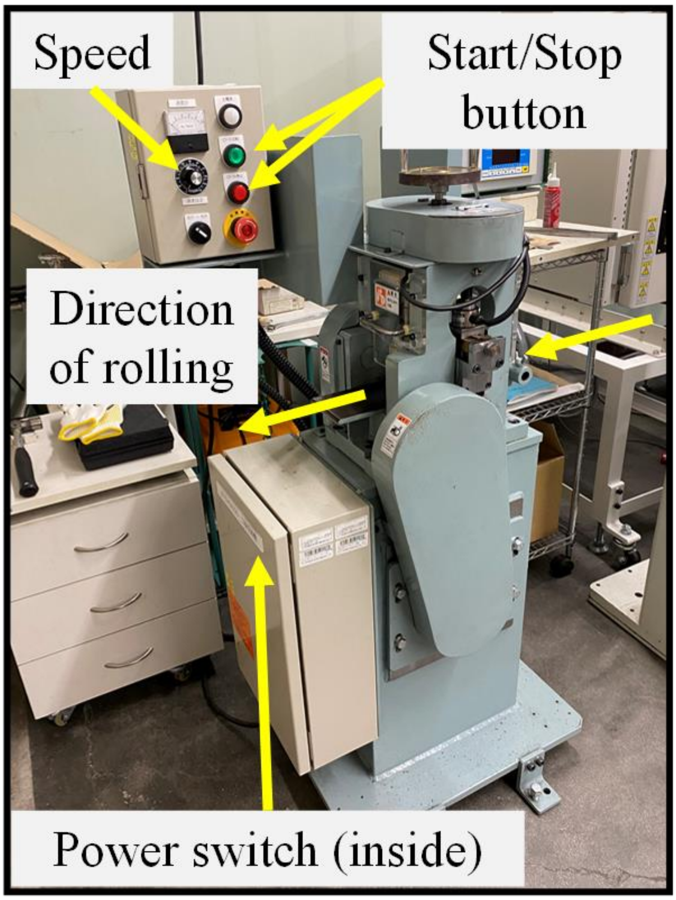
APPENDIX

Appendix 1. Cutting of TNTZ cylindrical rods

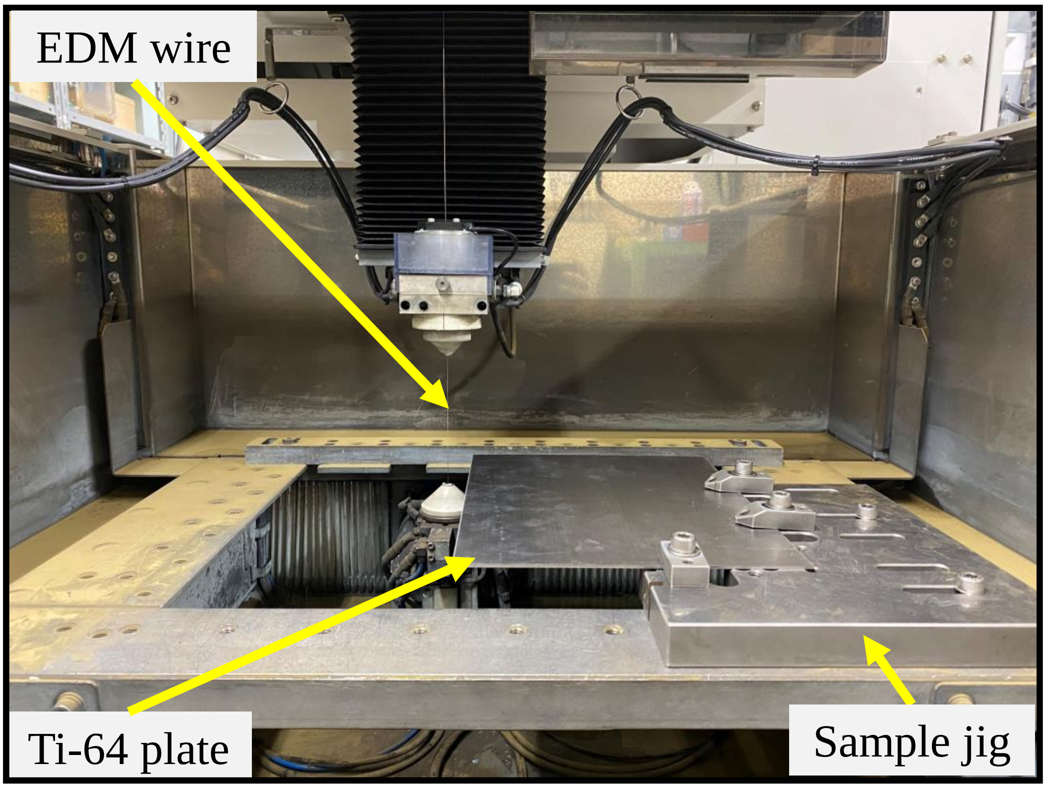


Appendix 2. Rolling mills

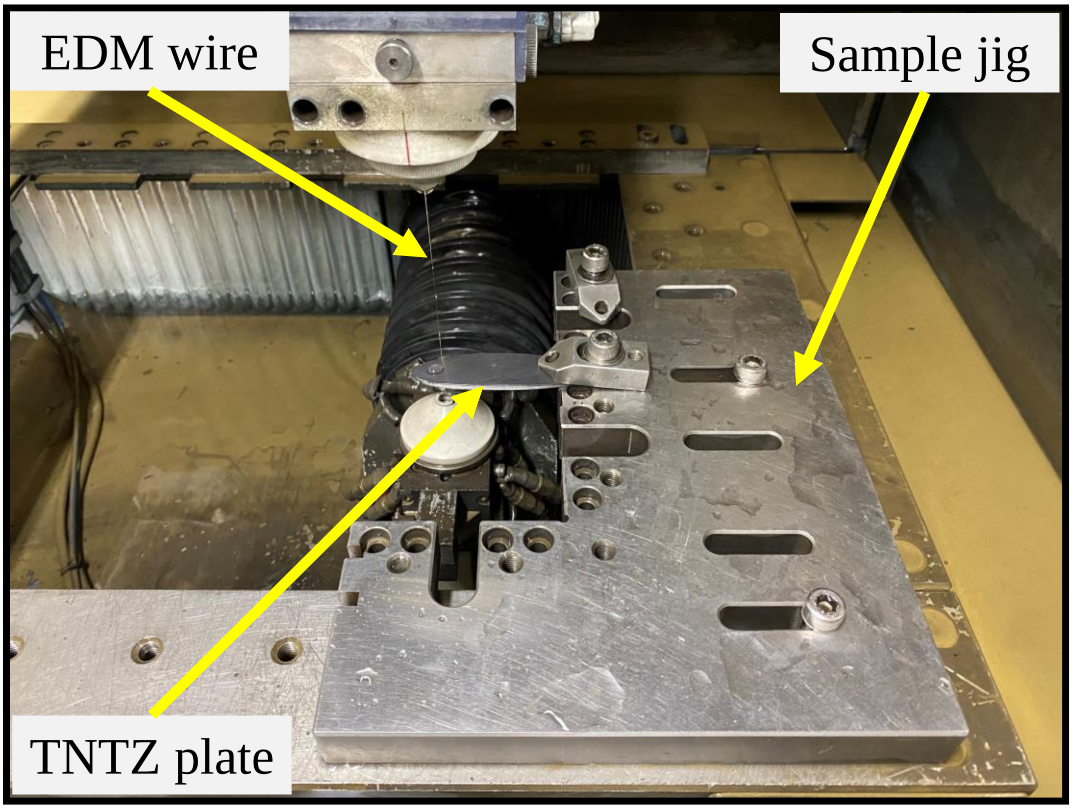


Appendix 3. Electric discharge machine cutting

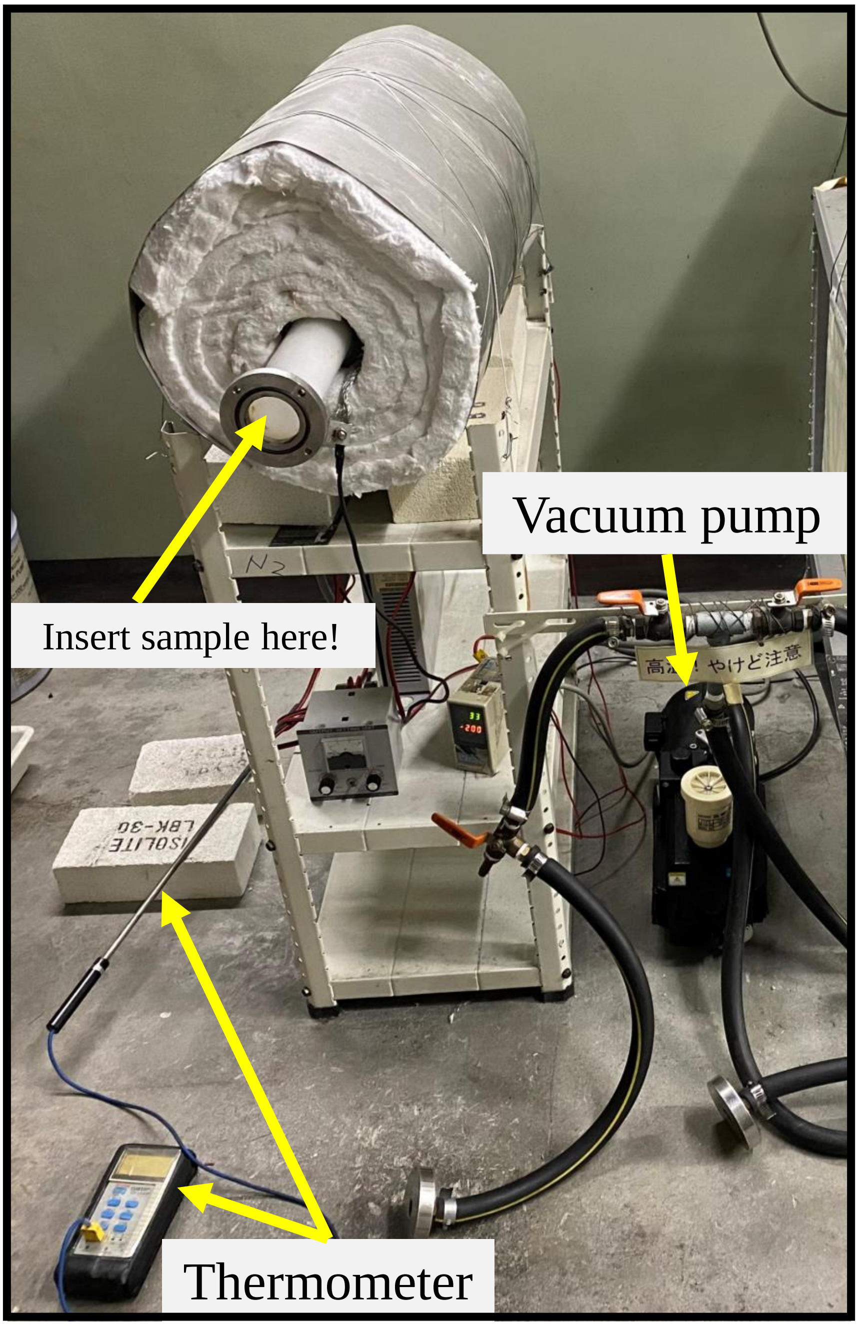
① Cutting of Ti-64 plate



② Cutting of TNTZ plate



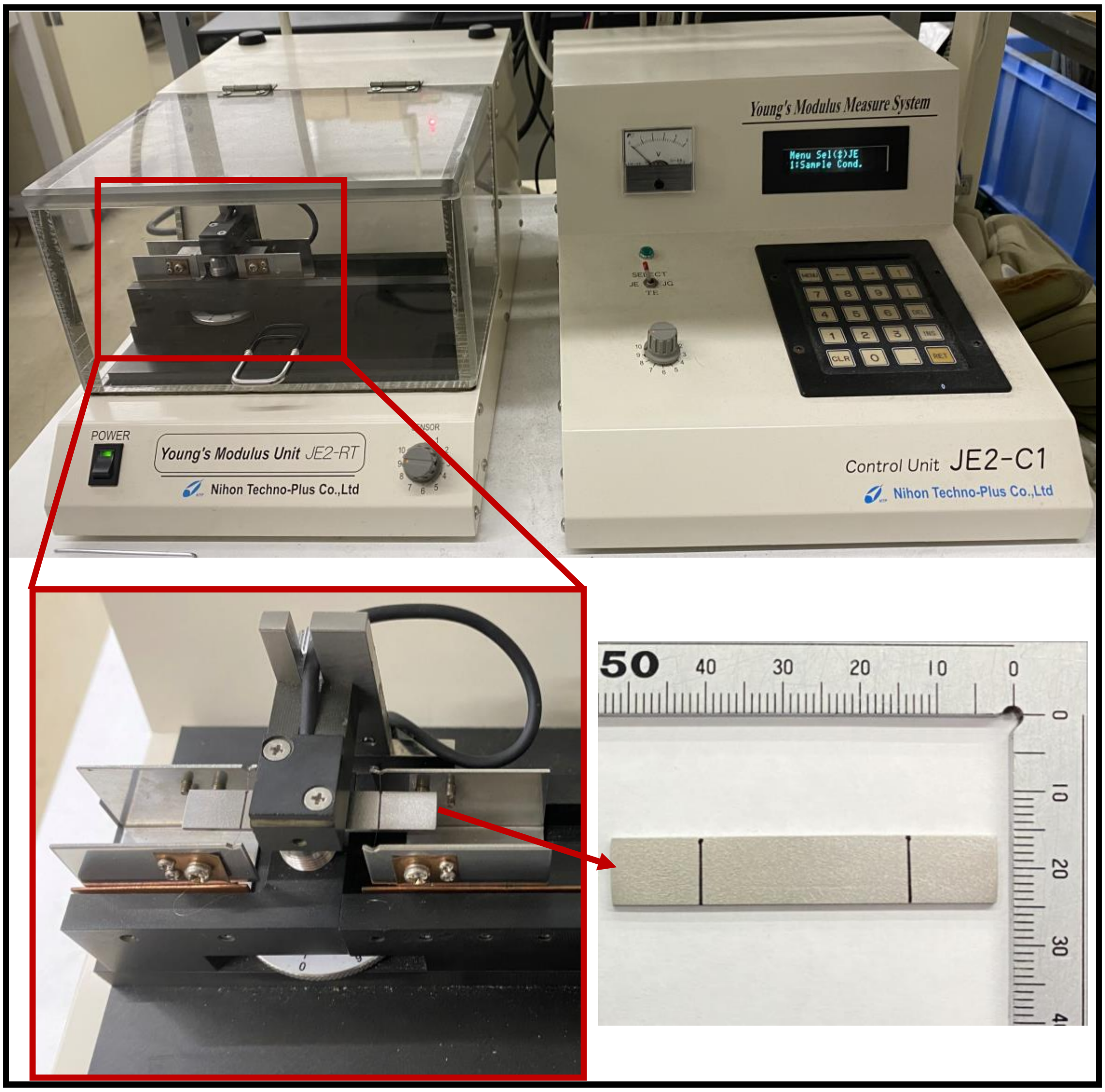
Appendix 4. Heat treatment chamber



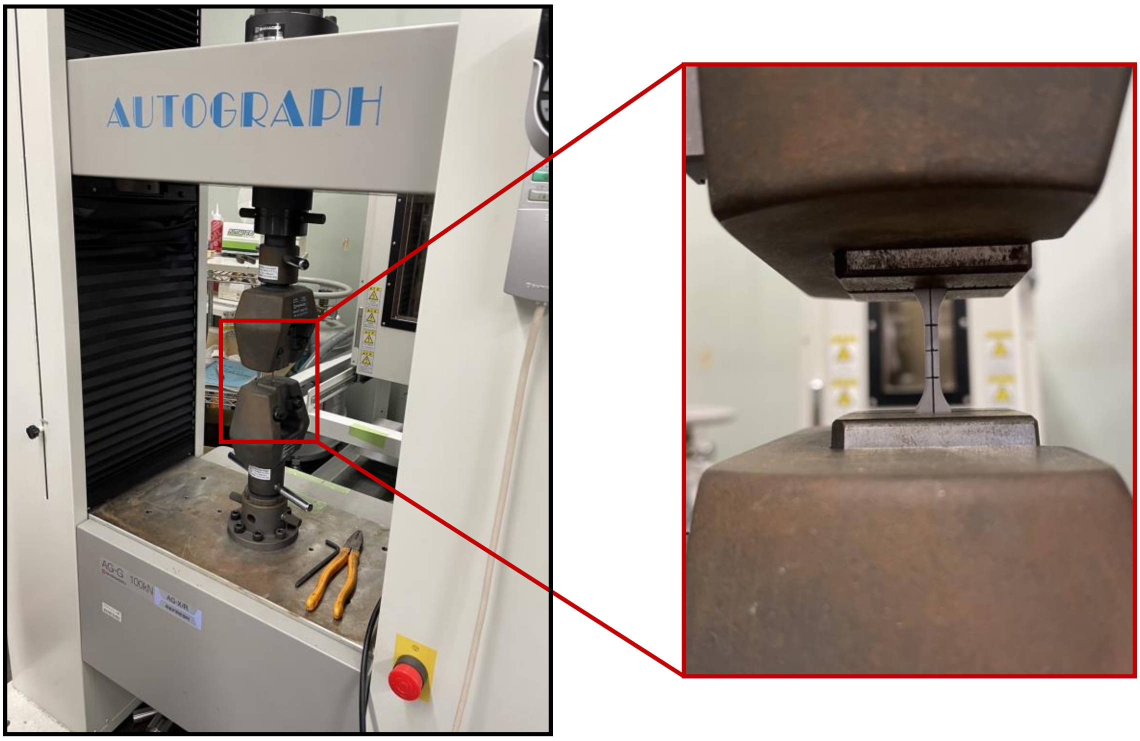
Appendix 5. Mechanical properties of implant plate

① Elastic modulus measurement

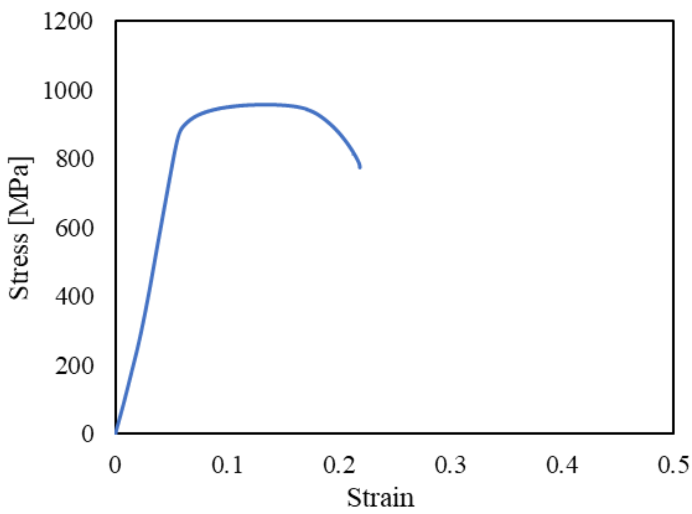
The value of TNTZ alloy measured is 61.63 GPa. It is concluded that the TNTZ regained its initial properties after solution treatment. On the other hand, the elastic modulus of Ti-64 alloy is 112.66 GPa. The value is about the same as stated in the previous study, so every calculation and discussion was made using 60 GPa, and 100 GPa, respectively.



② Tensile properties



Stress-strain curve of Ti-64 alloy



Stress-strain curve of TNTZ alloy

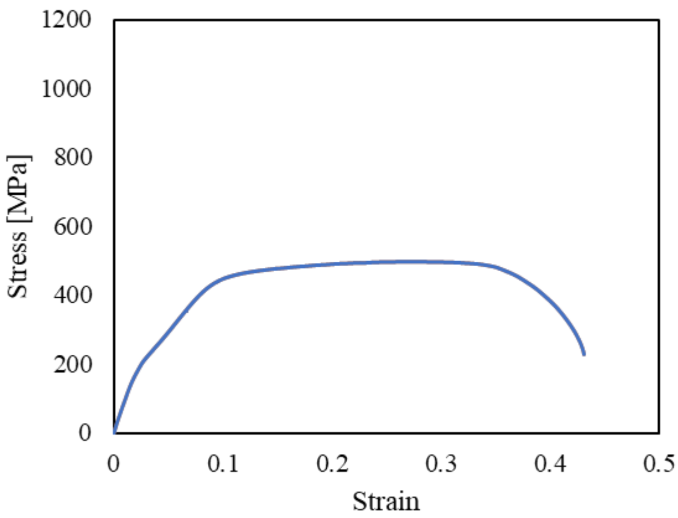
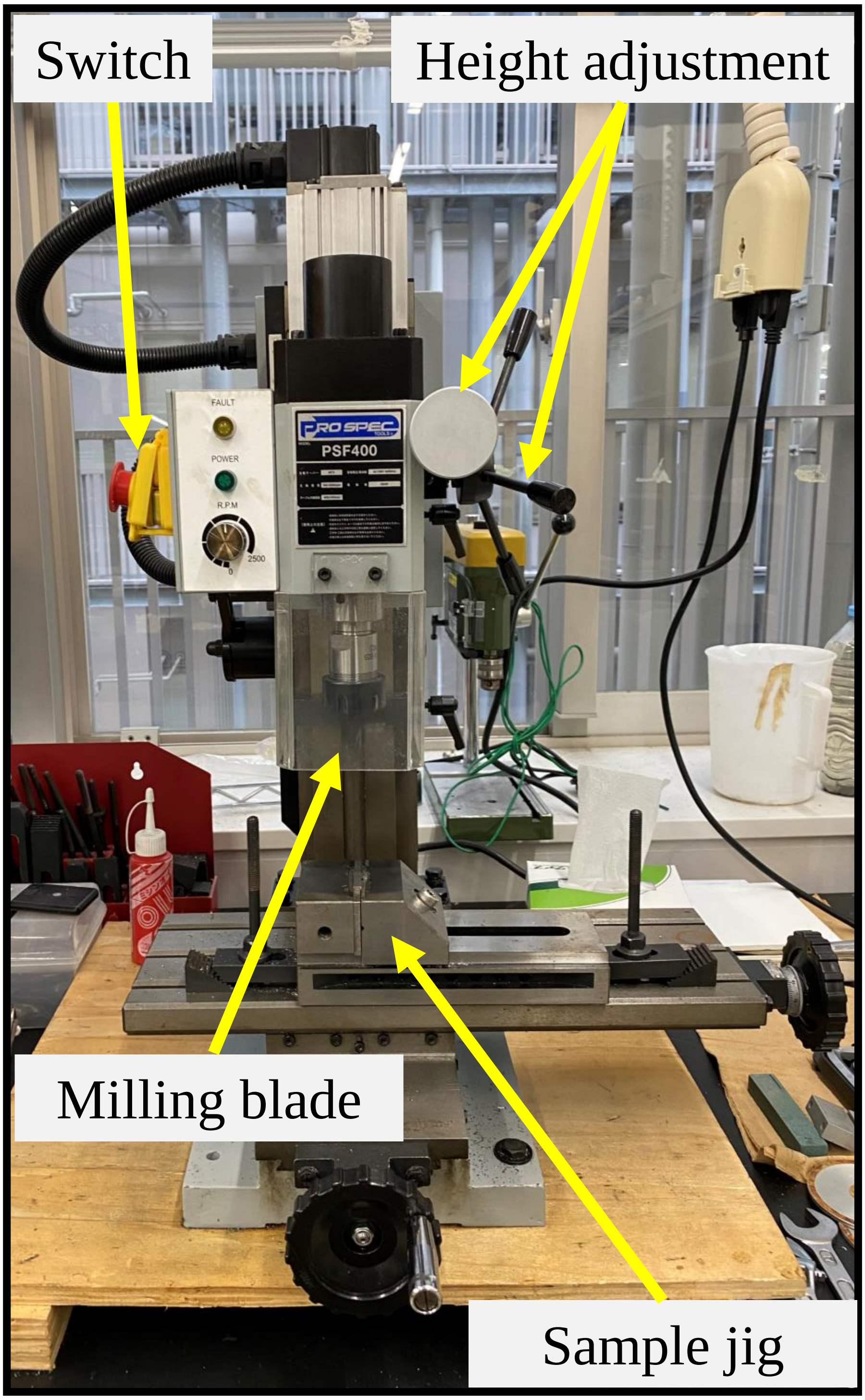


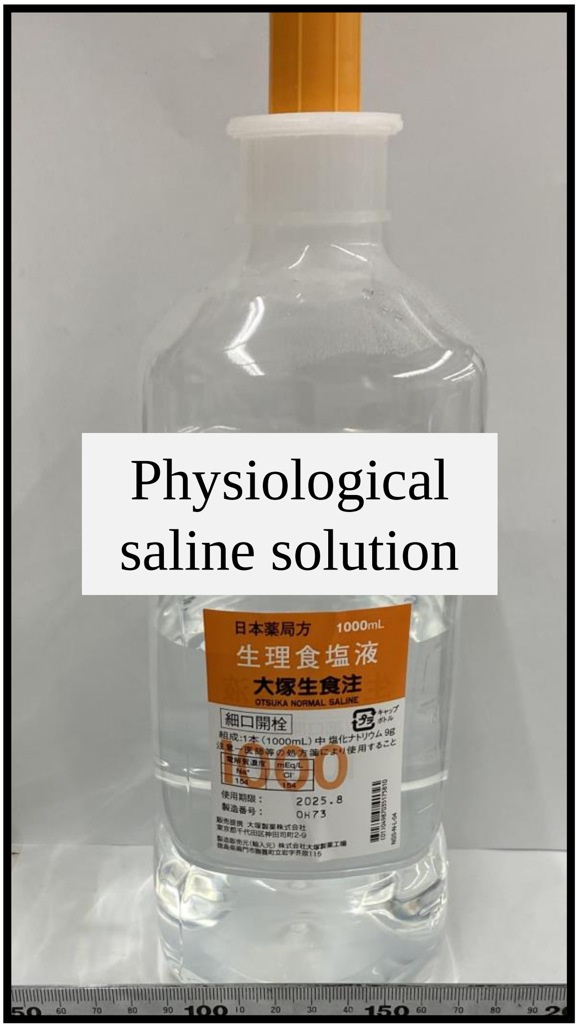
Table Tensile properties of Ti-64 alloy and TNTZ alloy			
Materials	Tensile strength [Mpa]	Elongation [%]	0.2% proof stress
Ti-64	958.0	16.20	884.5
TNTZ	496.9	35.23	423.5

The tensile properties for both alloys in present study in in the range of generally published in other studies. Due to this, the alloys in this study are eligible for comparison with other works.

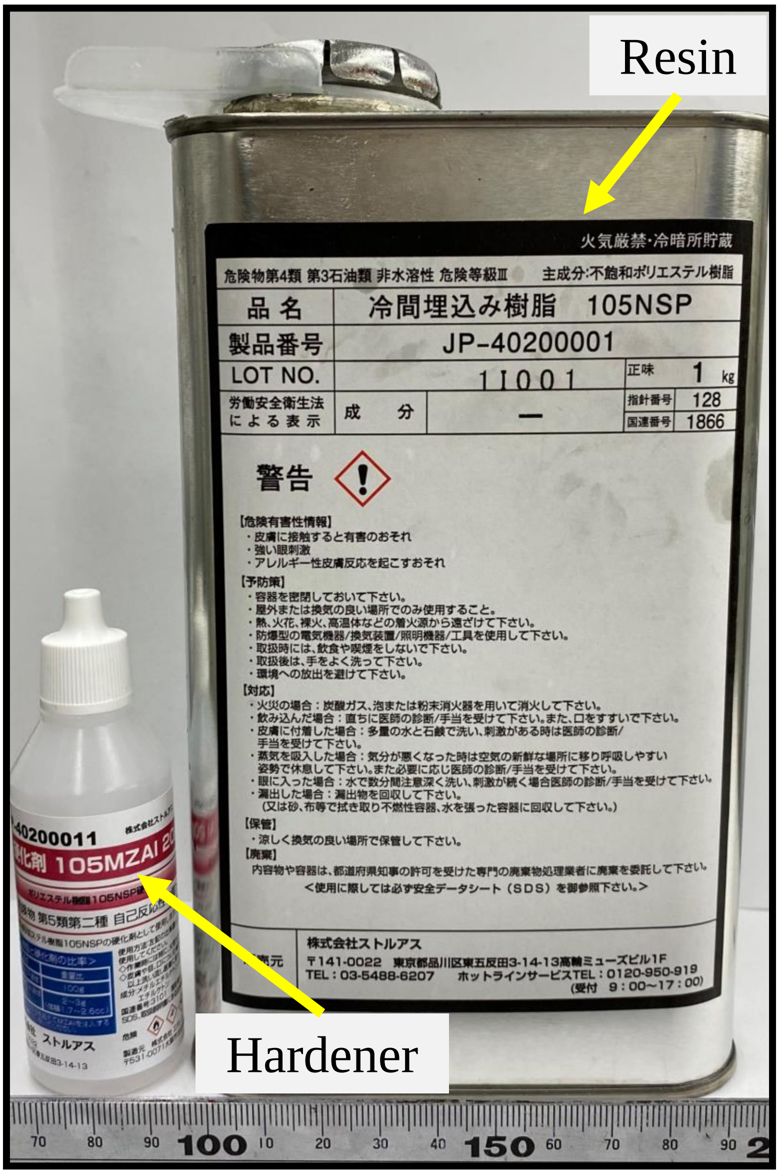
Appendix 6. Milling machine



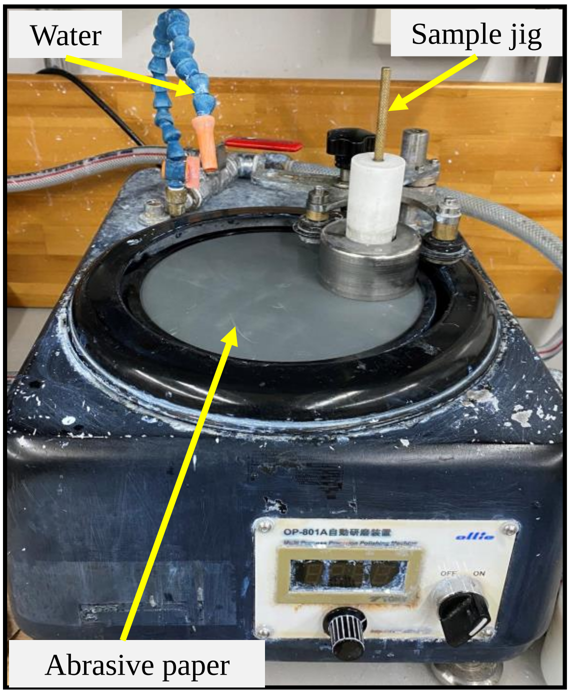
Appendix 7. Bone immersion liquid

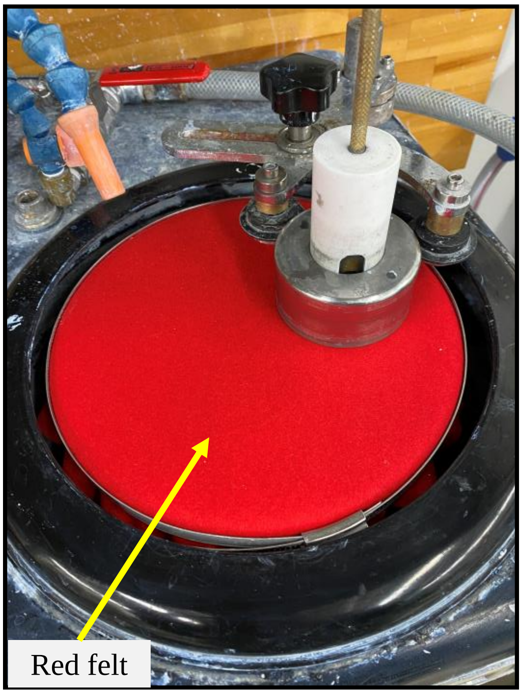


Appendix 8. Resin embedding



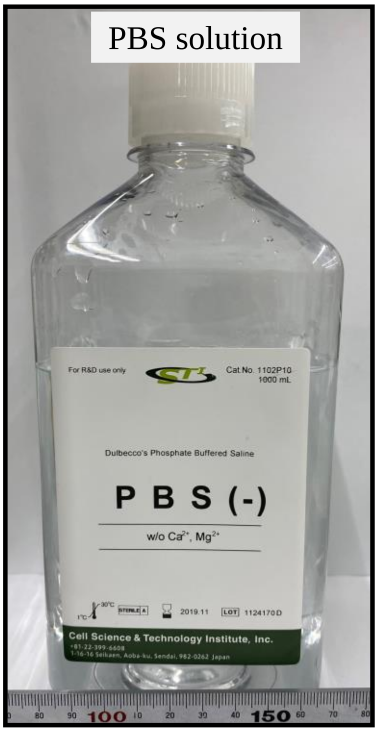
Appendix 9. Bone polishing sample

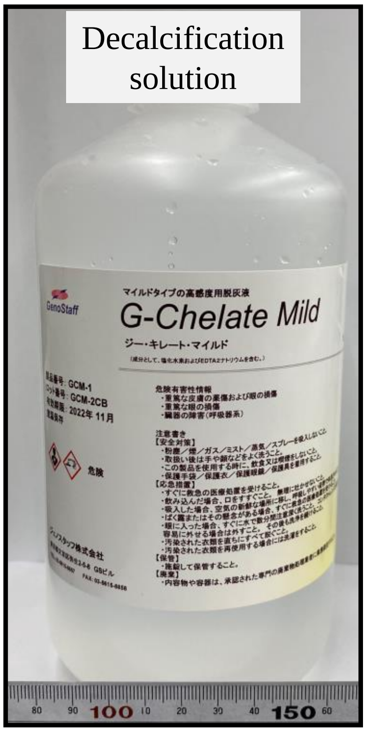




Appendix 10. Carbon coating machine

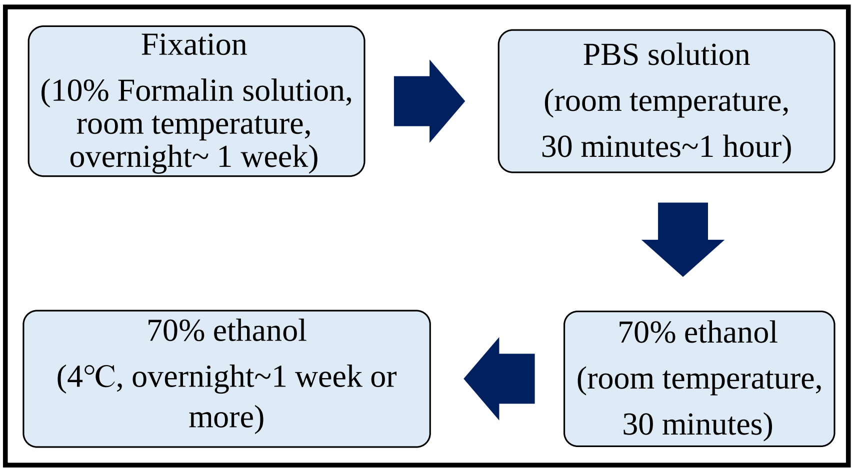
Appendix 11. Decalcification reagent etc.



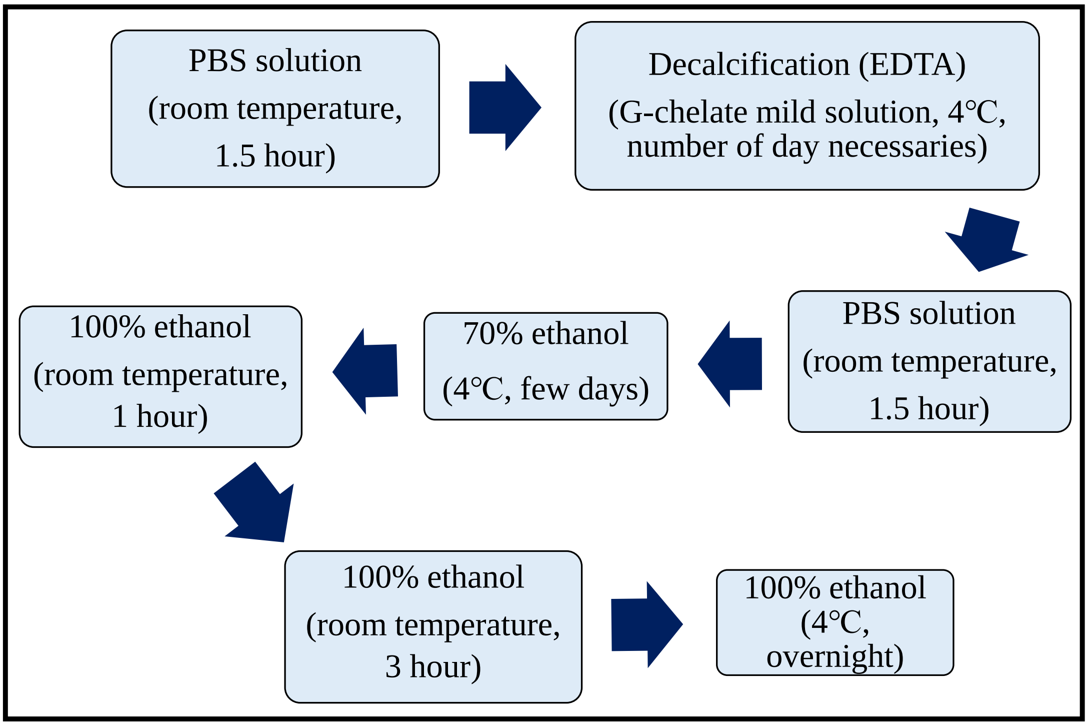


Appendix 12. Bone sample preparation protocol before staining

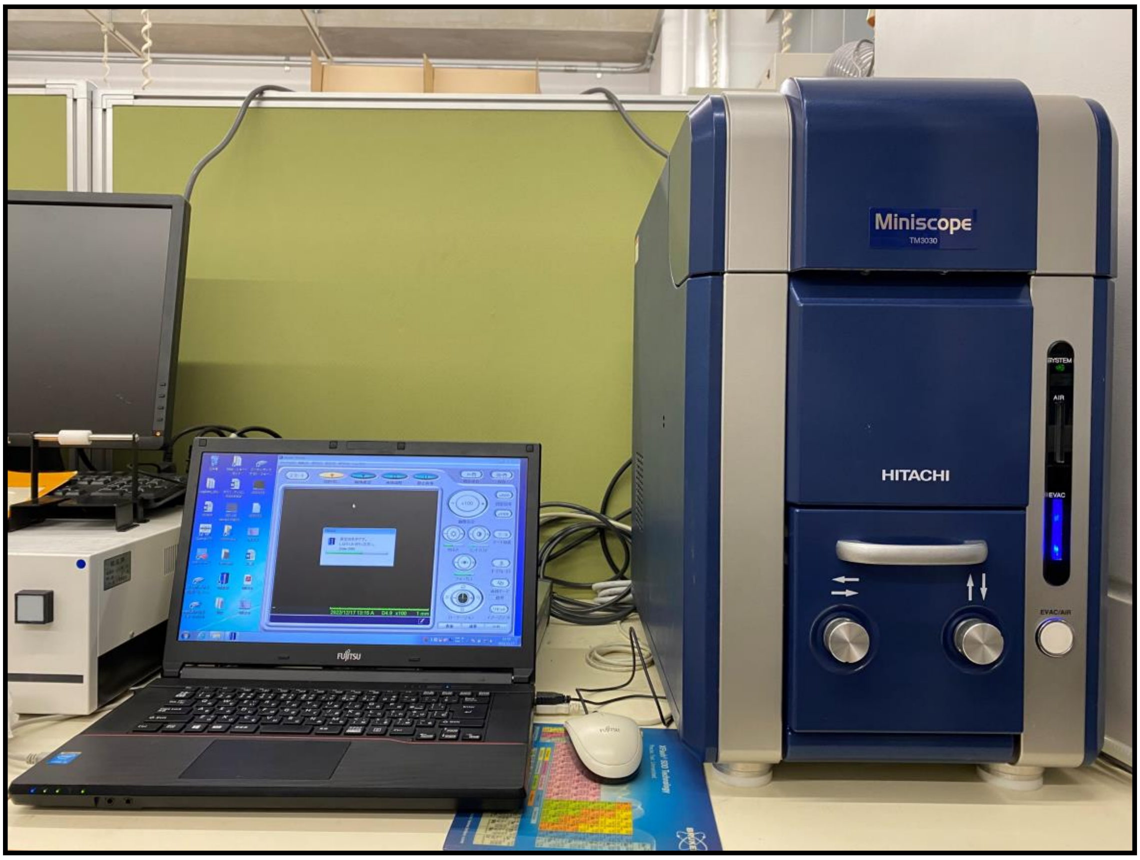
① Preparation



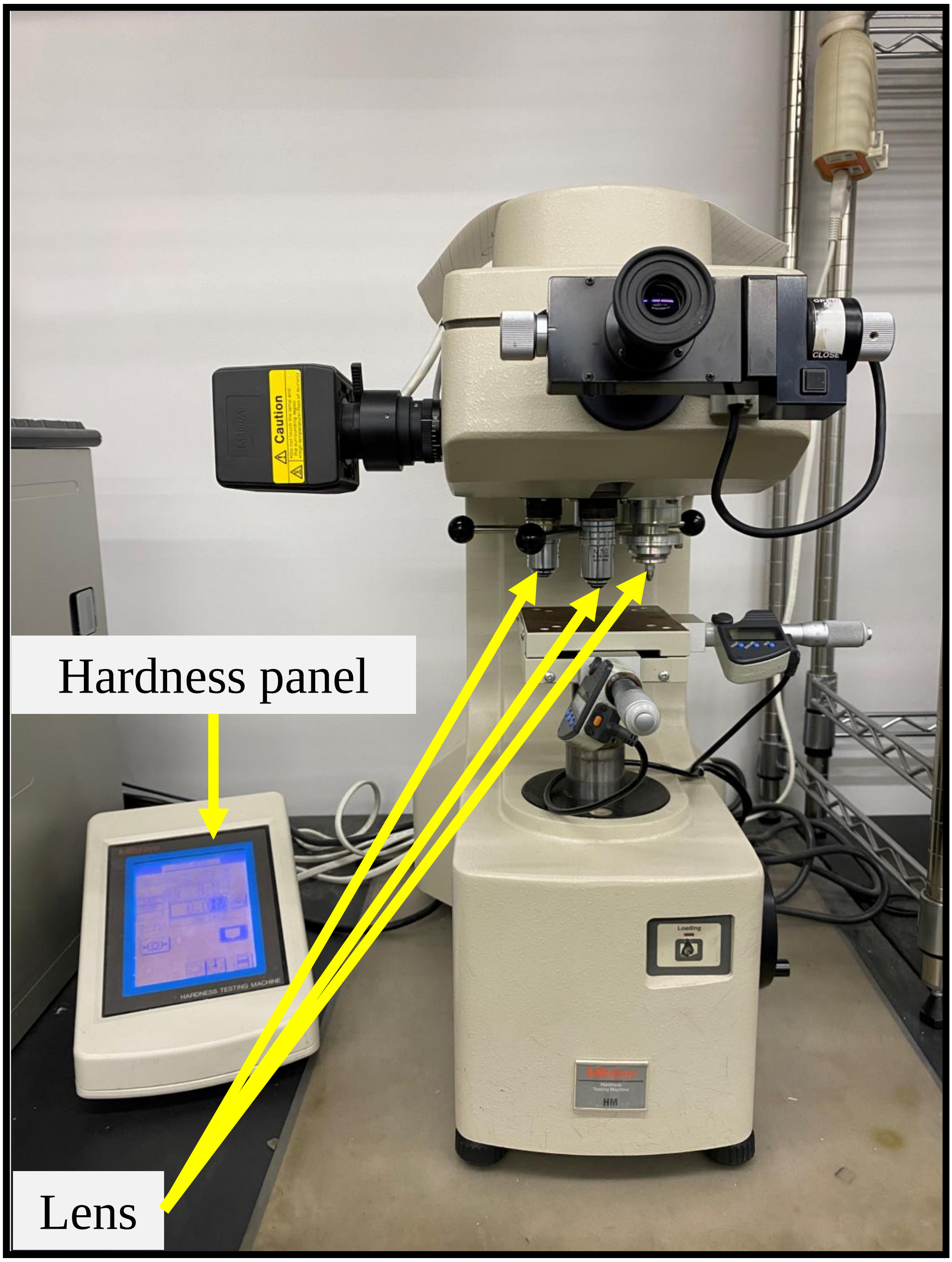
② Decalcification



Appendix 13. SEM machine

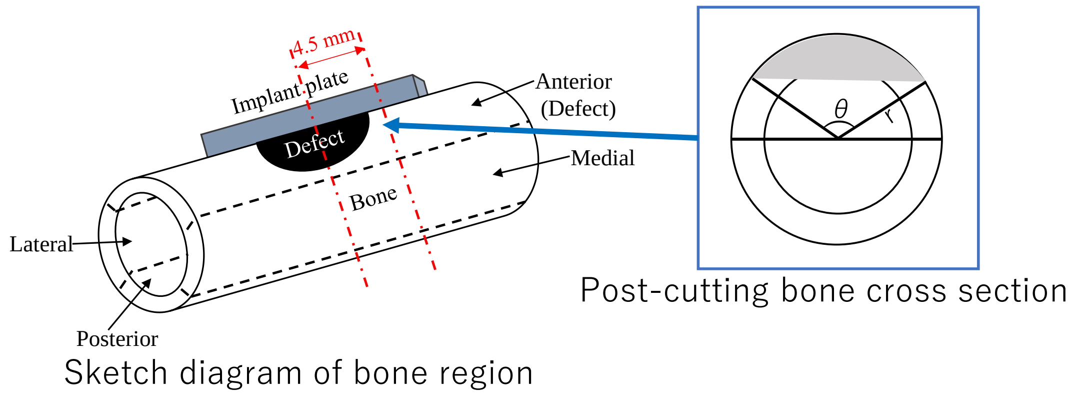


Appendix 14. Vickers hardness machine



Appendix 15. Vickers hardness machine

The stiffness of defect was calculated using the following mathematical formula. The elastic modulus of bone is assumed as 30 [GPa] and the radius of bone cross section is 6.0 [mm]. The angle was measured by Ansys 2022 R1 software which is 120°.



$$E\ x\ I = E\ x\ \frac{r^4}{8}\left(\theta - \sin\theta + 2\sin\theta\,\sin^2\frac{\theta}{2}\right)$$

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List of Research Activities

Conferences

International:

[1] Norain Binti Abdullah, Masaaki Nakai, Yuki Kawamura, Ei Yamamoto, Mitsuo Niinomi: Effect of titanium plate fixation on bone healing, 14th World Conference on Titanium, Nantes, France. 10th-14th June 2019. (Poster presentation, peer review)

[2] Norain Abdullah, Daisuke Miyazaki, Ei Yamamoto, Kosuke Ueki, Masaaki Nakai: Bone formation pattern after titanium alloy implantation in rabbit femur during early stage of healing, International Workshop on Advanced Experimental Mechanics for Students and Young Researchers 2022, Online. 25th-26th November 2022. (Oral Presentation, peer-review)

Domestic:

[1] Norain Binti Abdullah, Masaaki Nakai, Yuki Kawamura, Ei Yamammoto, Mitsuo Niinomi: Influence of Young's modulus difference of titanium alloys on bone healing, 2018 Autumn Annual Meeting of The Japan Institute of Metals and Materials, Sendai, Japan. 19th-21st September 2022. (Poster presentation, peer review)

[2] Norain Binti Abdullah, Masaaki Nakai, Yuki Kawamura, Ei Yamamoto: Evaluation of bone properties after titanium plate fixation, 2019 Masters in Graduate School of Science and Engineering, Osaka, Japan. 28th February 2019. (Poster presentation, no peer review)

[3] Norain Binti Abdullah, Masaaki Nakai, Yuki Kawamura, Ei Yamamoto: Effect of titanium plate fixation on bone formation during healing period, 2019 Spring Annual Meeting of The Japan Institute of Metals and Materials, Tokyo, Japan. 20th- 22nd March 2019. (Poster presentation, peer review)

[4] Norain Binti Abdullah, Masaaki Nakai, Kosuke Ueki, Yuki Kawamura, Ei Yamamoto, Mitsuo Niinomi: Effects of Young's modulus difference of titanium alloys and bone on bone formation, 2019 Autumn Annual Meeting of The Japan Institute of Metals and Materials, Okayama, Japan. 11th -19th September 2019. (Oral presentation, peer review)

[5] Norain Abdullah, Daisuke Miyazaki, Ei Yamamoto, Kosuke Ueki, Masaaki Nakai: Effects of titanium plate fixation on callus formation during early stage of bone healing, The 4th Meeting of The Japan Institute of Metals and Materials, Division 7: Biological Body, Medical Care and Welfare, Online. 11th December 2021(Poster presentation, no peer review)

[6] Norain Binti Abdullah, Daisuke Miyazaki, Ei Yamamoto, Kosuke Ueki, Masaaki Nakai: Effects of elastic modulus differences between implant plate and bone on bone formation during early stage of fracture healing, 2022 Spring Annual Meeting of The Japan Institute of Metals and Materials, Online. 22nd March 2022. (Poster presentation, peer review)

[7] Norain Binti Abdullah, Daisuke Miyazaki, Ei Yamamoto, Kosuke Ueki, Masaaki Nakai: Effect of titanium alloy fixation on new bone formation, 2022 Masters in Graduate School of Science and Engineering, Osaka, Japan. 16th -21st May 2022. (Oral presentation, no peer review)

[8] Norain Binti Abdullah, Daisuke Miyazaki, Ei Yamamoto, Kosuke Ueki, Masaaki Nakai: Effects of elastic modulus differences between titanium alloys and bone on new bone formation during early stage of healing, The 61st Annual Conference of Japanese Society for Medical and Biological Engineering, Niigata, Japan. 28th-30th June 2022. (Organized session, peer review)

[9] Norain Binti Abdullah, Daisuke Miyazaki, Ei Yamamoto, Kosuke Ueki, Masaaki Nakai: Effect of implant plate stiffness on callus formation pattern during bone healing, 2022 Autumn Annual Meeting of The Japan Institute of Metals and Materials, Fukuoka, Japan. 20th-23rd November 2022. (Oral presentation, peer review)

Awards

[1] Norain Binti Abdullah: Young Student Award, The Japan Institute of Metals and Materials & The Iron and Steel Institute of Japan, March 2018.

[2] Norain Binti Abdullah: Best Poster Award, Masters in Graduate School of Science and Engineering, February 2019.

[3] Norain Binti Abdullah: The Metals Best Poster Award, 2019 Spring Annual Meeting of The Japan Institute of Metals and Materials, March 2019.

[4] Norain Binti Abdullah: Best Poster Award, The 4th Meeting of The Japan Institute of Metals and Materials, Division 7: Biological Body, Medical Care and Welfare, December 2021.

[5] Norain Binti Abdullah: Outstanding Oral Presentations, International Workshop on Advanced Experimental Mechanics for Students and Young Researchers 2022, November 2022.