STUDY ON ELECTROMYOSTIMULATION INFLUENCING MECHANICAL AND MICROSTRUCTURAL PROPERTIES OF BONES BEYOND THE STIMULATED SITE

DISSERTATION

STUDY ON ELECTROMYOSTIMULATION INFLUENCING MECHANICAL AND MICROSTRUCTURAL PROPERTIES OF BONES BEYOND THE STIMULATED SITE

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Abstract

Electromyostimulation is a nonpharmacological prevention method for osteoporosis that is safe and feasible for the elderly and people with physical disabilities. In the previous study, the random pulse train (RdPT) electromyostimulation at the rat quadriceps induces the mechanical properties not only at the stimulated femoral neck but also the unstimulated contralateral femoral neck. This brought a new hypothesis about the possibility of electromyostimulation in inducing the mechanical properties of bones beyond the stimulated site. The aim of this study is finding the possibility if the electromyostimulation could induce the mechanical properties of bones beyond the stimulated site. In the first study, the RdPT electromyostimulation hadn't shown its effectivity in inducing the mechanical properties of the long bones' diaphyseal in a whole-body scale. In the second study, the RdPT electromyostimulation showed its capability to influence the mechanical properties of vertebra but it worked specifically. Only the stiffness of the L2 was increased. Additional comparator testing with μCT scan also shows the influence of the RdPT electromyostimulation on the mineral content or the bone volume of the L2, but not the bone mineral density. This influencing on distant bones suggests nerve involvement in this process. On the other hand, the PrPT electromyostimulation did not show any effect on these bones. In conclusion, the RdPT electromyostimulation is effective not only in the stimulated femur but also in the lumbar vertebrae depending on the vertebra's location.

Key terms: electromyostimulation, random pulse train, vertebral body, mechanical properties, bone microarchitecture

Chapter 1

Introduction

1.1 Background

1.1.1 Osteoporosis and Bone Fracture

Osteoporosis is the bone disorder that is characterized by low bone mass density or deterioration of bone's microarchitecture (figure 1.1). Generally, the bone mineral density (BMD) test result is the common testing to know the bone is osteoporotic or not by comparing the BMD with the average ideal BMD of the adult person. As the comparative score of BMD, the level of T-score will indicate the level of BMD or the level of osteoporosis. The T-score of osteoporosis is below -2.5 SD of the young adult.

a. Normal bone

b. Osteoporotic bone

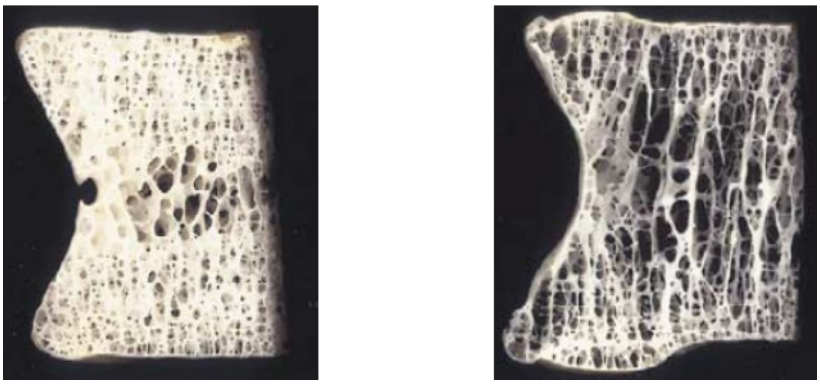

Figure 1.1 Comparison normal bone (a) and osteoporotic bone (b). The osteoporosis bone structure of 81-year-old-woman (b) is lesser dense and thinner than the normal bone structure of 37-year-old-woman (a) [1].

The osteoporosis can be occurred because of aging, diseases such as diabetes[2], lack of physical activity, disability, or long duration spaceflight[3] which results in bone fragile and easy to be broken. Because the osteoporosis symptoms are difficult to detect, the osteoporosis is not properly diagnosed until being checked by X-Ray when the fracture has occurred.

In the world, around nine million osteoporotic fractures were occurred in the year 2000 (figure 1.2). The most fracture was occurred at the forearm (1.7 million) and followed by the hip (1.6 million) and the vertebra (1.4 million)[4]. The fractures were occurred mostly because of falling and vertebrae collapsing progressively which were initialized by initial fracture and continued by deformation which resulted in the losses of vertebrae's weight.

Site of fracture	Men	Women	Total	Percentage	F/M
Hip	490	1,137	1,627	18.2	2.3
Forearm	332	1,328	1,660	18.5	4.0
Spine	554	862	1,416	15.8	1.6
Humerus	178	528	706	7.9	3.0
Other sites	1,909	1,641	3,550	39.6	0.9
Total	3,463	5,496	8,959	100	1.6

Figure 1.2 Estimated number of fractures (in thousands) at some sites in the year 2000.
The biggest concern of fractures was at hip and spine (beside of forearm)[4]. F/M is female to male ratio.

1.1.2 Bone Structure and Bone Quality

Bone is a composite material that is built from organic matrix protein (50% of volume) and mineral phase (50% of volume). The weight of bone is mostly influenced by the mineral phase (75%) than the organic matrix (25%). Specifically, 90% of the organic matrix is collagen type 1, and on the other hand, the minerals phase is composed of

mostly hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. In the structural level, the bone is divided into the cortical bone (compact bone) and the trabecular bone (cancellous) bone. The different construction of cortical and trabecular bone influences the mechanical quality of a bone. Figure 1.3 describes the structure of bone from at macro level until sub-nanostructure.

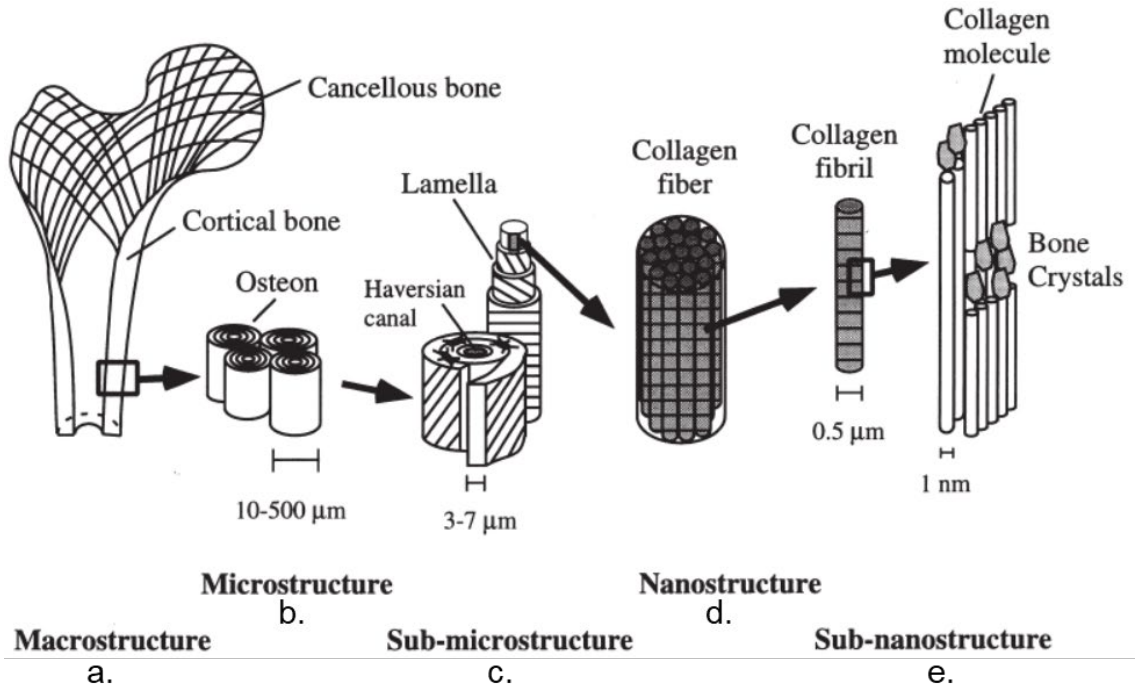

Figure 1.3 Hierarchy of a long bone structure.
It shows the structure of bone from macro level until sub-nanostructure[5].

The cortical bone is more stiff and solid than the trabecular bone. In the diaphysis, this bone more like groups of osteon shafts (figure 1.3b) where every osteon is formed by lamella cylinder with a haversian channel in the middle (figure 1.3c). The bone's mechanical properties and quality of this area are depended of these groups of osteons. In the middle of an osteon, there are channels which supply the blood and nerve, which are the Haversian channel (parallel direction with osteon) and the Volkmann channel

(perpendicular with osteon). Differently, in the diaphysis, the cortical bone in the epiphysis is more like thin shell which covers the trabecular (spongy) bone (figure 1.5) which is fulfilled the epiphysis.

Differently, with the cortical, the trabecular bone is composed of struts and bone marrow. The mechanical properties or the quality of a bone in the epiphysis area is depended on these struts' construction and these struts' mechanical properties. In an analysis, these struts are described as rods or plates (~5nm x ~5nm x ~40nm). The trabeculae in the bone influence 5-70% of the bone's density and 30-90% of the bone's porosity. Comparing the cortical bone in the diaphysis area, the post yielding of trabecular bone in the epiphysis area is more difficult to interpret because of the mechanical properties of trabecular bone decreases after the elastic region. The trabecular plays an important role in absorbing the mechanical energy[6]. In the mechanical testing result, the stress-strain curve of trabecular bone testing shows the mechanical quality of trabecular bone is depended on the trabeculae network and its materials properties.

The difficulties to understand the mechanical quality of a bone are coming from the point of view of bone tissue as a material and the point of view of bone as a structure. The quality of bone is coming from both as a material, which is reflected by the mechanical properties such as the strength, elasticity or toughness, and a construction which is reflected by geometry (size and shape). Because of that, it is complex to understand bone failure, such as which properties are more responsible for bone failure. Until now, it seems that the energy of failure (or toughness) is more dominant in the failure process[7].

Even though it is difficult to understand the bone fracture mechanisms, it is clear that the bone is damageable, a viscoelastic composite, and a living material that capable

of repairing by itself. Base on this characteristic of bone and its properties understanding, the bone disorder such as osteoporosis have capability to be prevent or to be cured.

1.1.3 Osteoporosis Treatment

Osteoporosis fracture, especially in elderly people, reduces the quality of life and life expectancy. This condition initiates a lot of researchers to investigate the way of prevention or treatment of osteoporosis. The most general way is consuming drugs and getting the result instantly, but unfortunately, the drugs have a side effect[8]. The high impact physical exercises such as walking, running or jumping, are often suggested in preventing osteoporosis, although the exercising effect is not as quick as pharmaceutical treatment.

However, exercising is not appropriate by people in special condition such as disability old people or bedridden patients. They have poor movement ability, or they have a special condition that forces them to stay at the bed in an all day. Plochoki (in vivo research experiment) found that the amount and the location of osteogenesis during the skeletal development were depended on by age, and the late adolescence is the optimal time to reach the maximum bone mass and strength[9]. More than that, it is difficult to maintain bone mass and strength. The other group is an astronaut in space that has a low gravity environment that causes bone loss. Therefore, before going to or after coming back from the outer space, an astronaut should do a routine load or low impact exercising to stimulate his bone, as well as in the shuttle space or outer space laboratory[10,11], to avoid bone fracture after landing (figure 1.4). It means they need to have a special time

and space in the space flight to do exercising. Therefore, an alternative method is required for people in these special conditions.

Figure 1.4 Exercising in the outer space.
Inconvenient exercising in the space with resistance piston-vacuum cylinder as weight substitute because of the absence of the gravity[10].

There are several alternative ways of preventing osteoporosis or bone loss, such as vibration, mechanical loading or magnetic field. The study of vibration showed the possibility of this treatment to prevent bone loss, but it still needs voluntary movement or a special place. On the other hand, in vivo study, the mechanical loading has shown its capability to induce osteogenesis through the mechanosensory system. The high bone strain is the key for the osteogenesis process. The loading at a bone also can influence the intramolecular pressure that influences the hormonal system which in influence the osteogenesis also. Unfortunately, this physiotherapy has an application limitation. The high strain in a bone because of mechanical loading is suspected enough to have capacity breaking the bone. The other physiotherapy for osteoporosis treatment is the magnetic

field, but this study hasn't shown the stability of the results of this physiotherapy to induce osteogenesis.

1.1.4 Electromyostimulation

Another alternative treatment is the electromyostimulation. It is described as an alternative to mechanical stimulation that can avoid bone fracture. The bone strain is occurred not because of stimulation but because of the mechanical force of muscles contraction at a bone, and this contraction is stimulated by electric current. It has been reported that this stimulation has the capability to induce muscle contraction and mechanical force in the bone via tendons[12], thus resulting in increasing bone formation[13], suppressing the bone loss of the osteoporosis model[14], or decreasing muscle mass loss in denervation conditions[15]. Although the interactions between bone and muscle are still unclear, previous studies demonstrated that muscle contraction influences not only mechanical conditions but also blood circulation[16] and endocrine activity[17] in bones and muscles. Our previous study demonstrated that electromyostimulation-induced contraction forces are influenced by the frequency and resting time of electrical stimulation[12]. This influence is related to muscle fatigue during stimulation[18]. A critical factor in eliciting significant osteogenesis is the stimulation pattern of bones by muscle contraction; however, this topic is controversial because it likely has strong nonlinearity between the stimulation amount and the effectiveness[19,20]. One of the electromyostimulation types is random pulse train ectromyostimulation[21].

As the new pattern of electromyostimulation, the random electrical pulse train (RdPT) indicated has capability in inducing the mechanical properties by increasing not

only the strain energy of the stimulation femoral neck but also of the unstimulated contralateral femoral neck after left quadricep stimulation at rats[22]. Unfortunately, it is unclear that the RdPT could induce osteogenesis or increase the mechanical properties of bones at locations beyond the stimulation site. This study would investigate this occurrence.

1.2 Originality and Significance of This Study

As a physiotherapy osteoporosis treatment, the electromyostimulation has shown its capability to influence the mechanical properties or to induce osteogenesis without voluntary movement. This stimulation also hasn't shown side effect. It is an appropriate therapy for people in disability movement such as an elderly and a bedridden patient, or an astronaut who is in the microgravity environment. Unfortunately, the study of this stimulation is only at the stimulated site, but the effect of this stimulation on the locations beyond the stimulated site is unknown. The originality of this research is discovering the effect of the electromyostimulation on the mechanical quality of bones at locations beyond the stimulated site.

The significance of this research that this research will suggest a new clinically physiotherapy that one position of the stimulation will prevent osteoporosis of all bones in a whole-body scale. Moreover, unlike the drugs, this treatment doesn't have side effects and it is important for disability movement people such as a bedridden patient or an elderly to increase their life expectancy.

1.3 Hypothesis

There is a possibility that electromyostimulation may influence the mechanical quality beyond the stimulated site. It will suggest a new clinically physiotherapy that one position of the stimulation will prevent osteoporosis at all bones in a whole-body scale.

1.4 Objective of The Study

The aim of this study is to investigate the possibility of electromyostimulation to stimulate bone's mechanical qualities and structure beyond the stimulated site.

1.5 Structure of The Thesis

The thesis is divided into five chapters. Chapter 1 describes the research background of this study, the originality and the significance of this study, the hypothesis of this study, and the purpose of this study.

In chapter 2, the previous studying of the electromyostimulation is described. It is started with the beginning idea of this stimulation. It described not only general research of this stimulation, but also the latest research, including the research in the Bioengineering Laboratory-Kanazawa University.

Chapter 3 explains the study of the effect of electromyostimulation on the mechanical properties of diaphyseal long bones. The purpose of this study to investigate the effect of this stimulation on the distant long bones. This chapter found that this stimulation hasn't shown its effectiveness to induce the mechanical properties of distant long bones, even though it could increase the mechanical properties of cortical bone that it is known hard to be influenced.

Chapter 4 explains the study of the effect of electromyostimulation on the mechanical properties of lumbar vertebra. This study tried to investigate at the location which has mostly trabecular bone. Finally, in this study, this stimulation could influence the mechanical properties.

Chapter 5 summaries all study in this research and suggests the recommendation for future work.

Chapter 2

Electromyostimulation

2.1 Introduction

Because of the side effects of using drugs[8], the alternative ways of preventing osteoporosis are being looked. Nowadays, one of applicable way is the high impact exercising such as walking, running or jumping. The exercising is not only can increase the muscle's mass and size[23] or the blood flow in a bone[24–26], but also can increase the BMD[27,28] (figure 2.1). Unfortunately, the exercising unable being performed by people in disability condition such as old people or bed rest patients.

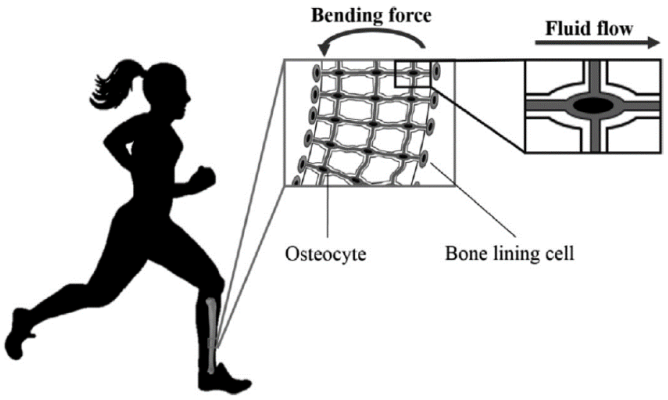

Figure 2.1 Bone strain and fluid flow because of exercising. Schematic representation of the effect of training not only inducing the mechanical loading at a bone cell but also causing interstitial flow[26].

Several researchers have studied to find alternative ways to prevent osteoporosis, such as vibration or mechanical stimulation. Oxlond et al., found that the low-intensity and high-frequency vibration could suppress the decrease in bone strength[29]. Robling et al.,

found that mechanical loading is giving more effect to the osteogenic response if the stimulation is divided into discrete loading bouts[30] (figure 2.2).

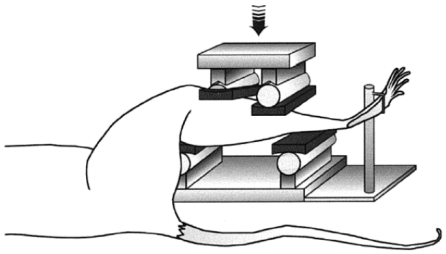

Figure 2.2 The mechanical stimulation at a rat's tibia. The discrete loading gives more effect to the osteogenic response[30].

Unfortunately, the mechanical stimulation has limitation base on Turner et al.'s research[31]. They reported 1050 μstrain of is the minimum value to induce osteogenesis. The implication of this limitation is applying this stimulation at the human with the risk of bone fracture in the stimulation application time. The limitation suggests finding the other alternative to prevent osteoporosis, such stimulate the muscle which is the muscle will give force loading to the bone via tendon connection.

This chapter describes the study of the electromyostimulation. It started with interaction bone and muscle as the beginning idea of electromyostimulation and followed by the study of electric stimulation. Finally, this chapter described the edge of previous research that will continue with this study.

2.2 Bone-Muscle Interaction

The correlation of bone and muscle was proposed by Burr[32], based on the cause and effect between muscle force and bone adaptation, and the relationship between

muscle mass and bone loss during aging. This analysis suggests the muscle strength as a factor of bone's gain and loss. Robling also proposed the possibility of muscle force as mechanical factor to induce osteogenesis[33]. On the other hand, by using an animal model, Nemirovskaya et al. showed that muscle contraction can prevent muscle atrophy[34]. Base on the theory that the biomechanical signal at the bone can be sensed because of muscle contraction via tendons (internal load) or load-bearing mechanism such as ground reaction force (external load), it suggests using of electric stimulation to stimuli the muscle and produces the mechanical stimulation to a bone.

2.3 Electromyostimulation; Electric Stimulation to Bone via Muscle

Previously, the idea of using electric stimulation was to cure mobilization because of Spinal Cord Injury (SCI). In the process, the electric stimulation not only has the capability to repair the injury but also can increase the muscle mass and its coordination. In parallel with muscle stimulation, the effect of electric stimulation on bone was started with studying the effect on this stimulation on the healing process on the fractured bone. Direct stimulation on the bone could induce the generation of new bone. The concept of electricmyostimulation was started when studying the effect of electrical stimulation on muscle and the effect of muscle stimulation on the bone. The muscle stimulation could induce muscle mass as well as bone mineral density. This coincident suggests the correlation between bone and muscle system. At the previous study of electromyostimulation, this stimulation not only can suppress bone loss[14,35,36] and muscle loss[15,37] but also induce the osteogenesis[13].

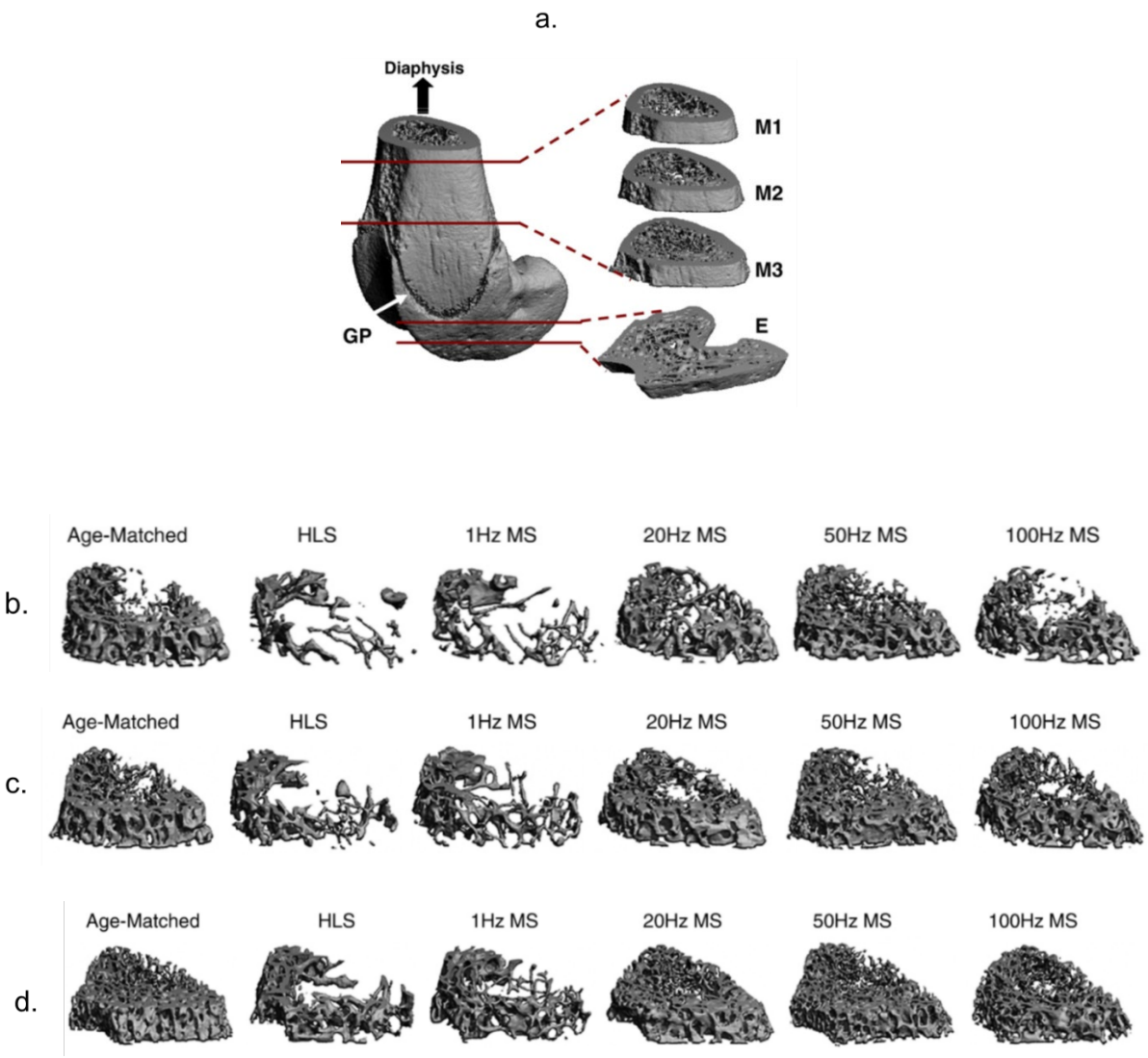

Figure 2.3 The effect of electromyostimulation on suppressing bone loss.
Trabecular bone with three metaphyseal sections (a) and the representative 3D μCT image of trabecular bone in M1 (b), M2 (c), and M3 (d) region[14].

The electric stimulation at the muscle in preventing bone loss was proposed by Lam and Qin by using 1 Hz, 20 Hz, 50Hz, and 100 Hz electrical stimulation[14]. The bone

loss was prevented by electric stimulation through muscle contraction (figure 2.3). The better result was shown by 20Hz, 50Hz and 100Hz[14].

Moreover, it has been demonstrated that muscle contraction because of electric stimulation can prevent muscle atropy[38]. Other researchers, Midura et al., by using 30Hz electrical stimulation, generated 200μstrain peak of dynamic compressive strain and suppressed tibia loss on hindlimb suspended rat[36].

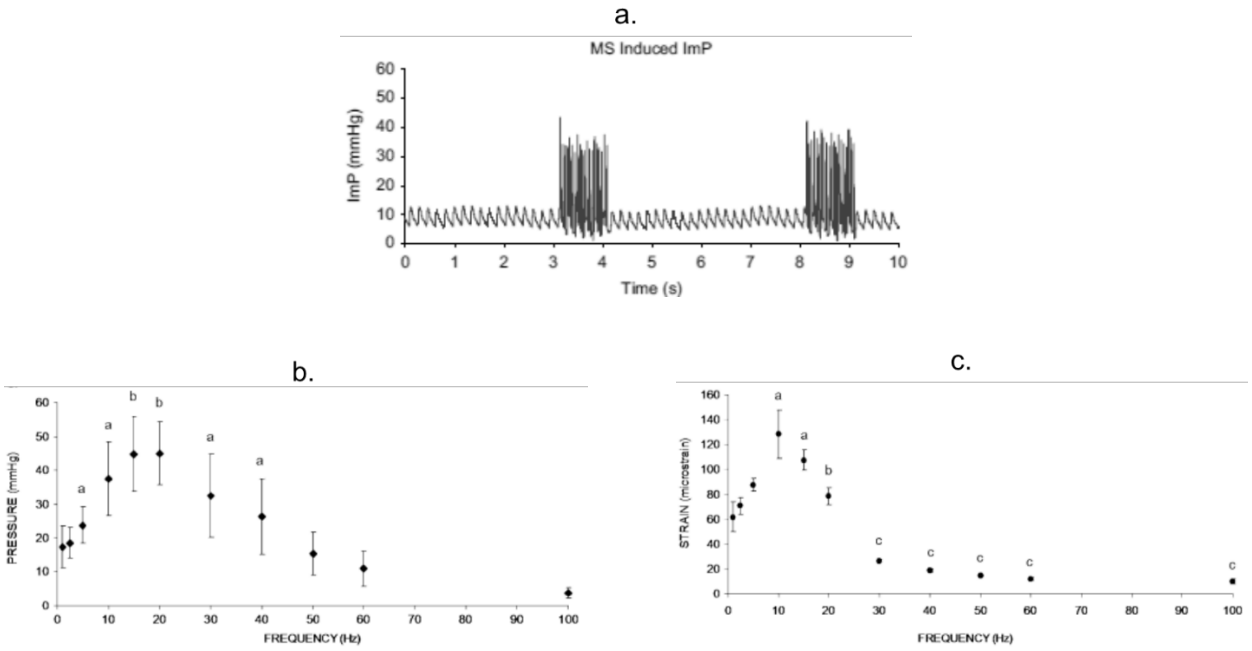

Figure 2.4 The effect of muscle stimulation on the Intramedullary Pressure (ImP) (a,b) and the matrix strain (c).
The 10-20Hz muscle stimulation increased the fluid flow's ImP and the bone matrix strain[39].

As well as the previous study, Qin et al., by using 1-100 Hz electrical stimulation, founded that the maximum Intramedullary Pressure (ImP) occurred at 20Hz and the maximum matrix strain occurred at 10 Hz[39] (figure 2.4). This founding confirms the

capability of electric stimulation to suppress bone loss (especially trabecular) and to maintain the bone microarchitecture at the osteoporotic model (figure 2.5).

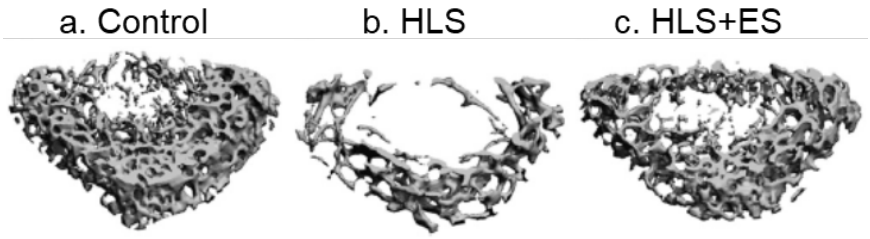

Figure 2.5 Comparison μCT images of trabecular bone of age-matched control (a), Hindlimb Suspension (HLS) (b), and HLS + 20 Hz muscle stimulation (c).
Muscle stimulation (ES) suppressed bone loss at Hindlimb Suspension model[39].

2.4 Noise Electromyostimulation

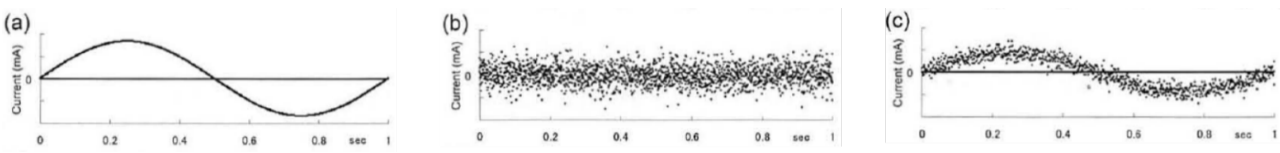

Figure 2.6 Study of electric muscle stimulation with noise stimulation.
Sinusoidal (a), noise (b), and sinusoidal + noise electric muscle stimulation[40].

The electromyostimulation has been also developed in our laboratory (Bioengineering Laboratory, Kanazawa University). In the beginning, as a prototype of stimulation, the noise electric stimulation (figure 2.6) showed its effectivity to prevent bone losses in the 4-weeks osteoporosis model comparing with sinusoidal electric stimulation[40](figure 2.7).

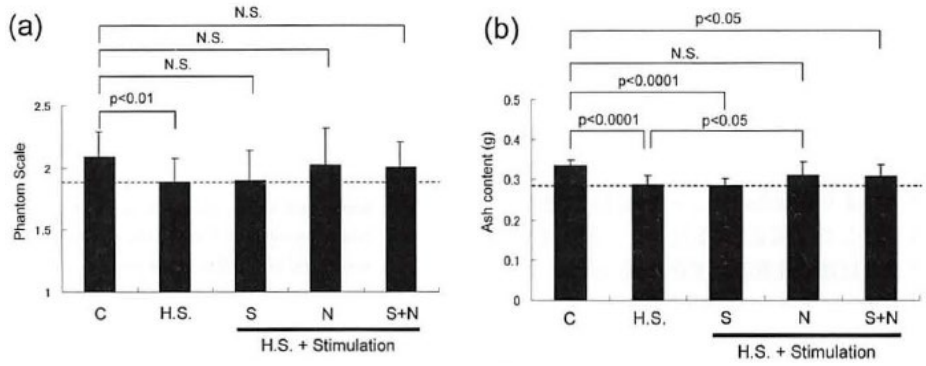

Figure 2.7 Effect of electric muscle stimulation on bone density (a) and bone mass (b).
Bone loss in osteoporosis model could be suppressed by giving a noise signal[40].

In the gene expression level, the electromyostimulation in a specific muscle contraction frequency could induce the expression level osteocalcin mRNA. Although, the highest number of muscle contraction was at the 40 Hz (figure 2.8 a) and the highest number of average peak-to-peak force was at 2 Hz (figure 2.8 b), the highest gene expression of osteocalcin was shown by 20 Hz contraction (figure 2.8 c) if compared with the other frequencies which are 2, 10, 40, and 80 Hz. These results show the 20 Hz muscle contraction is most appropriate muscle contraction to induce osteogenesis[13]. Furthermore, by using finite element analysis, the direct effect of muscle contraction on bone showed approximately 320 μstrain compression at the surface of midshaft and maximum 173 μstrain compression at the femoral neck[41].

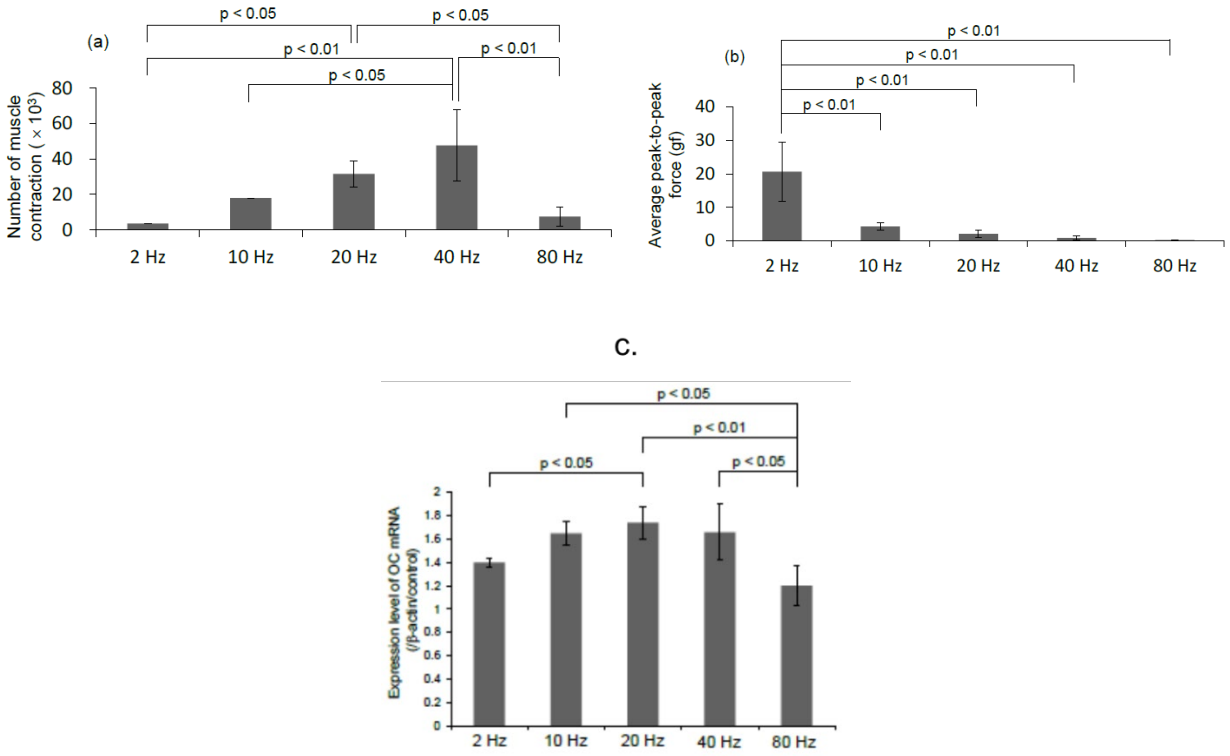

Figure 2.8 Effect of muscle contraction frequency on the number of muscle contraction, average peak-to-peak muscle force, and osteocalcin's gene expression.
The most number of muscle contraction was shown at 40 Hz, the highest peak-to-peak was shown at 2 Hz, but the biggest effect on osteogenesis was shown at 20 Hz[13].

Supporting the previous investigation, in in-vitro studying, the noise electric stimulation looked has the capability in inducing osteogenesis. The noise stimulation rose the alkaline phosphate (ALP) activity in regenerated bone compared with unstimulated one[21] (figure 2.9). This suggests the superior of this stimulation than periodic stimulation.

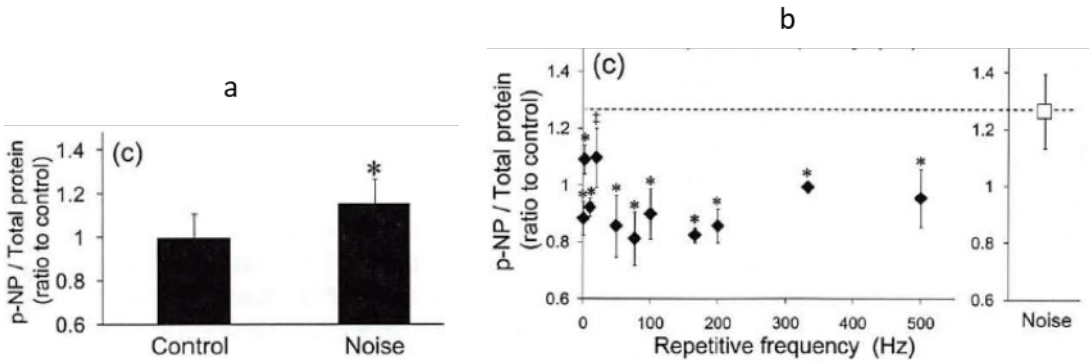

Figure 2.9 Comparison of ALP activity between control and noise stimulation (a); and between periodic stimulations and noise stimulation.
Noise stimulation gave more effect on producing new bone than control or periodic repetitive frequency[21].

2.5 Random Pulse Train Electromyostimulation

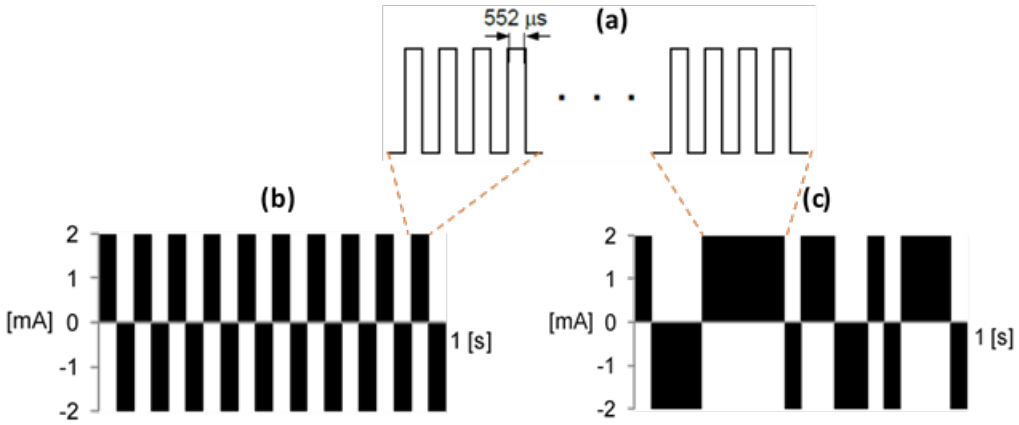

Figure 2.10 Periodic Pulse Train (PrPT) and Random Pulse Train (RdPT) Electromyostimulations.
Pulse train of electric stimulation (a), which then two type electric stimulations which are PrPT (b) and RdPT (c). Both PrPT and RdPT have same pulse train but different in the reversing polarity time[22].

There are two types of electromyostimulation that are developed at the Bioengineering Laboratory Kanazawa University. They are the periodic pulse train (PrPT),

which is common electric stimulation, and the random pulse train (RdPT) electrostimulation (figure 2.10) as developing research from noise stimulation. Both have similar pulse train but different in controlling the reverse polarity time.

Comparing to the periodic pulse train (PrPT) electromyostimulation, the polarity changing time is always changing and the period of a pulse train also is always changing. The duration of each RdPT pulse train appearance was determined from the probability of geometric distribution which followed the formula[22]:

$$\Pr(d) = p\,(1-p)^{k-1}$$

where: Pr is the appearance probability of train duration (figure 2.11)
d is function dependent of the polarity reversal (p)
p is 0.5
k is the positive integer (k = 1, 2, 3, …)

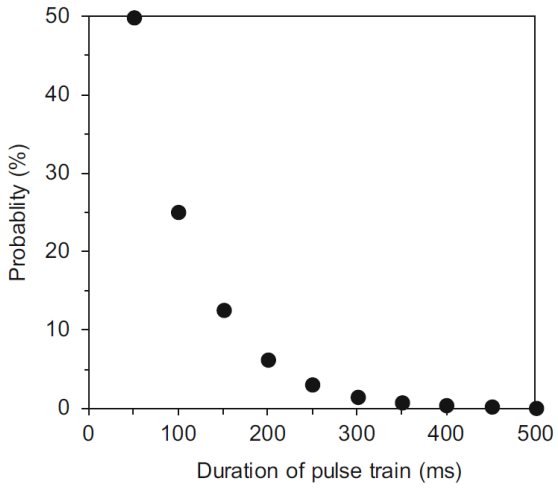

Figure 2.11 The appearance probability of every pulse train duration in RdPT[22].

The study, by using both PrPT and RdPT, showed the capability of both PrPT and RdPT inducing osteogenesis in the mid-diaphyseal (figure 2.12) and the femoral neck (figure 2.13). Base on the Turner et al.'s research that the 1050 μstrain is the minimum

requirement for inducing osteogenesis[31] and the previous study about electric induced muscle contraction at the Bioengineering Laboratory – Kanazawa University[41] show that the compressive strain that could be generated was 320 μstrain in average[41] but the stimulation could induce osteogenesis[13]. It suggests the bone strain is not only the primer cause of osteogenesis.

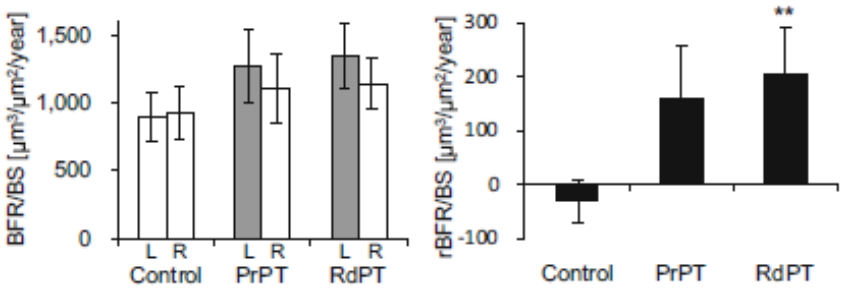

Figure 2.12 Effect of electromyostimulations on bone formation rate at mid-diaphysis.
Both electromyostimulations (PrPT and RdPT) increased the bone formation rate at stimulated diaphyseal area (mostly cortical bone)[22].

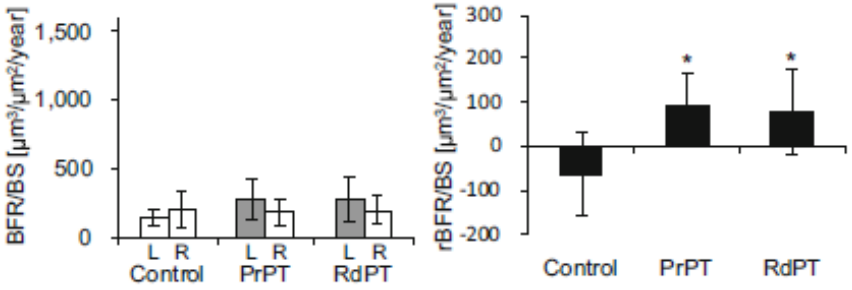

Figure 2.13 Effect of electromyostimulations on bone formation rate at femoral neck.
Both electromyostimulations (PrPT and RdPT) increased the bone formation rate at femoral-neck stimulated area (mostly trabecular bone)[22].

Furthermore, interestingly the RdPT have capability of increasing the mechanical properties of the femoral neck (figure 2.14). The maximum load and the energy of failure were increase because of RdPT stimulation, and it wasn't shown in the PrPT group. It suggests the capability of RdPT to influence the mechanical properties more than PrPT by influencing the maximum load and the energy of failure of bone matrix.

Moreover, the interesting phenomenon was found when the RdPT did not only induce the mechanical property that was the energy of failure at the stimulated femoral neck but also at the contralateral unstimulated femoral neck (figure 2.14). This phenomenon suggests the contribution of nerve in osteogenesis process. On the other hand, the PrPT didn't show the same phenomenon as the RdPT. It means that the RdPT possibly also induced the bone matrix at distant bones. Functional adaptation of bones by stimulating a single bone mechanically had ever been proposed by Sample et.al[42]. They suggested neuronally regulation in functional adaptation and it involves multiple bones. But it is still controversial. Differently with Sample et.al's research, Sugiyama et.al's research argued it and recommended the contralateral bone as the control[43] because it is not loaded so it doesn't have strain. Functional adaptation is locally phenomenon and could be extended depending on the strain intensity which will affect on.

Although it is still controversial, the mechanical test at the femoral neck (figure 2.14) and the bone histomorphometry at the femoral neck (figure 2.13) showed the possibility of RdPT to influence the mechanical properties more than PrPT. It showed also that the BMD is not the only determinant of bone quality and it gave a hypothesis that the RdPT might influence the osteogenesis by inducing generation of type I collagen fiber by

controlling the enzymatic crosslinking, lysyl oxidase, which is secreted by osteoblast. The RdPT electromyostimulation may stimulate the muscle spindle that generates the afferent neuron signal via the spinal cord. This signal may promote collagen fiber and its enzymatic crosslinking LOX.

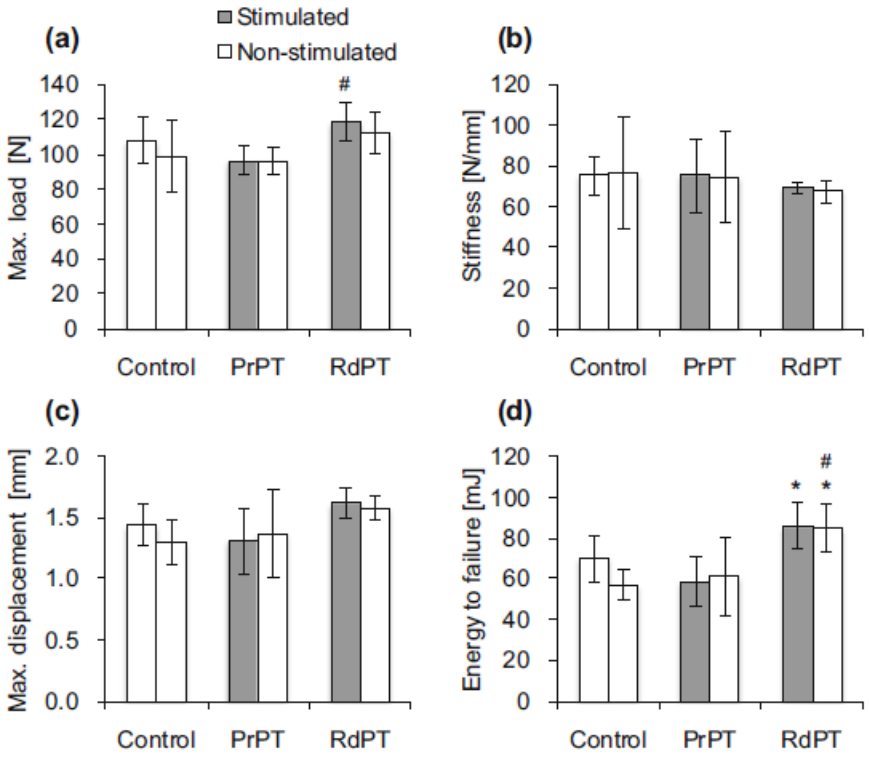

Figure 2.14 Effect of electromyostimulations on mechanical qualities of the femoral neck.
Only RdPT increased the mechanical qualities of the femoral neck stimulated area. Interestingly, the mechanical properties of the contralateral unstimulated femoral neck also were influenced by this stimulation[22].

2.6 Summary

By using the muscle-bone interaction, the electromyostimulatin not only showed its capability to suppress muscle loss or to induce muscle mass but also to suppress bone

loss or induce osteogenesis. Moreover, the RdPT made an impression that it could influence unstimulated the contralateral bone also. This RdPT phenomenon will bring this study to a new hypothesis that the bones could be stimulated by only one stimulation's location.

Chapter 3

Effects of Electromyostimulation on Mechanical Properties of Diaphyseal Long Bones Apart from the Stimulated Site

3.1 Introduction

The electromyostimulation has been introduced as a new treatment to prevent osteoporosis. In the stimulated site, it is clear that this stimulation has the capability to suppress bone loss[14,36,44], to induce osteogenesis[21,22,35], or to induce intramedullary pressure in a bone that can influence the osteogenesis[39]. On the other hand, the influence of this stimulation on the site beyond the stimulated site is still unclear[22].

The purpose of this study was to investigate the influence of the electromyostimulation on the mechanical properties of bones beyond the stimulated site which was in a whole-body scale. The object of this study were the long bones which was femora, tibiae, humerus, and ulnas-radii. This selection was based on the equality condition of the same testing that will be used. To get the mechanical properties results, the four-point bending test were chosen based on the heterogeneity structure of the long bones. In keeping the equality condition with the previous experiment, the procedures of sample preparation were the same as the previous study[22].

This chapter covers the influence of the electromyostimulation at the left quadriceps on the all long bones at a whole-body scale which was femora, tibiae, humerus, and ulnas-radii. It also reports the body weight as the control of the rat condition because of the stimulation.

3.2 Materials and Methods

3.2.1 Animals

A week before treatment, 7-weeks-old female Sprague-Dawley rats were purchased and housed under standard laboratory condition to adapt their environment including the 24°C laboratory temperature and the 12 hours day-night cycle. The rats were divided into three groups of treatment, which were Control group, PrPT group, and RdPT group. Age-matched and same-sex rats were used to maintain and to control the equality of the results. In the laboratory the rats were provided with free access to do a daily activity such as free access to get food or water.

After seven days in the laboratory, the rats received the electromyostimulation in three days continuously. Fifteen days after stimulation, the rats were sacrificed and their long bones were collected (figure 3.1).

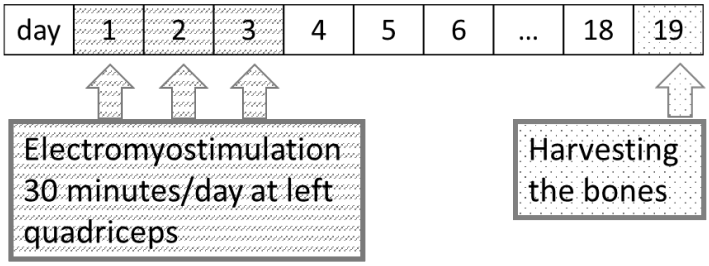

Figure 3.1 Experimental time schedule for animal handling.

Throughout the entire experiment period, the animals' body weights were monitored by measuring their weight at once in every week, prior to stimulating time, and

prior to be sacrificed. Moreover, prior to being stimulated and to being sacrificed, the rat was anesthetized by intraperitoneal injection with pentobarbital sodium (somnophentyl).

The animal treatment was performed according to guidelines from the Experimental Animal Institute of Kanazawa University and approved by the Committee on Animal Experimentation of Kanazawa University with Approval No. AP-173865.

3.2.2 Electromyostimulation to Rat Quadriceps

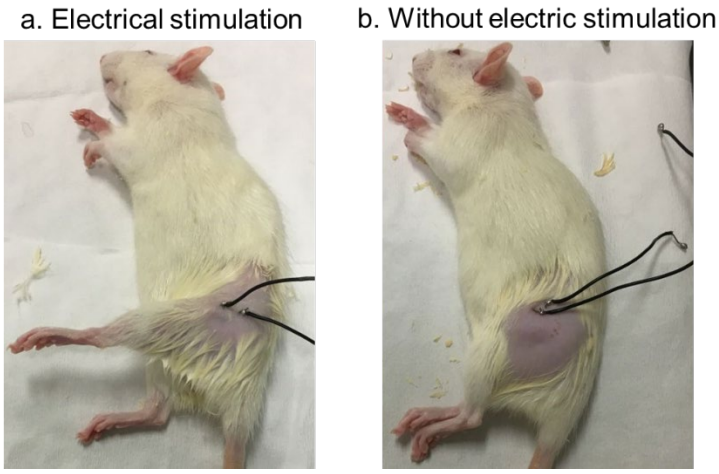

Figure 3.2 Insertion needle electrodes at the left quadriceps. The muscle was contracted because of electric stimulation (a) and not contracted because of no electrical stimulation (b).

Prior to the stimulation, an 8-weeks rat was anesthetized with an intraperitoneal injection of pentobarbital sodium (somnophentyl) with doze 40mg/kg which was diluted with saline water. After falling asleep, in the lateral decubitus position, the left quadriceps of anesthetized rat was injected by the stainless-steel needle L-electrodes (26G x ½" needle, Terumo NN-2613S). For the rat at the PrPT group or the RdPT group, the electromyostimulation was applied at the quadriceps 30 minutes/day in three days continuously (figure 3.2a). On the other hand, as the sham treatment, the rats at the

control group received the electrodes injection also, but without electric stimulation (figure 3.2 b). The purpose of this injection in the control group is to keep the equality treatment or to avoid misleading interpretation of the results, because of the effect of the electrode injection, such as inflammation, mechanical injury, or because of an injected stimulation[45].

Both the waveforms of electrical stimulation, PrPT or RdPT, were generated which were based on a Visual Basic program on a windows-personal computer and sent to the needle electrodes via a 16-bit AD/DA interface board (National Instruments, DAQ Card 6036E) (figure 3.3). The electric waveform consists of pulse trains with a current amplitude of 2 mA, 552 μs duration, and 50 % duty ratio. The PrPT has a repetitive of reverse-polarity constantly 20Hz and the RdPT has a random duration of reverse-polarity up to 20 Hz. Voltages and electrical stimulation patterns were monitored with the digital oscilloscope (Iwatsu, DS-5106).

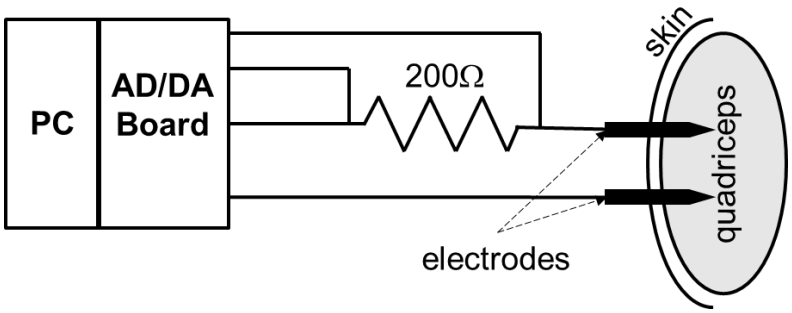

Figure 3.3 Electromyostimulation system.

3.2.3 Harvesting Bones

Fifteen days after the stimulation, prior to harvesting the bones, the rats were anesthetized with pentobarbital sodium 40 mg/kg and sacrificed with cervical dislocation. The femora, the tibiae, the humerus, and the ulna-radius were harvested immediately.

Their soft tissue was cleaned and immediately the bones were stored in the bottle that already inserted the saline water.

3.2.4 Bending Test

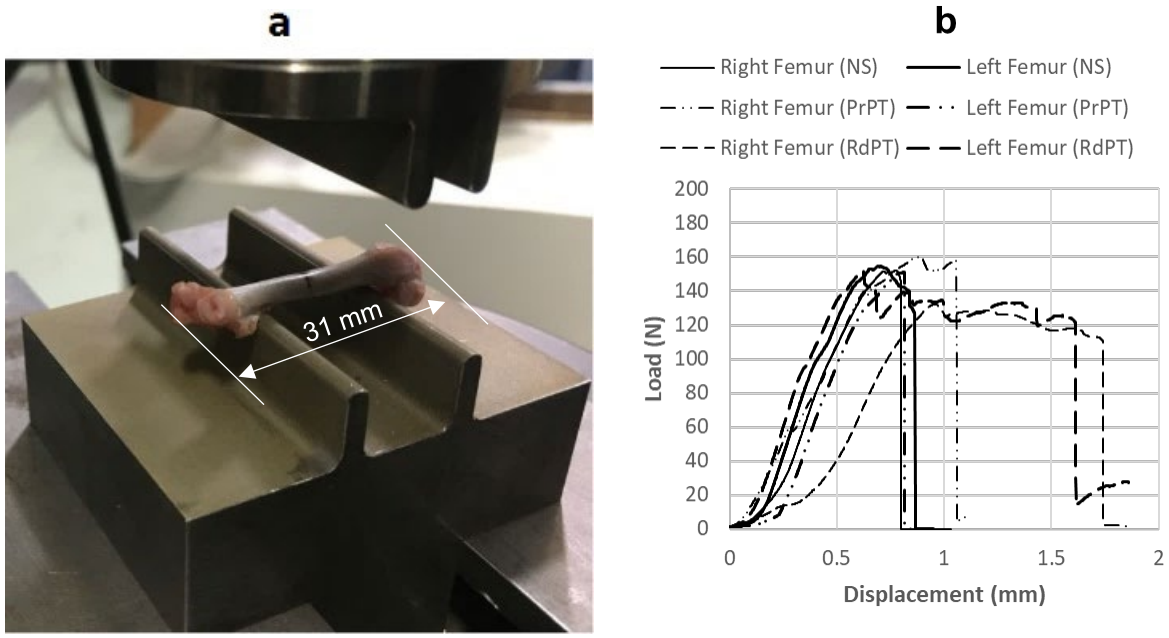

Figure 3.4 Bending test at the femur (a) and the load-displacement curve (b).

After harvesting the bone, immediately the mechanical qualities of the femurs, tibiae, humeri, and ulnas-radius were evaluated by four-point bending testing. The bones were placed specifically. Posterior of femurs (figure 3.4a), lateral of tibiae (figure 3.5a), medial of humeri (figure 3.5b), and lateral of ulnas-radius (figure 3.5c) were placed specifically on the 16mm-span bottom jig. By using the 8mm-span jig, the bones were loaded with displacement speed 1mm/minute until broken. The maximum load, strain energy, and stiffness were obtained from load-deformation curves (figure 3.7).

3.2.5 Statistic Analysis

To assess the statistical significance of the effectiveness of electromyostimulation treatment among the groups, the results were analyzed with Kaleidagraph Software (Version 3.6; Synergy Software, PA, USA). ANOVA with Dunnett's post-hoc tests was performed to compare the three experimental groups. Paired t-tests was conducted to compare the left and the right bones. A p value of 0.05 or lower indicated statistical significance.

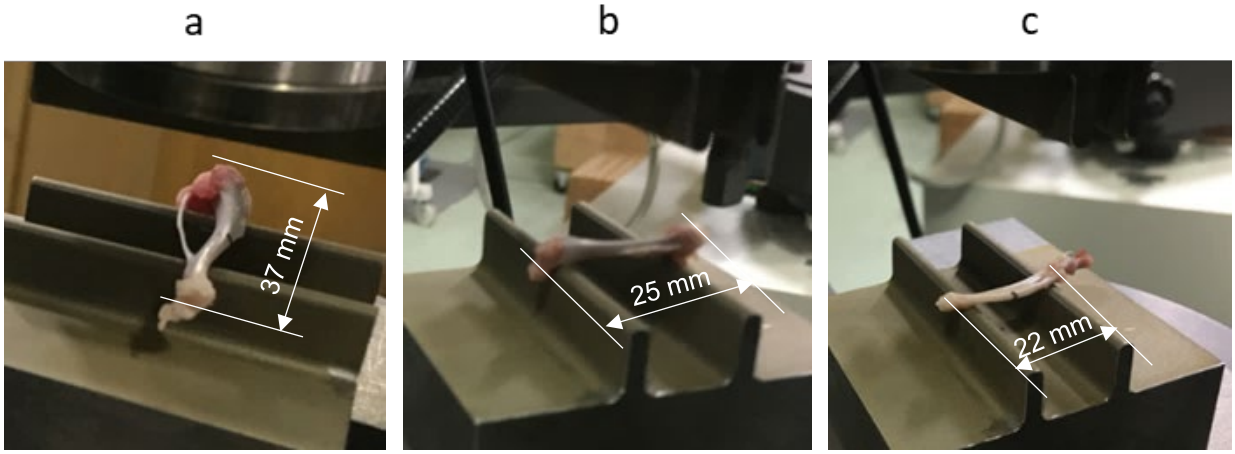

Figure 3.5 Position for the tibia (a), the humerus (b), and the ulna-radius (c) for bending tes.

3.3 Results

3.3.1 Body Weight

The body weight of the animals did not show any significant differences among the different treatment groups on the first day of stimulation, with average weights of 172.28 ± 13.92, 174.88 ± 5.68, and 172.96 ± 5.58 gram for the control, the PrPT, and the

RdPT groups, respectively. Furthermore, the body weights were not significantly different on the day of sacrifice which was 208.60 ± 18.89, 209.90 ± 3.80, and 206.94 ± 5.90 gram for the control, the PrPT, and the RdPT groups, respectively. These results show the equality of conditions of all rats even in different treatment groups and that electromyostimulation did not affect their body weights (Figure 3.6).

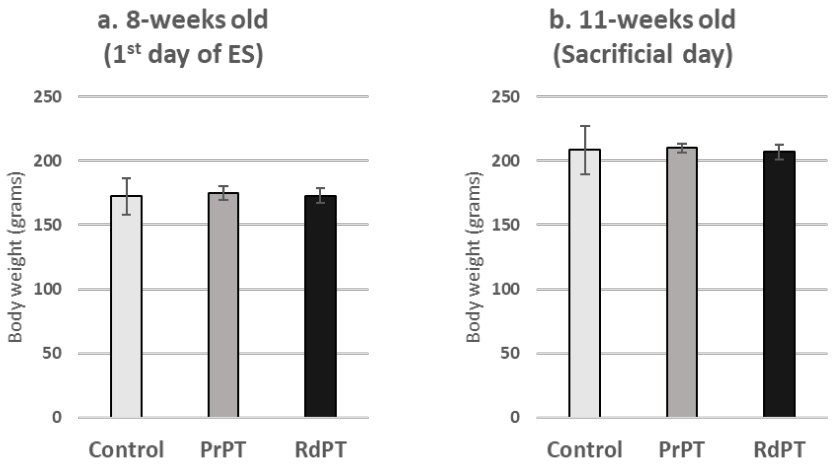

Figure 3.6 Comparing the rats' body weight because of electromyostimulation.
No significant differences among those groups at both on the 1st day of stimulation (a) and on the sacrificed day (b).

3.3.2 Bending Test

The mechanical test demonstrated significant increases of maximum strain and strain energy in simulated left femurs of RdPT, compared to the unstimulated right femurs. This result suggests the local effectiveness of RdPT electromyostimulation. However, the mechanical test didn't show any significant increases of the mechanical properties in all of the bones tested, compared to sham controls, regardless of the type of stimulation, except the femurs in RdPT stimulation (Table 3.1).

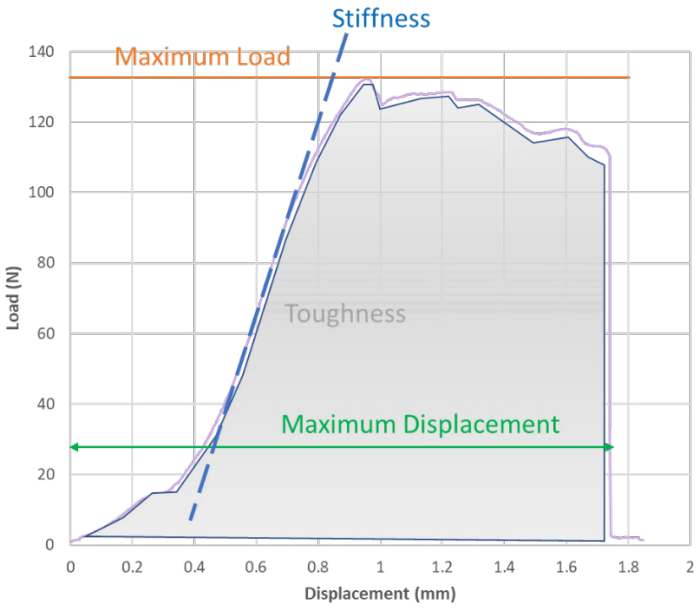

Figure 3.7 Bending test calculation to obtain maximum load, maximum displacement, toughness (strain energy) and stiffness.

Four-point bending testing on the diaphysis of the femurs showed that the strain energy of RdPT-stimulated left femurs has a significantly larger increase than that of unstimulated contralateral right femurs by 46.32% ($p < 0.05$). Other mechanical properties such as the maximum load and stiffness of the femur did not show significant differences between the left and right sides. Furthermore, electromyostimulation at the left quadriceps did not significantly influence the mechanical properties of the diaphysis of the other long bones, namely, the tibiae, humeri, and ulnas–radii (Table 3.1). However, in the case of an incomplete fracture of the ulna–radius, the strain energy could not be analyzed (data unavailable).

Table 3.1 Comparison of the mechanical properties of the femur, tibia, humerus, and ulna–radius diaphysis after the electromyostimulation at the left quadriceps.
The stimulation did not induce significant changes in the bone mechanical properties, except the strain energy of the stimulated left femur diaphysis. * $p < 0.05$ vs. control; ++ $p < 0.01$ vs. contralateral; N/A: not available because no fracture point was observed.

		Right	Left	Right	Left	Right	Left
Femur	**Max. Load (N)**	151.27 ±12.94	158.67 ±9.41	156.58 ±23.96	154.71 ±21.55	163.24 ±21.59	171.34 ±18.70
	Strain Energy (N.mm)	109.50 ±40.26	115.17 ±49.27	106.72 ±13.82	121.13 ±47.05	111.57 ±32.97	171.97 ±27.52*++
	Stiffness (N/mm)	308.84 ±72.27	361.23 ±41.44	357.01 ±125.71	335.64 ±60.03	403.42 ±117.74	394.38 ±52.84
Tibia	**Max. Load (N)**	110.22 ±27.97	113.42 ±17.15	126.39 ±33.80	122.13 ±16.41	125.99 ±17.94	116.36 ±17.24
	Strain Energy (N.mm)	120.12 ±56.19	95.39 ±48.33	170.05 ±75.55	125.16 ±40.72	111.35 ±31.58	108.88 ±34.36
	Stiffness (N/mm)	194.43 ±57.92	186.49 ±37.70	204.36 ±65.70	203.46 ±42.21	193.45 ±24.04	148.84 ±49.82
Humerus	**Max. Load (N)**	75.68 ±12.03	69.52 ±20.80	73.48 ±12.90	84.73 ±15.72	77.65 ±15.58	81.49 ±13.35
	Strain Energy (N.mm)	42.75 ±26.20	53.17 ±12.56	47.28 ±20.57	44.73 ±23.04	48.50 ±21.44	52.74 ±21.76
	Stiffness (N/mm)	165.51 ±46.93	118.30 ±57.70	144.12 ±63.38	185.73 ±38.63	163.06 ±41.87	157.28 ±69.37
Ulna-Radius	**Max. Load (N)**	57.12 ±3.44	51.31 ±7.44	58.53 ±9.14	50.23 ±7.21	56.97 ±1.74	55.73 ±6.94
	Strain Energy (N.mm)	N/A	N/A	N/A	N/A	N/A	N/A
	Stiffness (N/mm)	61.78 ±7.87	57.47 ±12.68	47.99 ±15.44	55.58 ±6.37	54.07 ±13.02	59.94 ±17.35

3.4 Analysis and Discussion

In this study, the stimulation only influenced the mechanical properties, which was strain energy, in the stimulated site and not the distant bones. Interestingly the location was in the femoral diaphysis, which mostly is cortical bone (figure 3.9). As well as Castillo et al. analysis, it is difficult to try to induce osteogenesis at a cortical bone. Mostly stimulations affect trabecular bone more than cortical bone even if it is investigated in microstructural level[46]. Rubin also showed that 30 Hz whole-body vibration could induce trabecular sheep, but not the cortical[47].On the contrary, Zhang et al. showed the possibility to induce osteogenesis at cortical by applying loading at knee mechanically 5 Hz or 10 Hz to induce osteogenesis at tibia and 15 Hz to induce osteogenesis at femur[48]. It could be possible maybe if the stimulation could induce the intramedullary pressure which is the interstitial fluid flow in the bone matrix, such as 20 Hz stimulation at turkey ulna[39]. This fluid flow circulation will increase osteogenic response in the bone. Differently with other studying, in this study, only RdPT can induce osteogenesis in the cortical bone. If it is related to mechano-sensitivity, the possible hypothesis that could be built is from the characteristic of RdPT. RdPT is not routine loading signal. Base on Turner review[20], the RdPT is not routine loading signal and it suggests that the bone cell is difficult to custom the mechanical loading environment which is internal strain from the muscle contraction.

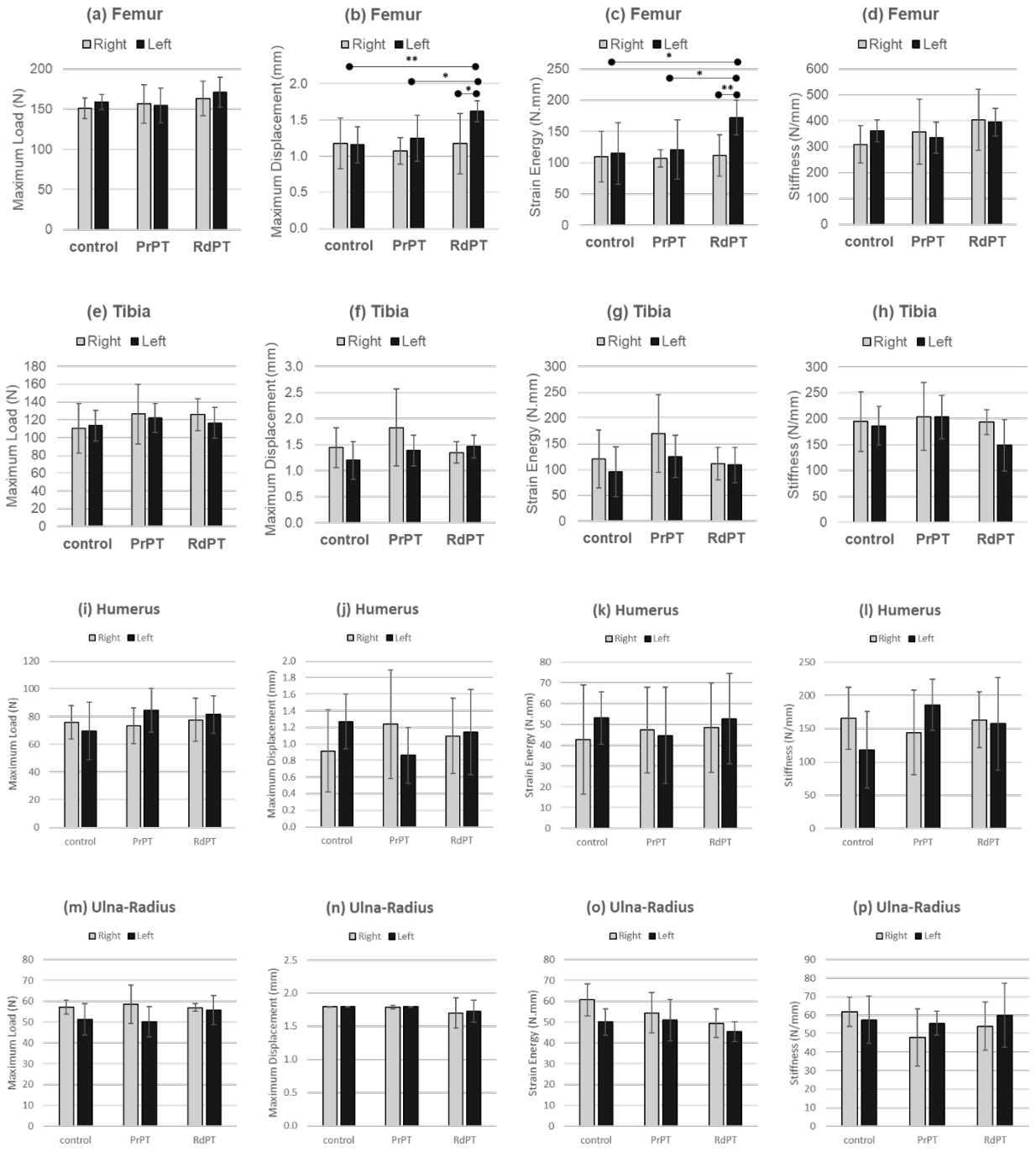

Figure 3.9 Comparison the mechanical properties among the groups of the femur (a-d), the tibia (e-h), the humerus (i-l), and the ulna-radius (m-p).
Only strain energy of stimulated diaphyseal femur was influenced by the RdPT.

Unfortunately, the mechanical result suggests that RdPT electromyostimulation do not influence osteogenesis to the midshaft area of the unstimulated contralateral femur (Fig. 3.9). However, on the other hand, the RdPT electromyostimulation induced the osteogenesis at the femoral neck of the unstimulated contralateral femur as well as at the site of the stimulated femur[22]. It's already known that the epiphysis area of the femoral neck is full of trabecular bone than cortical bone, but on the other, hand the midshaft is constructed mostly of cortical bone. Generally, trabecular bone is known to be more adaptively than cortical bone. This could explain the site-depending osteogenic effect of RdPT electromyostimulation. Additionally, the innervation density of nerve system is higher in the epiphysis area than diaphysis area[49], suggesting a possibility of signal transduction through the nerve system to stimulate the bone formation in the contralateral bone. This also implies a possibility of promotion of osteogenesis in other trabecular bones by one-site stimulating with the RdPT electromyostimulation. Base on the clinical experience that the osteoporosis fractures occurred at the femoral neck and the vertebra (figure 1.2), the next study was the study of the effect of electromyostimulation at left quadriceps as the site of stimulation on the mechanical properties of vertebrae as the unstimulated site.

3.5 Summary

This study has shown the capability of the Random Pulse Train electromyostimulation to influence the mechanical properties at diaphysis bone (which is constructed mostly cortical bone than trabecular bone) better than the Periodic Pulse Train electromyostimulation. But, on the other hand, the results haven't shown yet the

capability the electromyostimulation to influence the mechanical properties of the diaphyseal of long bones in a whole-body scale. This study shows the limitation of this study and suggests that the electromyostimulation is hard to influence cortical bone beyond the stimulation site. Base on clinical experience, the bone fractures were occurred at the vertebra and femoral neck, which are mostly have trabecular bone than cortical bone. The next work studied the influence of this stimulation at left quadriceps on the rats' vertebra.

Chapter 4

Effects of Electromyostimulation on Mechanical and Microarchitectual Properties of Lumbar Vertebrae

4.1 Introduction

In the previous chapter, although the electromyostimulation, especially the randomly pulse train (RdPT), has capability to influence the mechanical properties of bones in the stimulated site, the stimulation hasn't shown yet its capability to influence the mechanical properties of the sites beyond the stimulated location (figure 3.9). Based on the previous study at long bones which are mostly constructed by cortical bones, and the clinical experience that the osteoporosis fractures mostly occurred at femoral neck and vertebra (figure 1.2) which are mostly constructed by trabecular bones, the effect of these stimulation to influence the mechanical properties of bones beyond the stimulated site was studied in this chapter.

The purpose of this study was to investigate the effect of the electromyostimulation on the mechanical properties of bones, which were the vertebrae, beyond the stimulation place. The objects of this study were lumbar vertebra. The selection was based on the connection in neurologically of the lumbar vertebrae and the lumbar bones[50] such as femora or tibia. Based on Rigaud, et al.'s studied about the sciatic nerve anatomy at rodents[50], the object of this study was limited to the lumbar vertebra number 2 (L2), number 3 (L3), number4 (L4), and number 5 (L5). To get the mechanical properties result, the compression test was chosen based on the commonly mechanical

test for the vertebrae. By using micro CT scan, the microarchitecture of the vertebrae also was analyzed as the comparator for the mineral content and the bone structure. In keeping the equality condition with previous study, the procedures of sample preparation were performed like the previous experiment as well (chapter 3).

This chapter covers the influence of the electromyostimulation on the mechanical properties of the lumbar vertebra L2, L3, L4, and L5. It also reports the microarchitecture as the supporting and the comparator results for the mineral content and the bone structure. The body weight also is reported as the controlling of the rats' condition because of the stimulation.

4.2 Materials and Methods

4.2.1 Animals

The procedure of the animals' treatment was performed as the previous study (chapter 3) such as purchased 7-weeks-old female rats, let the rats for a week adaptation, divided into 3 groups (Control, PrPT, and RdPT), stimulated the rats for 3 days continuously, or collected the bones on the 19^{th} day since the 1^{st} day of the stimulation (figure 3.1). The anesthetic procedure also was followed by the previous procedure, somnophentyl doze was 40mg/kg rat's weight. As well as the previous study, this animals' treatment and handling also was performed according to our institutional guidelines from the Experimental Animal Institute of Kanazawa University and approved by the Committee on Animal Experimentation of Kanazawa University with Approval No. AP-173865.

4.2.2 Electromyostimulation to Rat Quadriceps

In keeping the equality of the study, the procedure of the electromyostimulation was performed by following the previous study's procedure (chapter 3), such as the 30 minutes/day electromyostimulation at the left quadriceps in three days continuously or the electrodes injection at the control group.

4.2.3 Harvesting Bones

As well as the previous study, fifteen days after the electrical stimulation, prior to harvesting the bones, the rats were anesthetized with pentobarbital sodium 40mg/kg and sacrificed with cervical dislocation. The lumbar vertebra number 2 to number 5 (L2-L5) were harvested and cleaned from their soft tissue and stored immediately in the six-well microplates that already inserted the saline water.

4.2.4 Micro Computed Tomography (μCT) Scan

Before mechanical testing, the lumbar vertebras L2-L5 were scanned by using micro-computed tomography system (μCT Scanning Machine, Shimadzu InspeXio SMX-90CT Plus with HPC InspeXio high-performance computing system, Shimazu, Kyoto, Japan). The scanning process was set 0.040 mm/pix for the images, and 90kV and 110 μA for the machine condition.

As an image calibrator, in every scanning day, the Ratoc Phantom system 1508-113_No06_U5D1mmH (Ratoc System Engineering, Tokyo-Japan) was scanned also. This phantom has five cylinders graduated mineral concentration, which are 100, 300,

400, 200, and 500 mg/cm3, and 1550mg/cm^3 aluminum rod. The phantom scanning condition and the machine setting were similar to the vertebra scanning condition.

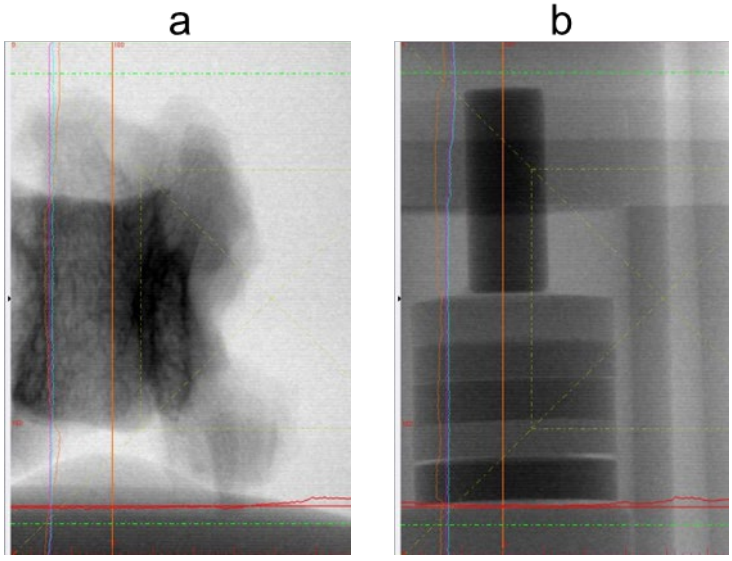

Figure 4.1 The image at micro CT of vertebrae (a) will be calibrated with the image of the cylindrical phantom (b) as the mineral density calibrator.

Finally, the gray images from micro-CT scan (figure 4.1a), were analyzed with TRI/3D-BON software (Ratoc System Engineering, Tokyo-Japan) with the Ratoc Phantom gray images (figure 4.1b) as the image calibrator. The gray image contrast was transformed into color images to show the density of the bone mineral (picture 4.2).

4.2.5 Compression Test

The lumbar vertebras L2-L5 were tested mechanically by the compression test. The inferior of lumbar vertebras was put on the bottom compression plate in a condition that their extended arches had been removed with the intention of flatting and balancing their superior-inferior position because of unsymmetrical shapes of the vertebra (figure

4.3a). The compression testing was stopped after 4 mm displacement (more than half of vertebra high) (figure 4.3b). The maximum load, 4 mm displacement's (half of vertebrae height) strain energy and stiffness were obtained from load-deformation curves (figure 4.4).

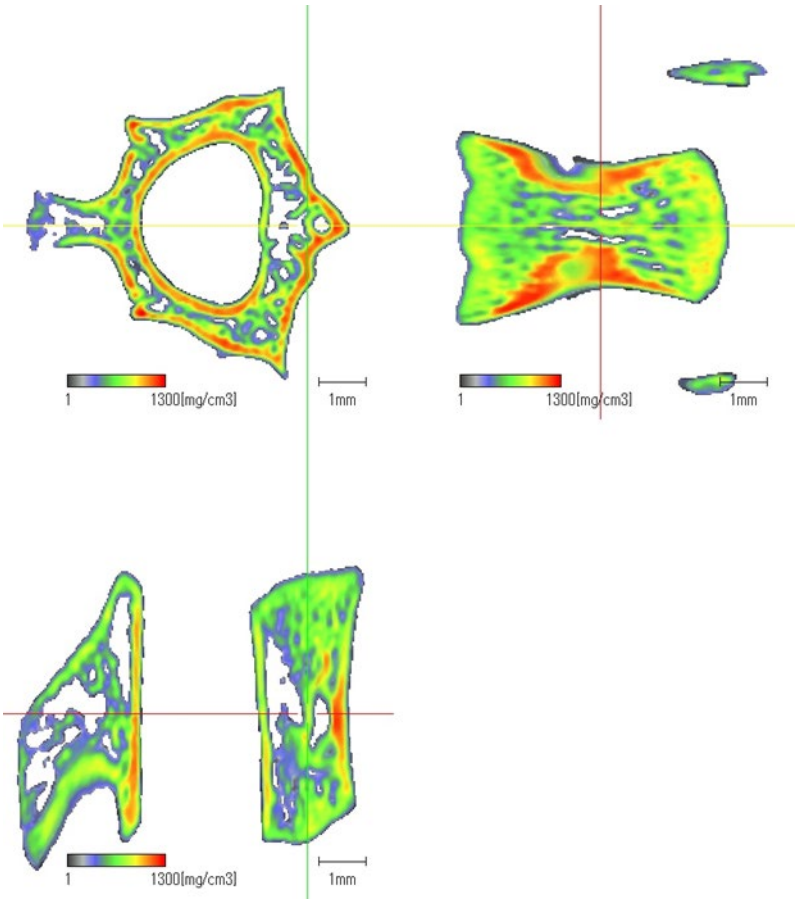

Figure 4.2 Color image shows the bone density after the calibration with phantom image scale.

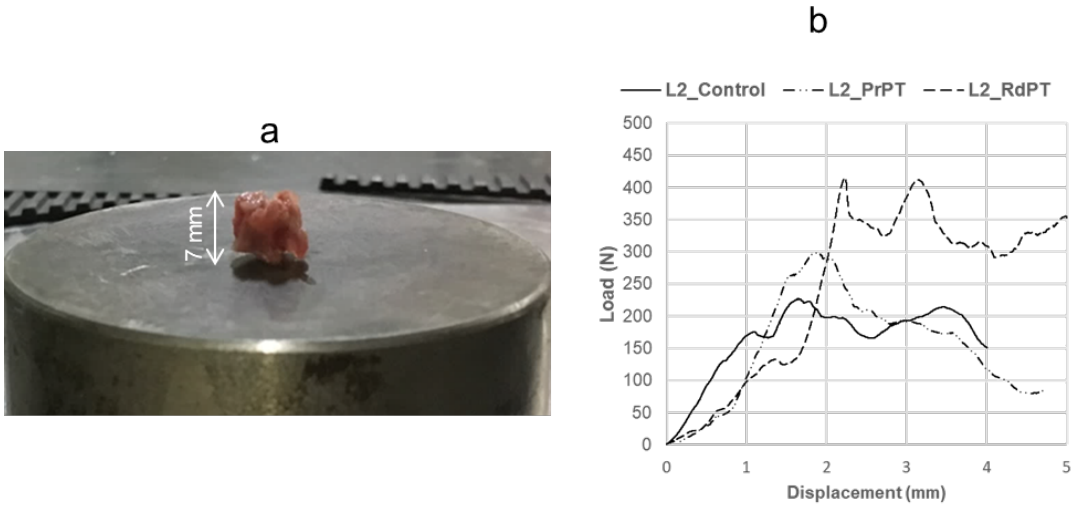

Figure 4.3 Compression test at the vertebra (a) and the load-displacement curve (b).

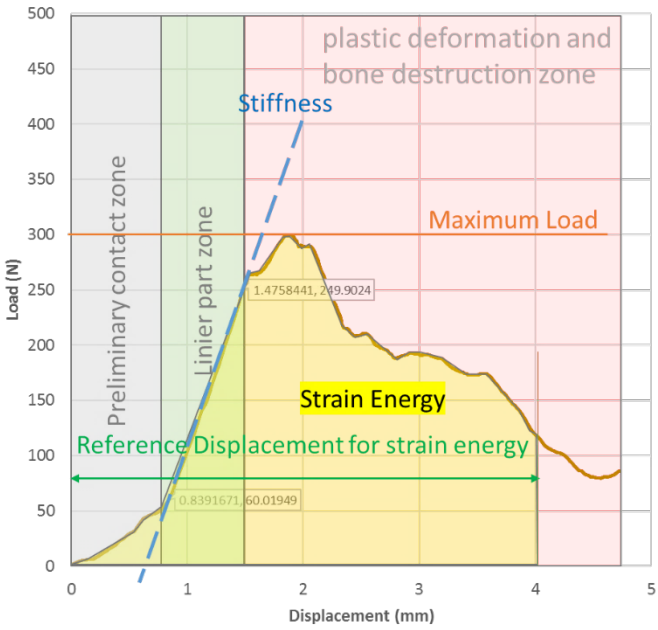

Figure 4.4 The calculation principles to obtain maximum load, maximum displacement, strain energy (toughness) and stiffness.

4.2.6 Statistic Analysis

As well as previous investigation (chapter 3), these investigation results were analyzed with Kaleidagraph Software (Version 3.6; Synergy Software, PA, USA). ANOVA with Dunnett's post-hoc tests was performed to compare the three experimental groups. A p value of 0.05 or lower indicated statistical significance.

4.3 Results

4.3.1 Body weight

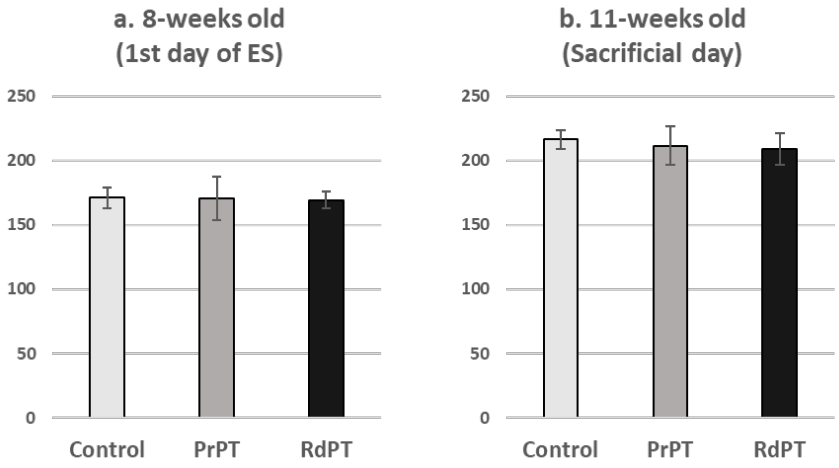

Figure 4.5 Comparison rat's weight in the 1st day of stimulation and in the sacrificial day.
No significant differences among those groups at both on the 1st day of stimulation (a) and on the sacrifice day (b).

The body weight of the animals did not show any significant differences among the different treatment groups on the first day of stimulation, with average weights of 171.22 ± 8.01, 170.52 ± 17.09, and 169.14 ± 6.52 gram for the control, PrPT, and RdPT groups, respectively. Furthermore, the body weights were not significantly different on the day of sacrifice: 216.55 ± 7.45, 211.62 ± 15.10, and 209.24 ± 12.15 gram for the control,

PrPT, and RdPT groups, respectively. These results show the equality of conditions of all rats even in different treatment groups and that electromyostimulation did not affect their body weights (Figure 4.5).

4.3.2 Mechanical Properties

Compression testing on the L2 demonstrated a significant increase in bone rigidity in the RdPT group by 84.62% ($p < 0.05$) compared with the control (Table 4.1). However, RdPT stimulation significantly decreased the stiffness of the L4 by 39.22% ($p < 0.05$) compared with the nonstimulated control. Furthermore, no significant changes in mechanical properties were observed in the L3, L4, or L5 after the stimulation.

Table 4.1 Comparison of the mechanical properties of the L2, the L3, the L4, and the L5.
The mechanical properties didn't change by the electromyostimulation to the left quadriceps, except the stiffness of the L2 and the L4 in RdPT group and the maximum load of theL4 in PrPT group. * $p < 0.05$ vs. control.

		Control	PrPT	RdPT
L2	**Max. Load (N)**	331.76 ±70.57	327.09 ±62.64	335.57 ±65.19
	Strain Energy (N.mm)	772.26 ±136.02	693.91 ±156.94	782.59 ±118.45
	Stiffness (N/mm)	312.97 ±86.22	367.96 ±77.31	481.72 ±159.19*
L3	**Max. Load (N)**	380.92 ±17.32	334.86 ±66.69	334.57 ±70.40
	Strain Energy (N.mm)	890.00 ±89.03	758.20 ±164.68	743.22 ±111.44
	Stiffness (N/mm)	403.51 ±75.81	351.22 ±136.85	333.58 ±137.95
L4	**Max. Load (N)**	403.97 ±20.80	350.21 ±44.87*	370.13 ±57.51
	Strain Energy (N.mm)	799.11 ±41.45	770.37 ±100.21	808.90 ±146.88
	Stiffness (N/mm)	476.26 ±120.45	423.42 ±109.89	289.46 ±112.66*
L5	**Max. Load (N)**	349.01 ±31.10	351.93 ±23.62	361.24 ±19.84
	Strain Energy (N.mm)	778.27 ±93.70	825.20 ±30.53	791.76 ±111.89
	Stiffness (N/mm)	319.06 ±28.69	393.75 ±121.60	327.48 ±72.81

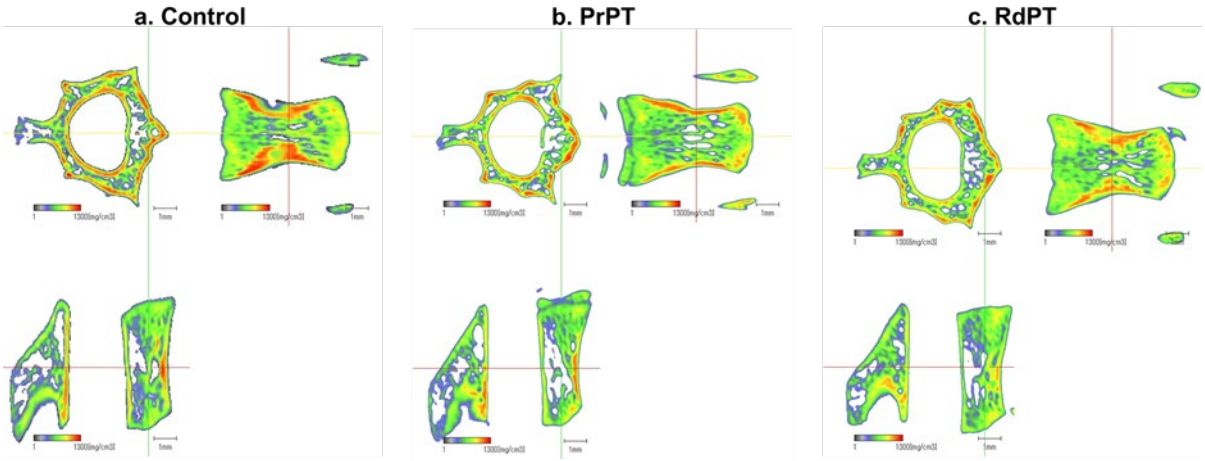

Figure 4.6 Typical the L2's μCT image in the Control group, the PrPT group, and the RdPT group.
No remarkable difference of μCT images of among the Control group, the PrPT group, and the RdPT group.

4.3.3 Bone Microarchitecture

The μCT images in figure 4.6 show that no remarkable differences existed among the groups. On the other hand, table 4.2 shows that the electromyostimulation influence L2 at the microstructural level. The RdPT and the PrPT reduced the BMC of the L2 by 6.90% ($p < 0.05$) and 7.30% ($p < 0.05$) respectively, compared with the control. The decreasing were also observed in the BV and TV of the L2. On the other hand, there was no significant difference between the BMD and vBMD in the L2. On the contrary, there were no significant differences in the other lumbar vertebrae when observing the microstructural parameters among those groups (Table 4.2).

Table 4.2 Comparison of the microarchitecture of the L2, the L3, the L4, and theL5.
The stimulation influenced only the microarchitecture of the L2 but not those of the L3, the L4, and the L5. * $p < 0.05$ vs. control.

		Control	PrPT	RdPT
L2	**BMD (x 10^2 mg/cm^3)**	7.48 ±0.15	7.36 ±0.19	7.38 ±0.07
	vBMD (x 10^2 mg/cm^3)	4.89 ±0.23	4.81 ±0.18	4.70 ±0.23
	BMC (x 10 mg)	5.81 ±0.19	5.38 ±0.21*	5.41 ±0.28*
	BV (x 10^{-2} cm^3)	7.76 ±0.24	7.31 ±0.19*	7.33 ±0.38
	TV (x 10^{-1} cm3)	1.19 ±0.02	1.12 ±0.04*	1.15 ±0.02*
	BV/TV (%)	65.4 ±2.4	65.4 ±1.4	63.7 ±2.9
L3	**BMD (x 10^2 mg/cm^3)**	7.43 ±0.13	7.33 ±0.21	7.38 ±0.08
	vBMD (x 10^2 mg/cm^3)	5.01 ±0.25	4.91 ±0.19	4.94 ±0.26
	BMC (x 10 mg)	5.93 ±0.18	5.81 ±0.21	5.91 ±0.51
	BV (x 10^{-2} cm^3)	7.97 ±0.14	7.93 ±0.39	8.01 ±0.68
	TV (x 10^{-1} cm3)	1.18 ±0.03	1.18 ±0.07	1.20 ±0.07
	BV/TV (%)	67.3 ±2.3	67.0 ±1.6	66.9 ±2.9
L4	**BMD (x 10^2 mg/cm^3)**	7.45 ±0.18	7.41 ±0.14	7.39 ±0.10
	vBMD (x 10^2 mg/cm^3)	5.17 ±0.30	5.12 ±0.10	5.08 ±0.25
	BMC (x 10 mg)	6.26 ±0.23	6.11 ±0.25	6.27 ±0.46
	BV (x 10^{-2} cm^3)	8.41 ±0.25	8.25 ±0.29	8.49 ±0.59
	TV (x 10^{-1} cm3)	1.21 ±0.04	1.19 ±0.04	1.23 ±0.06
	BV/TV (%)	69.3 ±2.6	69.1 ±0.7	68.6 ±2.5
L5	**BMD (x 10^2 mg/cm^3)**	7.20 ±0.17	7.29 ±0.18	7.27 ±0.05
	vBMD (x 10^2 mg/cm^3)	5.19 ±0.27	5.26 ±0.16	5.25 ±0.21
	BMC (x 10 mg)	6.48 ±0.35	6.28 ±0.13	6.49 ±0.47
	BV (x 10^{-2} cm^3)	9.00 ±0.45	8.63 ±0.29	8.92 ±0.61
	TV (x 10^{-1} cm3)	1.25 ±0.06	1.20 ±0.05	1.23 ±0.05
	BV/TV (%)	72.0 ±2.2	72.1 ±1.3	72.1 ±2.4

4.4 Analysis and Discussion

In this investigation, it seems that the electromyostimulation at the left quadriceps could influence the mechanical properties of the lumbar vertebra. The stiffness of the L2 and L4 were changed as the response of the stimulation (figure 4.7). It was different than the previous study at the diaphyseal long bones' diaphyseal (chapter 3) that this

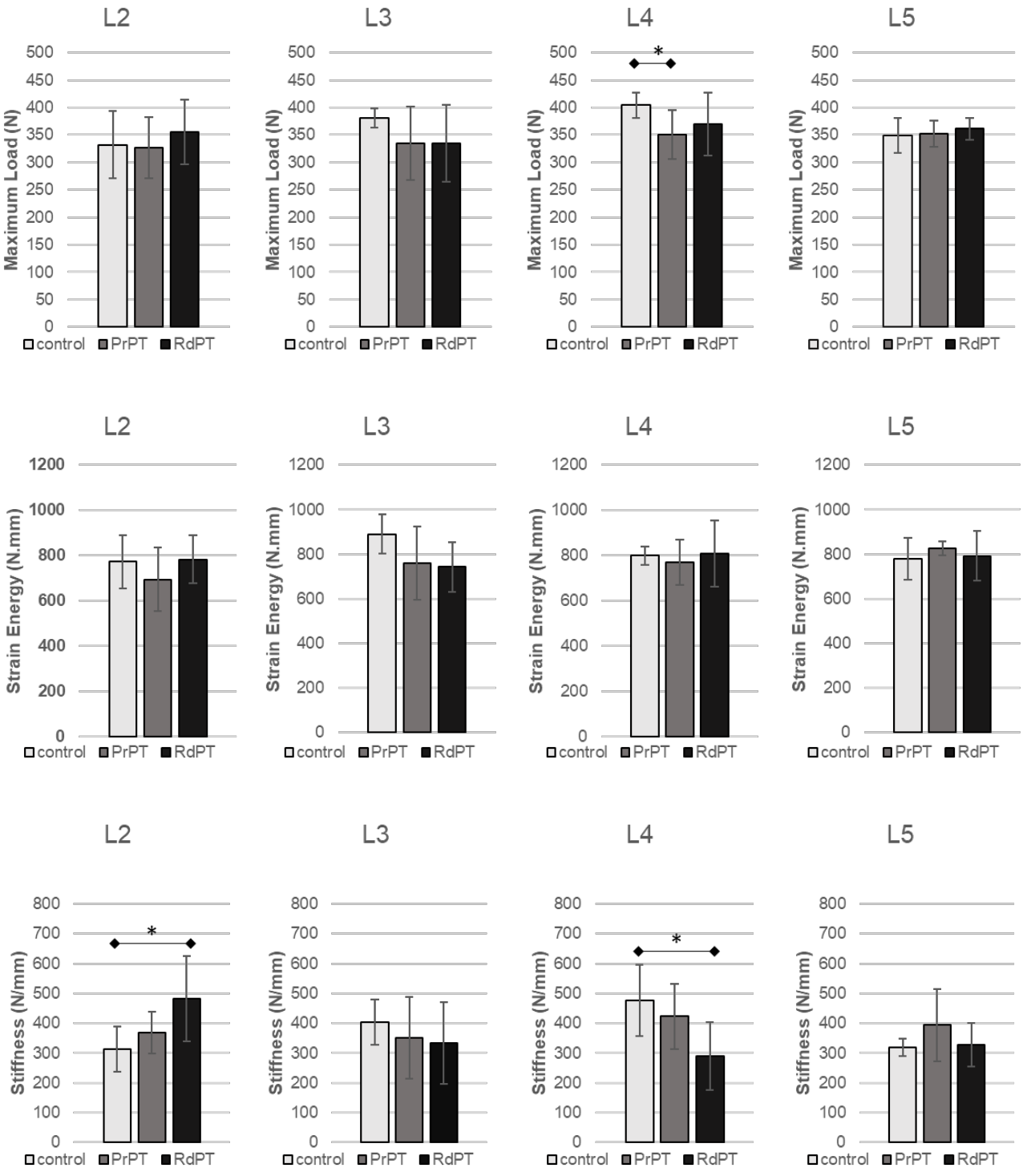

Figure 4.7 Comparison of the maximum load, strain energy, and stiffness of the L2 until the-L5.
The effect of stimulation occurred at the L2 and the L4.
The stiffness of L2 and L4 were influence by electromyostimulation.

stimulation hadn't shown its capacity to influence the mechanical properties of the long bones which were distant from the stimulated site. It is possible that nerve played an important role in this adaptation. It has been know that the trabecular bone at the femoral neck is rich of nerve network[49] as well as at the lumbar vertebra[51]. Beside of that, there is nervous network connectivity among lumbar vertebra and the bones at the lumbar area[50]. Figure 4.8 shows that the possibility the stimulation at the quadriceps is selectively sense by the nerve. It has been known that osteogenesis could be modulated by neurotransmitter from the sympathetic nervous system[52]. The sensory nervous system also influences bone formation through the neuropeptides[53]. It was possible that the nerve system in the periosteum and the bone marrow could be induced by an electric current from the needle electrode of electromyostimulation[54], so the neurotransmitter and the neuropeptide were induced in the lumbar vertebrae. This signal could be contributed in changing the mechanical properties at the lumbar vertebra.

Even though the general electromyostimulation has the capability to influence the osteogenesis[14,35,44], but the RdPT electromyostimulation has the capability to reinforce the bone mechanical properties. It has been known that the bone mechanical properties are not only depending on the mineral, but also the collagen and the enzymatic crosslinking[55,56]. The stiffness increasing at L2 suggests the RdPT electromyostimulation induced the neural signal to promote the enzymatic cross-linking, despite the BMD didn't change because of this stimulation. We suspicious the RdPT electromyostimulation controls the Lysyl oxidase secretion at osteoblast via sensory nervous system. The RdPT electromyostimulation generates the afferent sensory signals due to muscle contraction. These signals were transmitted to the spinal cord via Ia and II sensory fibers and reach

the unstimulated area and finally influencing the lysyl oxidase secretion and promoting the enzymatic cross-linking which is influencing the vertebra stiffness. Unfortunately, this hypothesis is applicable only for L2 and not for L3-L5 where the mechanical properties were ineffective or decrease. Further investigation is needed to understand fully about this result.

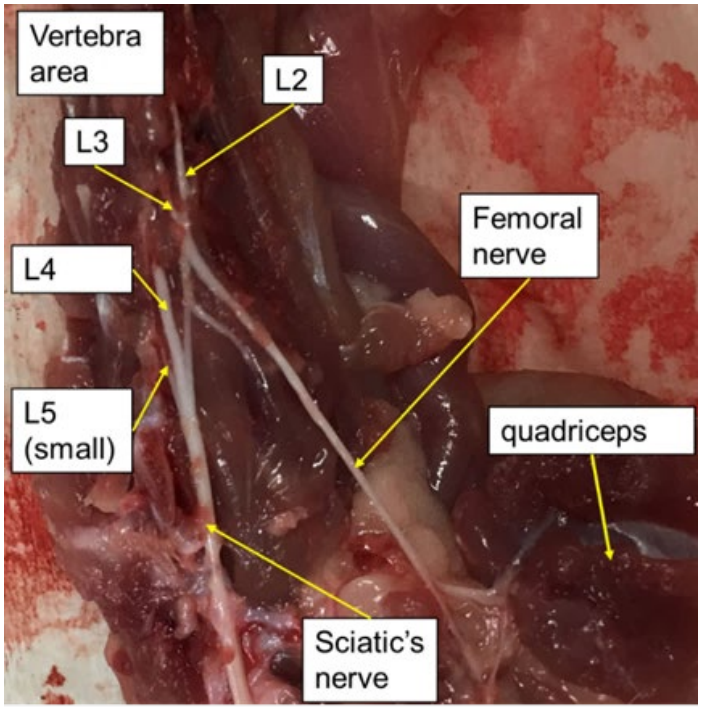

Figure 4.8 Lumbar nerves networking.
It can be seen the network between the L2 and the quadriceps.

4.5 Summary

The results of the effect of the capability of electromyostimulation to influence the mechanical properties of bones beyond the stimulated site are shown in this study. This study finally shows the RdPT electromyostimulation's capability to influence the mechanical properties of lumbar vertebra, but not all mechanical properties, and suggests

a specific mechanism such as how this stimulation control the enzymatic crosslinking. It supports also with the μCT result where the BMD didn’t change because of the stimulation. This study shows that these should have a correlation and a special connection with the nervous system.

Chapter 5

Conclusion and Future Work

5.1 Conclusion

The aim of this study is to investigate the possibility of electromyostimulation to stimulate bone's mechanical quality beyond the stimulated site. This study shows that the RdPT electromyostimulation has capability better to influence the mechanical properties of bones not only in the stimulated site but beyond the stimulated site through the nervous system. Moreover, both electromystimulations, PrPT and RdPT, influence the structure of distant bones.

It has been reported that the multiple distant bones were neutrally adapted by mechanical stimulation at a single bone[42,57,58], even though it needs further investigation[43]. The investigation at the long bone showed the difficulties of the electromyostimulation to influence the mechanical properties of the diaphyseal bones beyond the stimulated site. It was probably the diaphyseal is constructed mostly from cortical bone, which is built from the osteon structure, is difficult to sense small stimulation, while the osteon in the diaphyseal is the primary sensor in adaptation mechanism. On the other hand, the investigation at the vertebra shows that the stimulation at the quadriceps could influence the mechanical properties of the lumbar vertebra, but it is depended on the location of the vertebra. It suggests the nerve network plays an important role to control this phenomenon. It also shows that the trabecular bone, which is rich with nerve and blood vessel, is more adaptable with their environment than cortical bone. It can explain also

the effectiveness of the electromyostimulation to influence the mechanical properties of the unstimulated femoral neck[22].

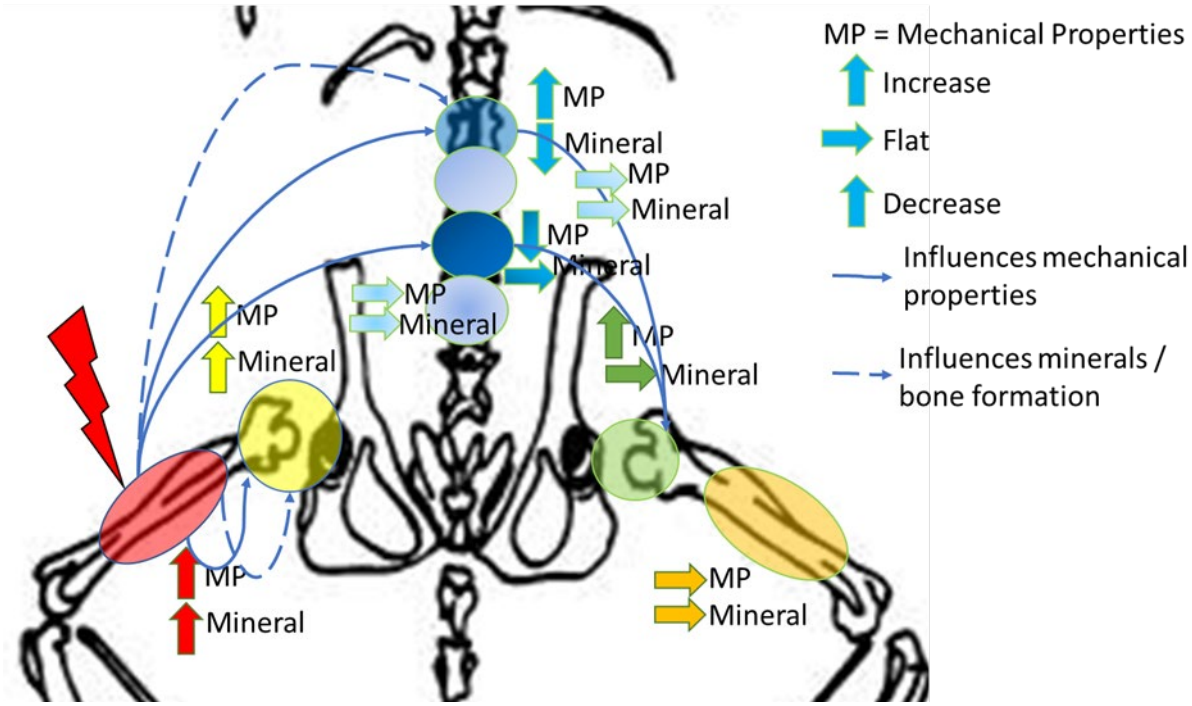

Figure 5.1 The pathways influence of the electromyostimulation that related to stimulation at the left quadriceps. The RdPT electromyostimulation at the left quadriceps influences the mechanical properties at the L2, the L4 and the right femoral neck. On the other hand, both the PrPT and the RdPT electromyostimulations influence bone mineral at the L2.

In influencing the mechanical quality, it looks the RdPT electromyostimulation works specifically in controlling the mechanical properties. Only strain energy in femoral neck[22] or the stiffness at the vertebra. It suggests that the electromyostimulation control the mechanical properties specifically or special component of bone such as the collagen or collagen crosslink. Relating to the nervous system, it is suspicioned that the RdPT electromyostimulation controls the secretion of enzymatic crosslink via the sensory

system. In this investigation, an electromyostimulation at left quadriceps induced neural signal that promoted enzymatic at vertebra and femoral neck (figure 5.1). Moreover, in influencing the mechanical qualities, both the PrPT and the RdPT electromyostimulation influence the bone mineral at vertebra and stimulated femur.

There are several limitations to this study. Firstly, the growing rats were used in this study, which has dominant bone formation than bone resorption. Secondly, the effect of this stimulation on the bone's mechanical qualities was only be investigated at the diaphyseal area for the long bones. It needs to be studied the effect of this stimulation on the other location which mostly has a trabecular bone. Thirdly, the investigation was seen only from the neural network. It needs to be investigated the other possible effect factors, such as hormonal system, circulation system, immune system, or bone growth.

Finally, this new finding which is the capability of the electromyostimulation on influencing the mechanical qualities of bones beyond the stimulated site, suggests the RdPT electromyostimulatioin , which has potential, as physical therapy for osteoporosis, even though it still needs optimization for clinical applications.

5.2 Future Work

It still needs an investigation to find the most appropriate condition for the RdPT electromyostimulation as the new physiotherapy for osteoporosis. It needs to know the mechanism that influences the mechanical properties, especially the effect of the stimulation on the collagen or its enzymatic cross-linking. It needs to know the role of the nervous system in inducing the mechanical properties beyond the stimulated site, and

most important thing, the effect of this stimulation to prevent bone loss in osteoporosis model as the final target of this stimulation.

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Appendix

Appendix A : Rats' Body Weight

1. Long bones' Project

1st day of ES (gram)	long bones					Average	SD
	Batch #1	Batch #3	Batch #4	Batch #5	Batch #6		
Control	192.1	181.7	162	164.8	160.8	**172.28**	**13.92**
PrPT	178.6	180.5	177.5	167.3	170.5	**174.88**	**5.68**
RdPT	170.3	181.7	172.5	173.7	166.6	**172.96**	**5.58**

Sacrificial day (gram)	long bones					Average	SD
	Batch #1	Batch #3	Batch #4	Batch #5	Batch #6		
Control	232.6	224	187.6	200.9	197.9	**208.60**	**18.89**
PrPT	213.9	214.1	206.1	207.6	207.8	**209.90**	**3.80**
RdPT	203	213.9	200	212	205.8	**206.94**	**5.90**

2. Vertebra's Project

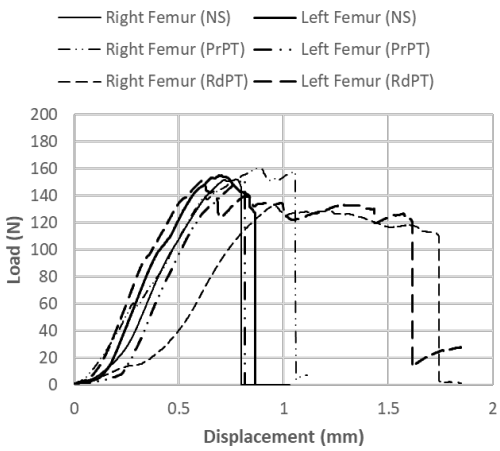
1st day of ES (gram)	vertebrae					Average	SD
	Batch #2	Batch #3	Batch #4	Batch #5	Batch #6		
Control	174.7	175.5	157	173.2	175.7	**171.22**	**8.01**
PrPT	185.9	174.8	141.7	179.6	170.6	**170.52**	**17.09**
RdPT	173.9	172	161.5	175.5	162.8	**169.14**	**6.52**

Sacrificial day (gram)	vertebrae					Average	SD
	Batch #2	Batch #3	Batch #4	Batch #5	Batch #6		
Control	222.9	222.1	214.1	207.1		**216.55**	**7.45**
PrPT	234.3	206.3	196.8	218.9	201.8	**211.62**	**15.10**
RdPT	225.4	198.1	215.4	210.9	196.4	**209.24**	**12.15**

Appendix B : 4-P Bending Test

1. Femur

Batch 1



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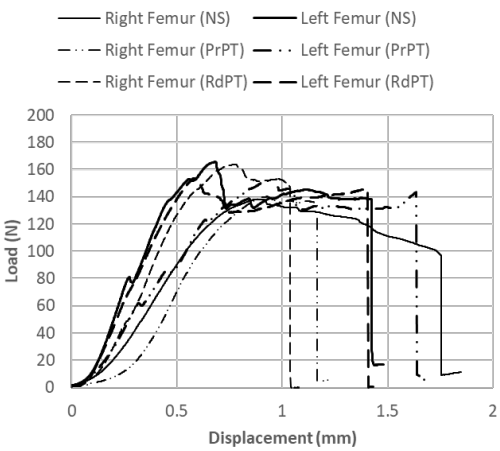

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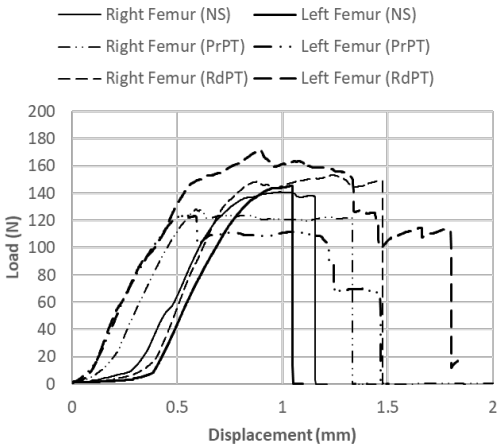
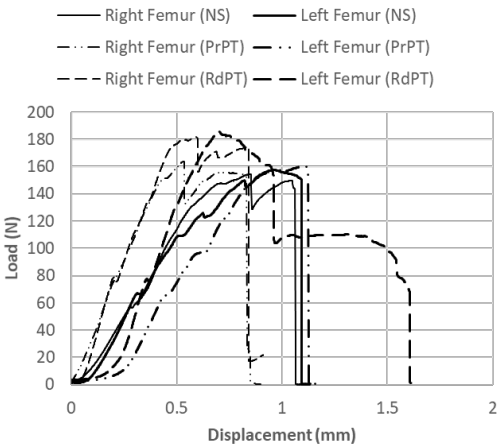
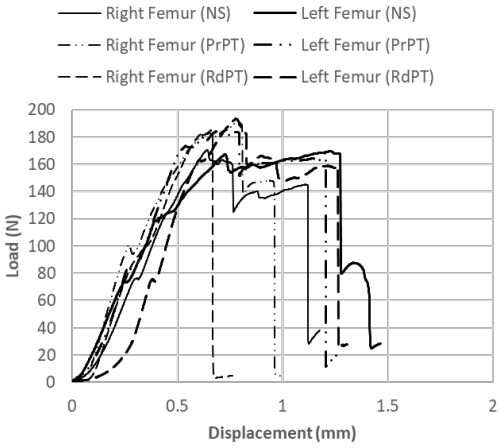


Batch 4

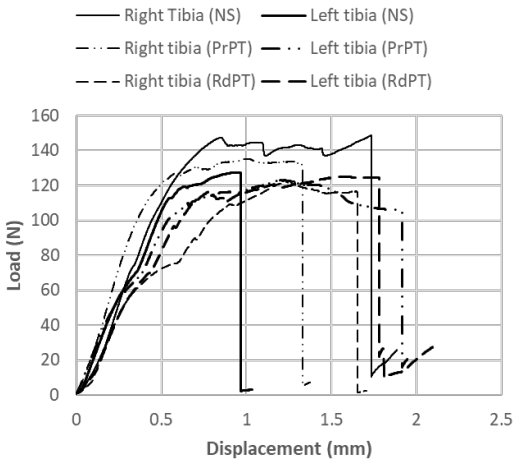


Batch 5



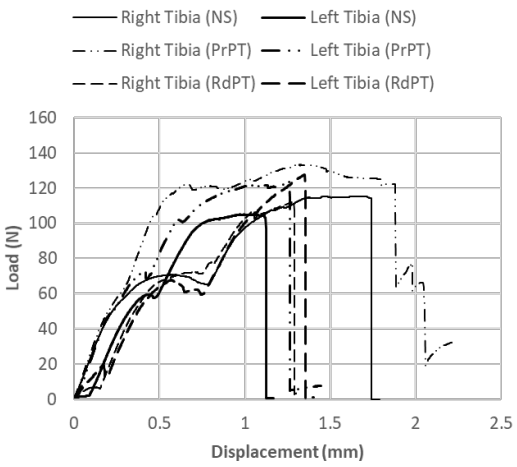
2. Tibia

Batch 1

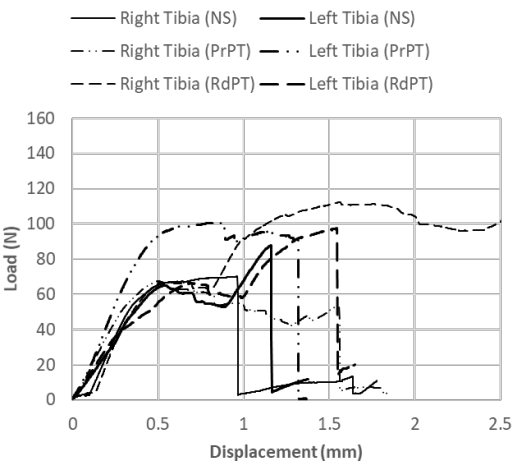


Batch 2

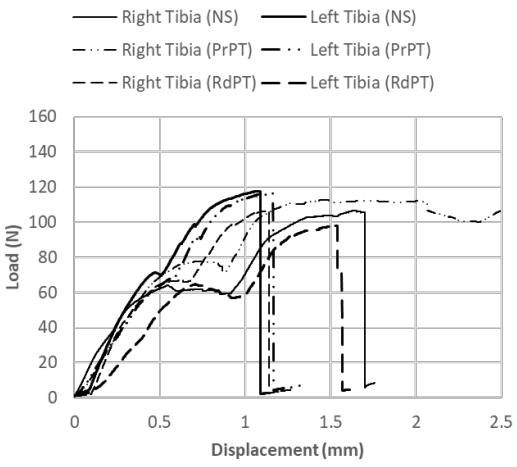
Batch 3



Batch 4

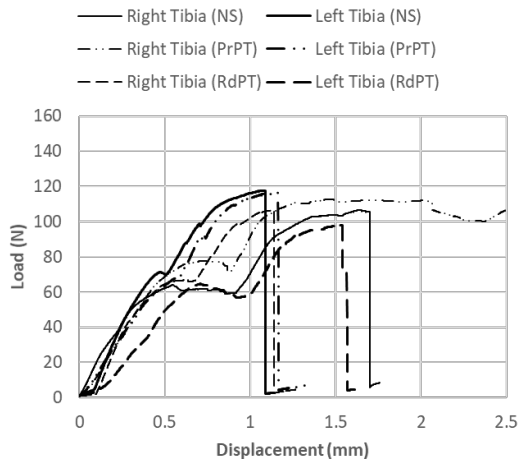


Batch 5

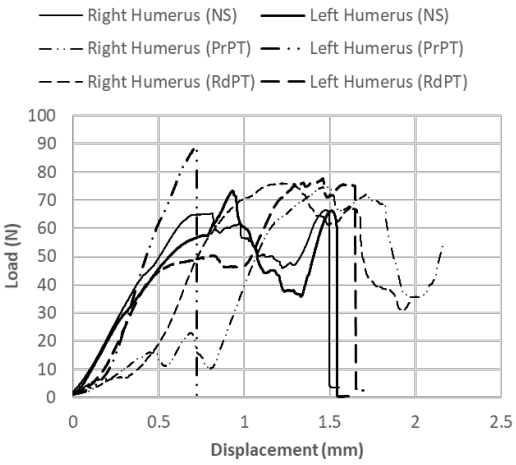


3. Humerus

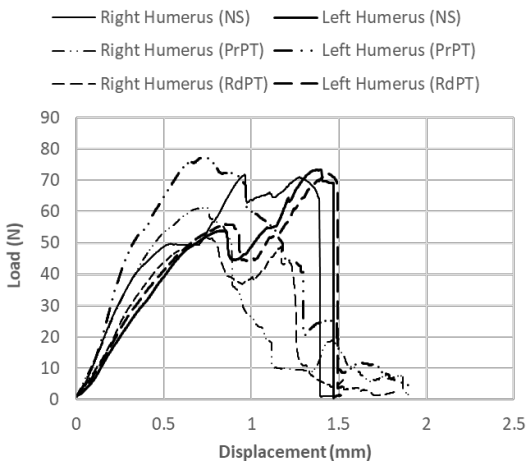
Batch 1



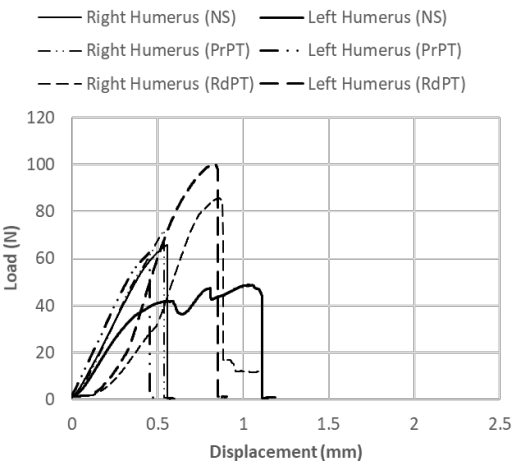
Batch 2



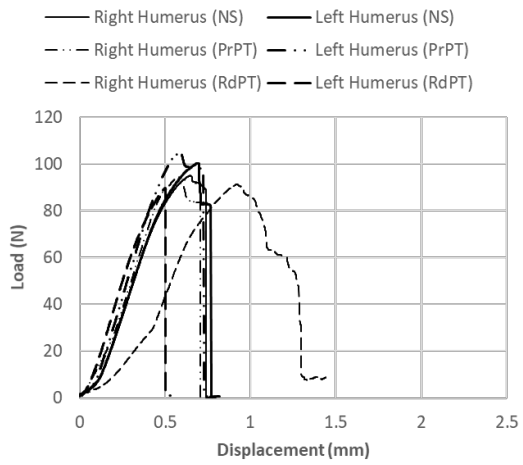
Batch 3



Batch 4

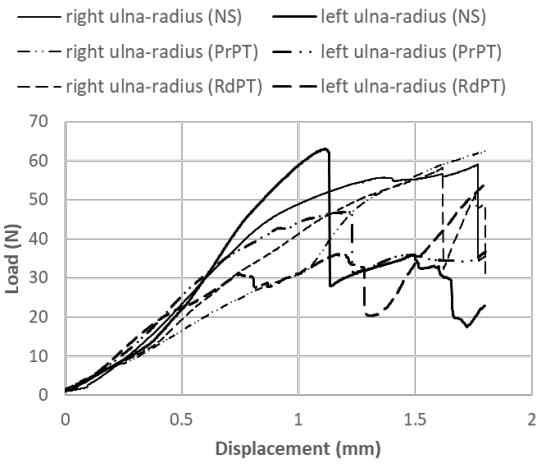


Batch 5

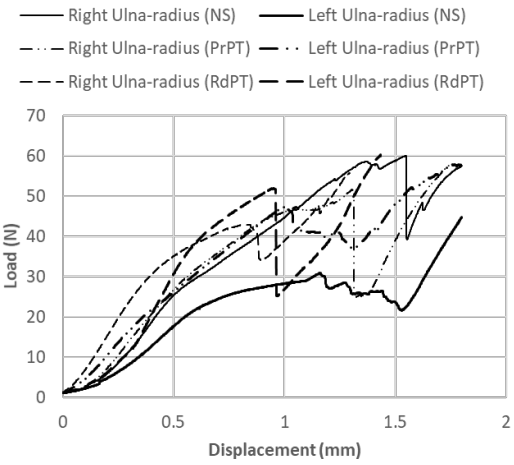


4. Ulna-Radius

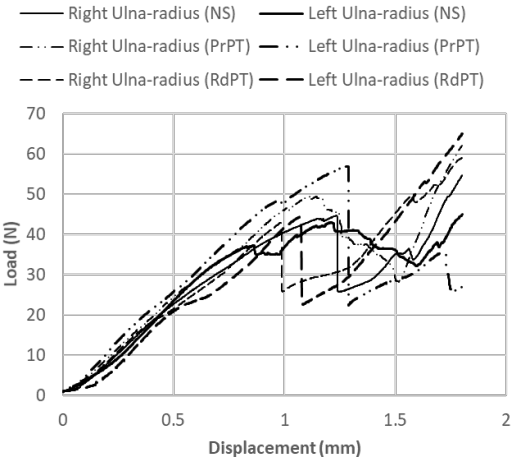
Batch 1



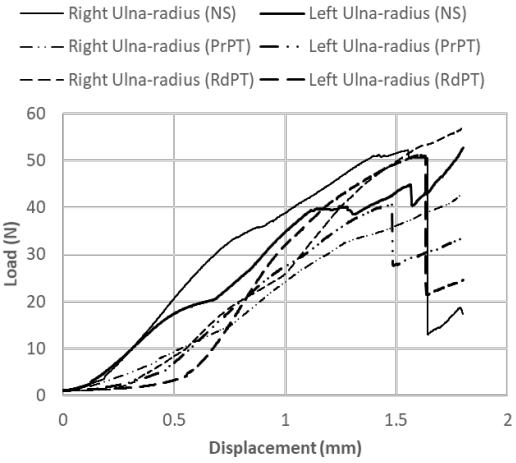
Batch 2



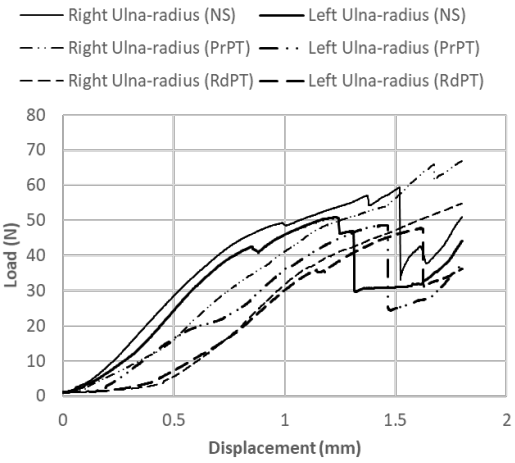
Batch 3



Batch 4



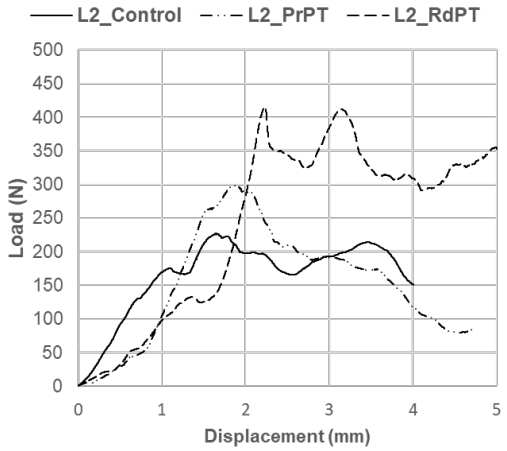
Batch 5



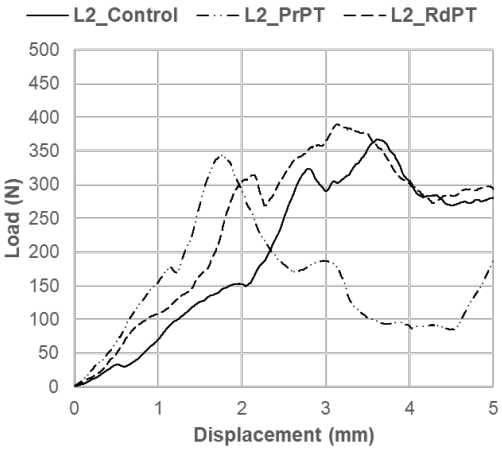
Appendix C : Compression Test

1. Lumbar Vertebra No. 2

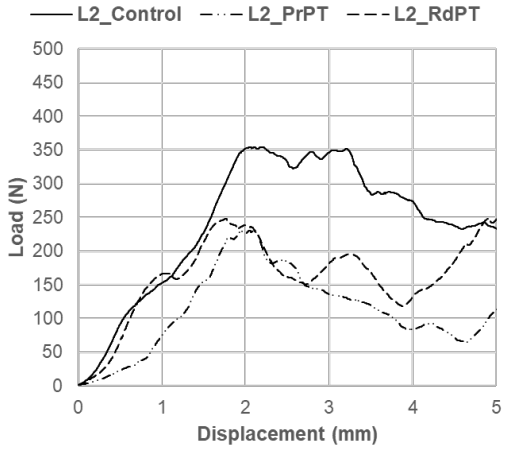
Batch 1



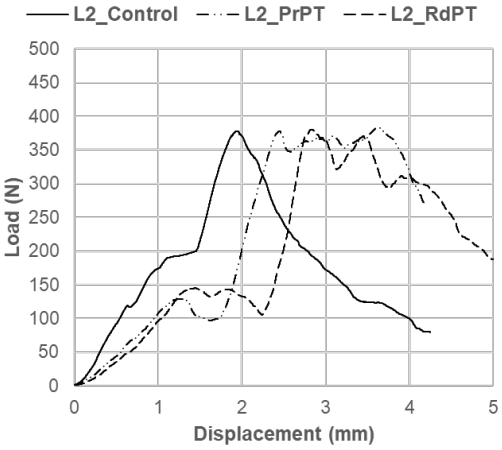
Batch 2



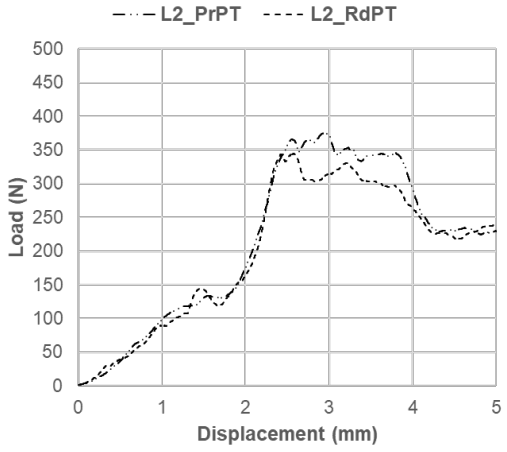
Batch 3



Batch 4

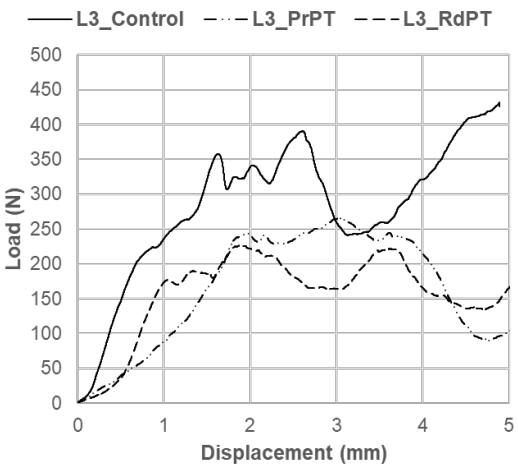


Batch 5

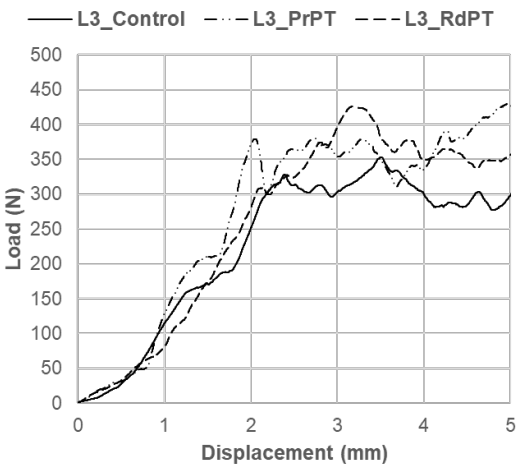


2. Lumbar Vertebra No. 3

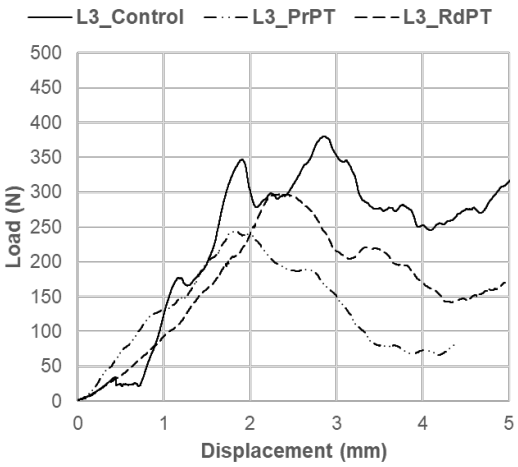
Batch 1



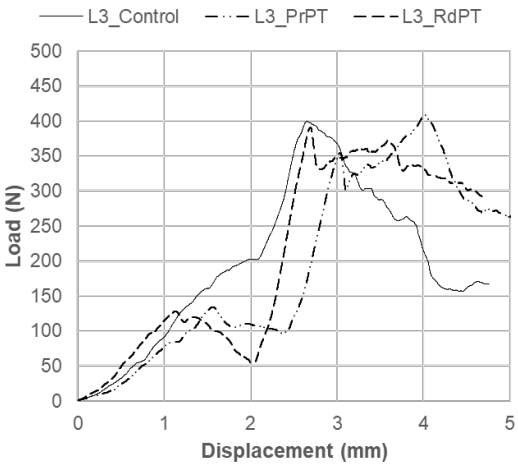
Batch 2



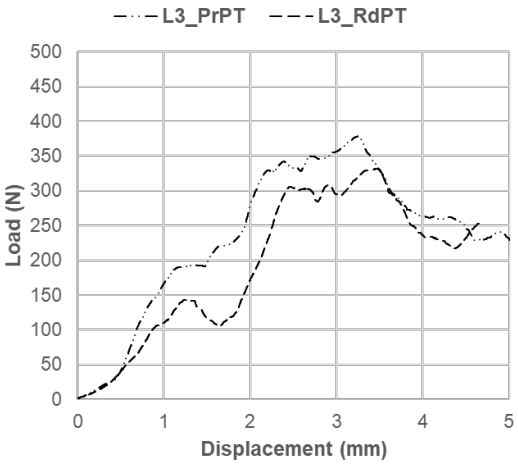
Batch 3



Batch 4

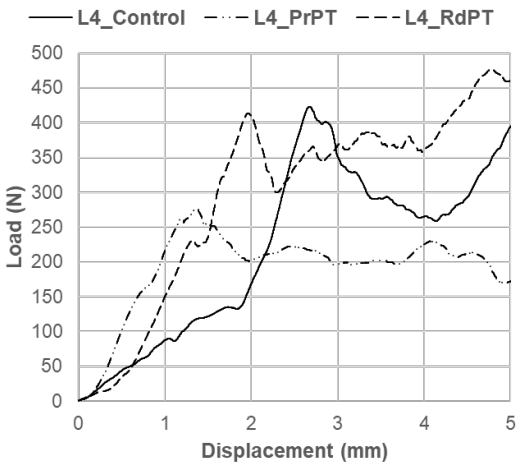


Batch 5

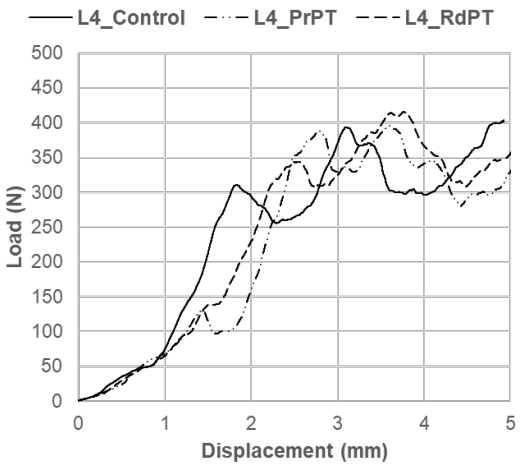


3. Lumbar Vertebra No. 4

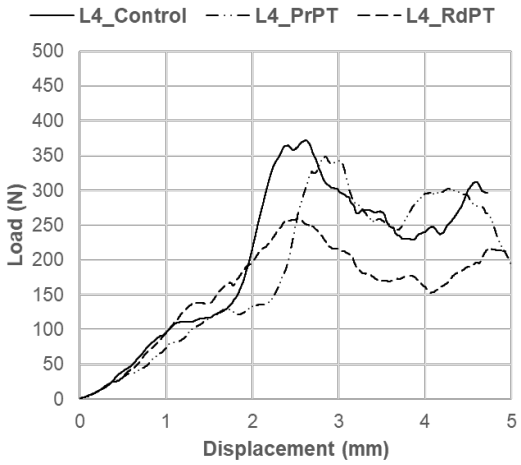
Batch 1



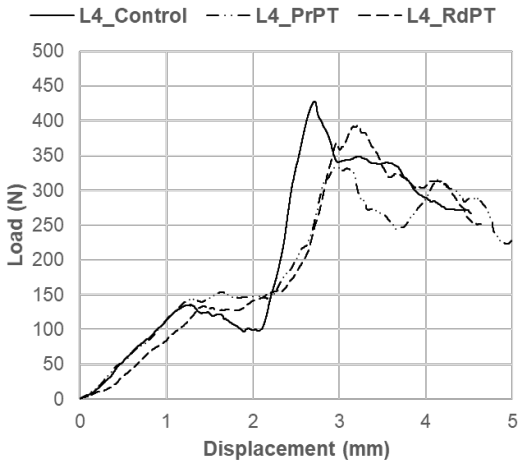
Batch 2



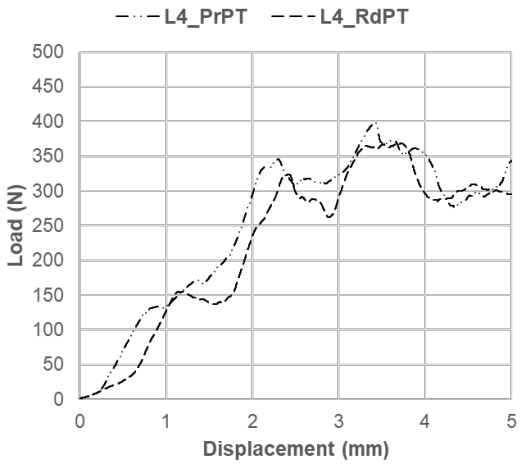
Batch 3



Batch 4

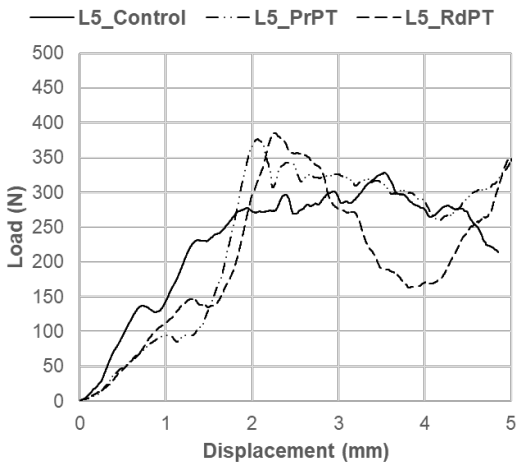


Batch 5

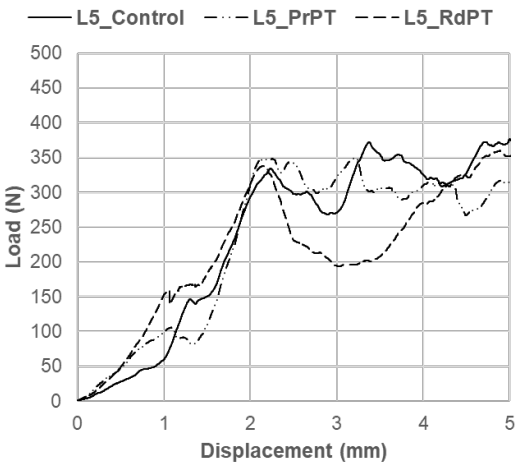


4. Lumbar Vertebra No. 5

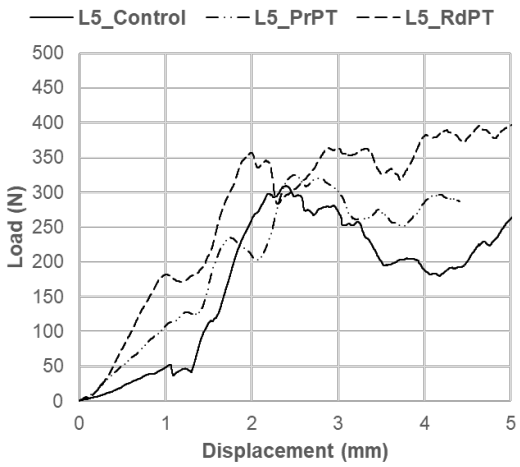
Batch 1



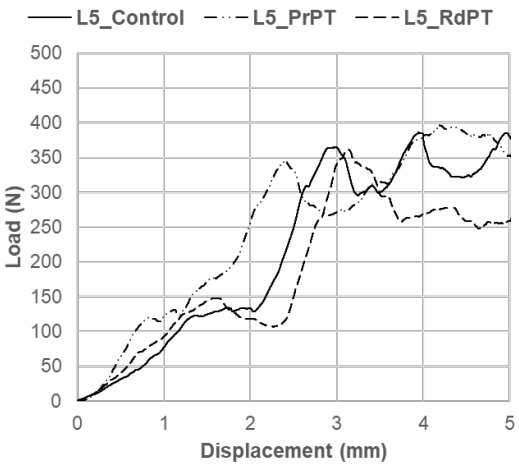
Batch 2



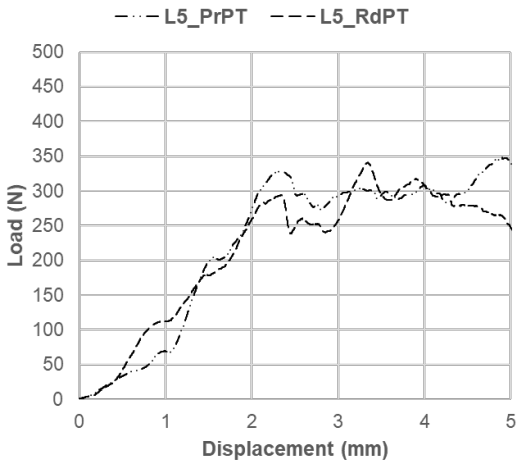
Batch 3



Batch 4



Batch 5



Appendix D : Micro-CT

1. Lumbar Vertebra No. 2

BMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	735.60	758.90	730.50	768.10		**748.28**	15.67
PrPT	737.80	753.40	700.00	748.80	741.10	**736.22**	18.93
RdPT	744.40	744.90	726.20	734.10	742.30	**738.38**	7.22

BMC (mg)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	58.68	55.89	56.74	61.07		**58.09**	1.99
PrPT	56.31	53.31	50.12	55.65	53.87	**53.85**	2.17
RdPT	56.94	55.53	53.25	55.77	48.94	**54.09**	2.84

BV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	7.98E-02	7.36E-02	7.77E-02	7.95E-02		**7.76E-02**	2.5E-03
PrPT	7.63E-02	7.08E-02	7.16E-02	7.43E-02	7.27E-02	**7.31E-02**	2.0E-03
RdPT	7.65E-02	7.45E-02	7.33E-02	7.60E-02	6.59E-02	**7.33E-02**	3.8E-03

BV/TV (%)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	66.60	62.50	63.7	68.7		**65.38**	2.43
PrPT	66.50	66.50	64.4	63.1	66.70	**65.44**	1.44
RdPT	67.50	65.30	63	64	58.70	**63.70**	2.92

TV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	1.20E-01	1.18E-01	1.22E-01	1.16E-01		**1.19E-01**	2E-03
PrPT	1.15E-01	1.06E-01	1.11E-01	1.18E-01	1.09E-01	**1.12E-01**	4E-03
RdPT	1.13E-01	1.14E-01	1.16E-01	1.19E-01	1.12E-01	**1.15E-01**	2E-03

vBMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	490.10	474.50	465.40	527.70		**489.43**	23.80
PrPT	490.90	500.80	450.50	472.60	494.00	**481.76**	18.20
RdPT	502.50	486.10	457.60	469.60	435.40	**470.24**	23.09

2. Lumbar Vertebra No. 3

BMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	733.80	745.20	730.10	765.20		**743.58**	13.67
PrPT	738.30	765.50	698.90	734.90	730.90	**733.70**	21.23
RdPT	750.30	746.20	726.40	735.10	734.00	**738.40**	8.68

BMC (mg)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	59.47	58.32	57.25	62.16		**59.30**	1.83
PrPT	61.53	55.46	57.19	59.64	56.69	**58.10**	2.19
RdPT	65.09	56.50	60.56	62.97	50.66	**59.15**	5.12

BV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	8.10E-02	7.83E-02	7.84E-02	8.12E-02		**7.97E-02**	1.4E-03
PrPT	8.34E-02	7.25E-02	8.18E-02	8.12E-02	7.76E-02	**7.93E-02**	3.9E-03
RdPT	8.67E-02	7.57E-02	8.34E-02	8.57E-02	6.90E-02	**8.01E-02**	6.7E-03

BV/TV (%)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	66.90	65.30	66.00	71.30		**67.38**	2.34
PrPT	67.50	67.60	67.00	63.90	69.00	**67.00**	1.69
RdPT	71.30	67.60	65.90	67.50	62.20	**66.90**	2.94

TV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	1.21E-01	1.20E-01	1.19E-01	1.14E-01		**1.18E-01**	3E-03
PrPT	1.24E-01	1.07E-01	1.22E-01	1.27E-01	1.12E-01	**1.18E-01**	7E-03
RdPT	1.22E-01	1.12E-01	1.26E-01	1.27E-01	1.11E-01	**1.20E-01**	7E-03

vBMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	491.20	486.80	482.00	545.60		**501.40**	25.73
PrPT	498.00	517.70	468.10	469.30	504.50	**491.52**	19.69
RdPT	534.90	504.70	478.90	496.20	456.80	**494.30**	26.09

3. Lumbar Vertebra No. 4

BMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	718.00	748.70	744.20	769.50		**745.10**	18.33
PrPT	739.20	762.30	725.20	747.20	733.00	**741.38**	12.71
RdPT	755.40	740.10	728.00	746.60	728.70	**739.76**	10.51

BMC (mg)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	61.01	59.96	63.91	65.79		**62.67**	2.31
PrPT	64.78	61.40	57.42	62.49	59.61	**61.14**	2.50
RdPT	66.37	60.93	64.76	67.26	54.67	**62.80**	4.61

BV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	8.50E-02	8.01E-02	8.59E-02	8.55E-02		**8.41E-02**	2.4E-03
PrPT	8.76E-02	8.05E-02	7.92E-02	8.36E-02	8.13E-02	**8.25E-02**	3.0E-03
RdPT	8.79E-02	8.23E-02	8.90E-02	9.01E-02	7.50E-02	**8.49E-02**	5.6E-03

BV/TV (%)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	68.10	66.90	68.6	73.8		**69.35**	2.64
PrPT	69.70	69.70	68.6	67.9	69.90	**69.16**	0.78
RdPT	72.20	68.80	67.6	70.1	64.70	**68.68**	2.51

TV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	1.25E-01	1.20E-01	1.25E-01	1.16E-01		**1.21E-01**	4E-03
PrPT	1.26E-01	1.16E-01	1.15E-01	1.23E-01	1.16E-01	**1.19E-01**	4E-03
RdPT	1.22E-01	1.20E-01	1.32E-01	1.29E-01	1.16E-01	**1.23E-01**	6E-03

vBMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	488.70	501.10	510.30	568.00		**517.03**	30.41
PrPT	515.10	531.00	497.80	507.30	512.20	**512.68**	10.88
RdPT	545.70	509.00	492.40	523.20	471.70	**508.40**	25.35

4. Lumbar Vertebra No. 5

BMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	707.90	720.80	705.30	748.50		**720.63**	17.13
PrPT	741.60	754.50	703.10	731.20	716.80	**729.44**	18.07
RdPT	737.30	727.40	722.40	728.40	723.90	**727.88**	5.20

BMC (mg)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	68.31	60.67	62.04	68.44		**64.87**	3.54
PrPT	63.25	62.08	63.77	64.55	60.70	**62.87**	1.35
RdPT	70.15	65.43	65.21	67.59	56.21	**64.92**	4.71

BV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	9.65E-02	8.42E-02	8.80E-02	9.14E-02		**9.00E-02**	4.5E-03
PrPT	8.53E-02	8.23E-02	9.07E-02	8.83E-02	8.47E-02	**8.63E-02**	2.9E-03
RdPT	9.51E-02	9.00E-02	9.03E-02	9.28E-02	7.76E-02	**8.92E-02**	6.1E-03

BV/TV (%)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	72.10	71.10	69.50	75.60		**72.08**	2.24
PrPT	71.30	73.80	73.10	70.00	72.50	**72.14**	1.35
RdPT	75.80	72.90	70.90	72.70	68.40	**72.14**	2.44

TV (cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	1.34E-01	1.18E-01	1.27E-01	1.21E-01		**1.25E-01**	6E-03
PrPT	1.20E-01	1.11E-01	1.24E-01	1.26E-01	1.17E-01	**1.20E-01**	5E-03
RdPT	1.26E-01	1.23E-01	1.27E-01	1.28E-01	1.13E-01	**1.23E-01**	5E-03

vBMD (mg/cm^3)	Batch #1	Batch #2	Batch #3	Batch #4	Batch #5	Average	SD
control	510.20	512.20	490.40	565.50		**519.58**	27.85
PrPT	528.80	557.10	513.90	512.00	519.40	**526.24**	16.50
RdPT	558.60	530.40	512.20	529.90	495.20	**525.26**	21.14

Appendix E : Anesthetic Procedures

1. Preparing 1ml syringe, 26G-needle, somnopenthyl, alcohol 70%, and saline water.
2. Measuring the rat's weight.
3. Calculating the amount of somnophentyl's volume that will be injected into the rat. Volume of somnophentyl (ml)= 6.2×10^{-4} x weight of the rat (gr).
4. Making the anesthetic solution by absorbing the amount of somnophentyl, to be calculated, into syringe and adding the saline water until the mixture fluid is 1ml.
5. Cleaning the needle of the syringe with alcohol.
6. Taking the rat by holding its tail. After that, covering fully the rat's body with a small towel and holding the rat at its tail while its body is being clamped with middle and ring finger.
7. Spraying the anesthetic injection's location (around peritoneal cavity) with alcohol.
8. Inject the anesthetic solution into the peritoneal cavity.
9. Release the rat at the cage.

Appendix F : Electromyostimulation Procedures

1. Preparing and assembling the electromyostimulation device by connecting the computer, I/O board, circuit board (resistor) and cables.
2. Preparing 26G-needle, black and red cable, plier, solder and soldering iron.
3. Cutting and bending the needle with the plier.
4. Creating the electromyostimulation electrodes by connecting and soldering the bended-needle with the cable.
5. Putting the rat at the operation table which has been covered with tissue papers.
6. If it is the first time for stimulation, removing the rat's hair with the hair clipper at around stimulating site.
7. Cleaning the left quadriceps and the electrodes with alcohol.
8. Injecting the electrode into the left quadriceps with distance 1 cm between both electrodes.
9. Connecting the electromyostimulation device to the electrodes.
10. Running the electromyostimulation program.

Appendix G : Harvesting Bone Procedures

1. Preparing syringe 1ml, 26G-needle, somnopenthyl, alcohol 70%, saline water, bone's container (such as bottles or six-well plate), and operation tools such as a clamp, scissors, knife leaf, etc.
2. Measuring the rat's weight.
3. Calculating the amount of somnophentyl volume that will be injected into the rat. Volume of somnophentyl (ml)= 6.2×10^{-4} x weight of the rat (gr).
4. Making the anesthetic solution by absorbing the amount of somnophentyl, as to be calculated, into a syringe and adding the saline until the mixture fluid is 1ml.
5. Cleaning the needle of syringe with alcohol.
6. Taking the rat by holding its tail. After that, covering fully the rat body with a small towel and hold the rat at its tail while its body is being clamped with middle and ring finger.
7. Spraying the anesthetic location around the peritoneal cavity with alcohol 70%.
8. Injecting the anesthetic solution into the peritoneal cavity.
9. Release the rat at the cage and wait until it sleeps.
10. Preparing the bone container and filling the container with saline water.
11. Removing the skin at the bone location.
12. Harvesting the bone.
13. Cleaning the bone from soft tissue with tissue paper.
14. Put the bone into the container.

Appendix H : Mechanical Testing Procedures

1. Preparing the bending jigs or compression jig by installing at the universal testing machine.
2. Setting the numbering system of the testing and the speed of testing at the testing machine's computer.
3. Taking the bone from the container and cleaning the bone with tissue paper.
4. Especially for long bone: Measuring the bone's length and marking the center of the bone with a small marker.

   Especially for vertebra: Removing the inferior extended arch which makes the position unbalance or not flat.
5. Put the bone at the bottom jig:

   - Bending test: placing the bone in such a way that the position of the mark is right in the middle.

     Femur: put the posterior side at the bottom jig.

     Tibia: put the lateral side at the bottom jig.

     Humerus: put the medial side at the bottom jig.

     Ulna-radius: put the lateral side at the bottom jig.

   - Compression test: placing the bone in such a way that the vertebra's inferior side is on top of the middle of the bottom jig.
6. Moving the upper jig until 1-2mm before touching the bone.
7. Running the mechanical test.
8. Returning the broken bone into its container and continuing the test with the other bones.

Appendix I : μCT Scanning Procedures

1. Preparing the μCT machine by warming it up.
2. Installing 0.1 mm filter.
3. Taking the bone from its container and cleaning it with tissue paper.
4. Put the bone into a container for scanning.
5. Put the container at the scanner's table.
6. Close the scanner's door.
7. Running the X-Ray.
8. Moving the scanner's table by controlling it from the computer until the bone picture size is appropriate with the monitor.
9. Setting the pixel dimension (512x512 or 1024x1024) and changing the pixel's size by moving the scanner's table.
10. Set the center of the bone by rotating it and centering it in several times.
11. Set the saving folder location for the scanning result.
12. Running the scanner.
13. After finish in scanning, putting the bone back into its container.
14. Continuing the scanning with other bones with the same procedure except changing the position of the scanner table. The scanner's table should be in the same position in all scanner day.
15. Scanning the phantom after scanning all bones.