論　文

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Study on Structural Changes in Tibial Cancellous Bone with Mechanical Loading After Short-term Immobilization in Rats

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Summary

This study aimed to investigate effects of exercise and immobilization just before the exercise on bone remodeling processes.

In experiment 1, rats were used as materials, and were divided into three groups: an exercise after immobilization group that performed 4-week-exercise after 4-day-immobilization (IM-EX), an exercise group that accomplished 4-week-exercise (EX), and a control that was fed normally for four weeks (CO). In experiment 2, rats were also divided into three groups: an exercise after immobilization group that performed 1-week-exercise after 2-day-immobilization (IM-EX), an exercise group that accomplished 1-week-exercise (EX), and a control that was fed normally for one week (CO). Rats in each group were euthanized at the end of experimental period, tibiae were excised from them and those specimens were observed histologically and morphometrically.

Density and thickness of the bone trabeculae increased at primary cancellous bone from immediately after starting of experiments in IM-EX of the experiment 1 and 2. As for secondary cancellous bone, IM-EX of experiment 1 showed remarkable decrease of bone mass once after four days immobilization, and it was necessary for four weeks to reached to the same level as EX by exercise. On the other hand, in IM-EX of experiment 2, the bone mass hardly changed by two-day-immobilization but decreased temporarily after 2 and 4 days of exercise period, and then, it already recovered up to level than EX after 7 days. Many osteoblasts showed positive reaction to immunostaining of RANKL, and many TRAP positive cells were also recognized, in IM-EX of experiment 2. And, bone matrixes indicated positive reactions to immunostaining of TGF-β, in each groups.

Through the results mentioned above, it was suggested that active bone formation could be brought by the exercise following the short-term immobilization, because factors like TGF-β that promoted differentiation and activation of the osteoblasts were released from the bone matrix by acute bone resorption.

Keywords: immobilization, jumping exercise, bone structure

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1． Introduction

Bone morphological changes were divided into two bone metabolic processes, one was bone modeling, that is, forming process of cortical and cancellous bone accompanied with growing, and the other was bone remodeling, that is, structural changing process without changes in size and morphology. Mechanical stress affected to both the bone modeling and the remodeling. It was reported, from studies by hind limb suspension or casting that simulated weightlessness in space or immobility, that decrease in mechanical stress caused loss of bone mass[1-3]. It was determined that cause of bone mass loss by decreasing of mechanical stress was mainly inhibition of bone formation. On the other hand, many reports indicated that exercise like walking, running and jumping improved the bone mass due to increase in mechanical stress[4-7]. In these reports, it was described that bone formation was promoted and resulted in bone mass enhance. The present writers already recognized that many cells showing positive reaction to osteocalcin appeared at primary cancellous bone of tibial proximal metaphysis, and the bone formation started from the portion near growth plate[8]. Also, the effects of immobilization just before exercise period were also investigated in that study, and it was observed that the bone mass of the cancellous bone couldn't recovery to normal level with exercise for the same period as immobilization. However, it was shown that osteoclasts increased but the bone mass hardly decreased by one or two days immobilization[9].

It was demonstrated, by experiment in vivo, that the osteoclasts were differentiated by RANKL and they didn't appear in mouse lacked RANKL[10]. Therefore, RANKL was often used as differentiation marker of the osteoclasts. TGF-β and BMP were embedded in bone matrix. They played a role of differentiating and activating factor of the osteoblasts, and promoted bone formation by the osteoblasts when they were released from the bone matrix with bone resorption[11, 12]. However, it was understood that the osteoclasts increased through the immobilization treatment, but the relationship between that treatment just before exercise and the bone formation had not been cleared.

Then, the aim of this study was to compare and investigate the effects of exercise and immobilization just before the exercise, by preparing the exercise group and exercise after short-term immobilization group.

2． Materials and Methods

2．1． Experimental protocol

Experiment 1:

Twenty four male rats (wistar strain) were used as materials, and were divided into an exercise group (EX), an exercise after immobilization group (IM-EX) and a control (CO), randomly. Knee joints of hind limbs were immobilized for four days just before the exercise period in IM-EX. Rats of EX and IMEX performed jumping exercise (45cm height jumping, 40 times/day, 5days/week) for 1, 2, 3 or 4weeks (EX-1W, 2W, 3W, 4W and IM-EX-1W, 2W, 3W, 4W, respectively). CO was also grouped into CO-1W, 2W, 3W and 4W, in the same way.

Tibiae were excised from rats after each experiment period. Then, the specimens were prepared by using them, and were observed and measured histologically and morphometrically, by following methods.

Experiment 2:

Twenty four male rats (wistar strain) were used as materials and they were divided into three groups: exercise after immobilization group (IM-EX), exercise group (EX) and control (CO). Rats of EX and IM-EX performed the jumping exercise similar to the experiment 1 for 2, 4 or 7 days (EX-2D, 4D, 7D and IM-EX- 2D, 4D, 7D, respectively). And, hind limbs were immobilized for two days just before the exercise period, in IM-EX. CO were divided similarly into CO-2D, 4D and 7D.

Methods of preparation and observation of specimens were also similar to experiment 1.

2.2. Morphometric measurements

The specimens were embedded in rigolac resin without decalcification, ground, stained by toluidine blue and observed by light microscope. Further, bone structures were measured at secondary cancellous bone in each groups by bone histomorphometric analysis method, and then those data were shown as Mean ± standard deviation. The data were assayed by Mann-Whitney U test for independent samples.

2.3. Macroscopic analyses

The specimens cut in sagittal direction were immersed in 20% KOH solution and their cleaved faces were observed macroscopically.

2.4. Histological analyses

Other specimens were decalcified by EDTA. They were dehydrated, cleared, and embedded in paraffin wax. Perfect serial sections were cut, using the paraffin blocks. Furthermore, the other specimens that were embedded in rigolac resin without decalcification were ground until thickness of about 100 micrometers. Those paraffin sections and ground specimens were stained by various methods and were observed with light microscope. The specimens were prepared by the method similar to macroscopic analyses. And, they were dehydrated, were evapolated with carbon and platinum, and were observed by scanning electron microscope (SEM).

２．５．immunohistological analyses

The paraffin sections were cut by the same methods described above. They were stained about RANKL and TGF-β immunohitochemically, and were observed by light microscope.

３．Results

3.1. Experiment 1

The primary cancellous bone existed in narrow space only just under growth plate, and the secondary cancellous bone occupied wide extent under the primary cancellous bone, when observing dried tibial specimens of IM and CO. Width of the primary cancellous bone of IM was the same as CO, but the bone trabeculae of the secondary cancellous bone decreased at the mid portion especially.（Fig. 3, 4）

When observing magnified images of the primary cancellous bone of CO-4W, EX-4W and IM-EX-4W, following observations were obtained. Thin bone trabeculae existed densely and arranged in superior and inferior direction in CO-1W. The same thickness and arrangements of the bone trabeculae were also seen in CO-4W, but thick bone trabeculae existed at lower portions of the primary cancellous bone.（Fig. 5-1-4）Slight thick bone trabeculae already appeared at the lower portion of the primary cancellous bone in EX-1W.（Fig. 5-5-8）Thickness of bone trabeculae already increased near the growth plate and the density of them was higher than other groups wholly, in IM-EX-1W.（Fig. 5-9-12）

The bone volume of the secondary cancellous bone increased in CO-4W, though it didn't show little changes in CO-2W. The bone volume value of EX-2W was lower than EX-1W, but it ascended accompanied with progressing of experimental protocol. The bone volumes of IM-EX-1W and 2W were lower than CO-1W and 2W, respectively, but those of IM-EX-4W indicated higher value than CO-4W and EX-4W.（Fig. 6）

The bone trabeculae were thick and arranged in superior and inferior direction at the lower portion of the secondary cancellous bone, when observing them with SEM. It was often found, in EX-1W, that neighbor bone trabeculae fused in each other, and resulted in thicker and plate-like bone trabeculae, compared with CO-1W. The bone trabeculae decreased remarkably in IM-EX-1W, but a few bone trabeculae showing plate-like shape were also observed. The number of the bone trabeculae decreased but their thickness increased conversely in CO-4W, compared to CO-1W. It was often observed that the thickness of the bone trabeculae increased and they arranged from anterior superior to posterior inferior direction. The bone trabeculae of IM-EX-4W existed densely than CO-4W, corresponded to data of the bone morphometry, but they arranged in superior and inferior direction mainly.（Fig. 7）

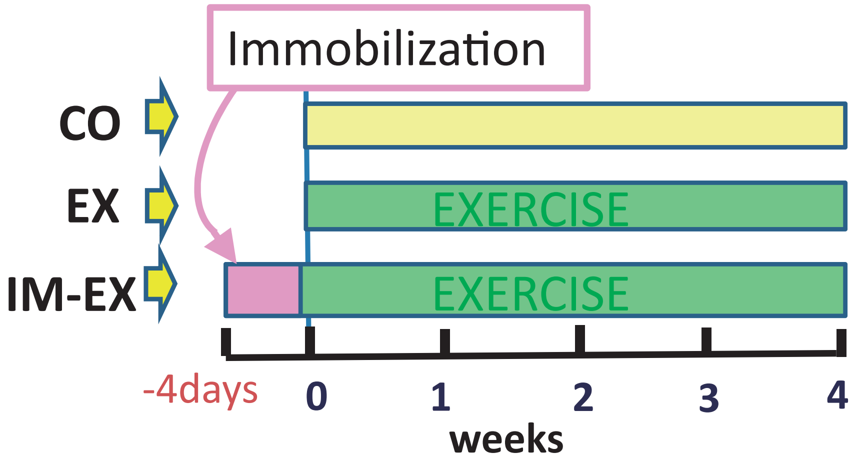

Fig. 1 Experimental protocol in each group of Experiment 1

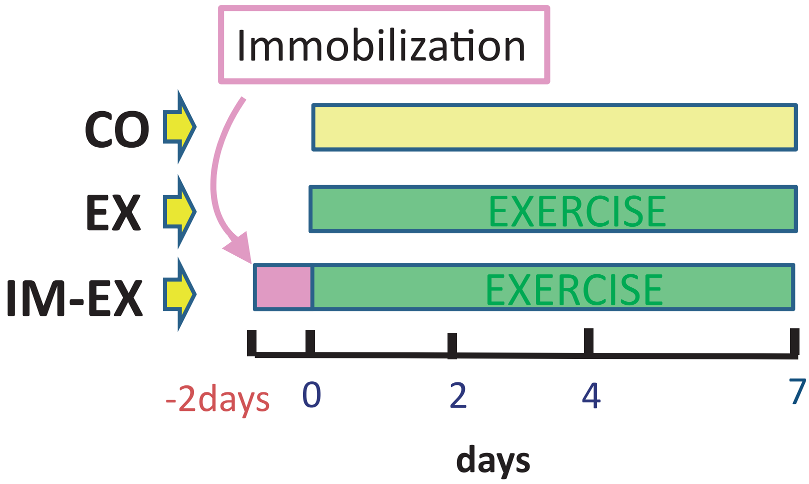

Fig. 2 Experimental protocol in each group of Experiment 2

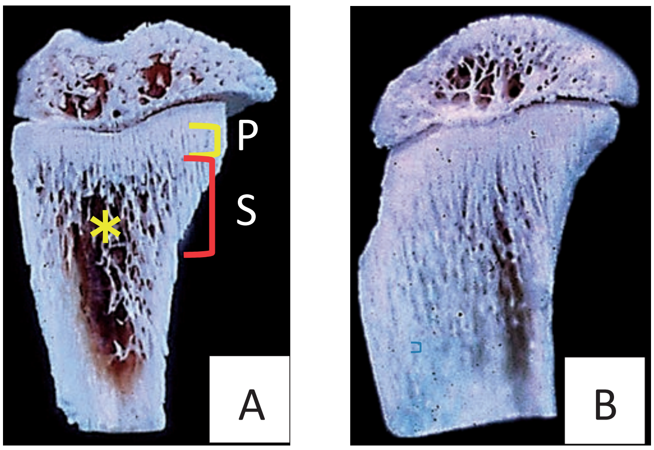

Fig. 3 Changes in bone structure with four days immobilization
(Dried bone specimens treated by KOH)
A: IM, B: CO, P: Primary cancellous bone, S: Secondary cancellous bone

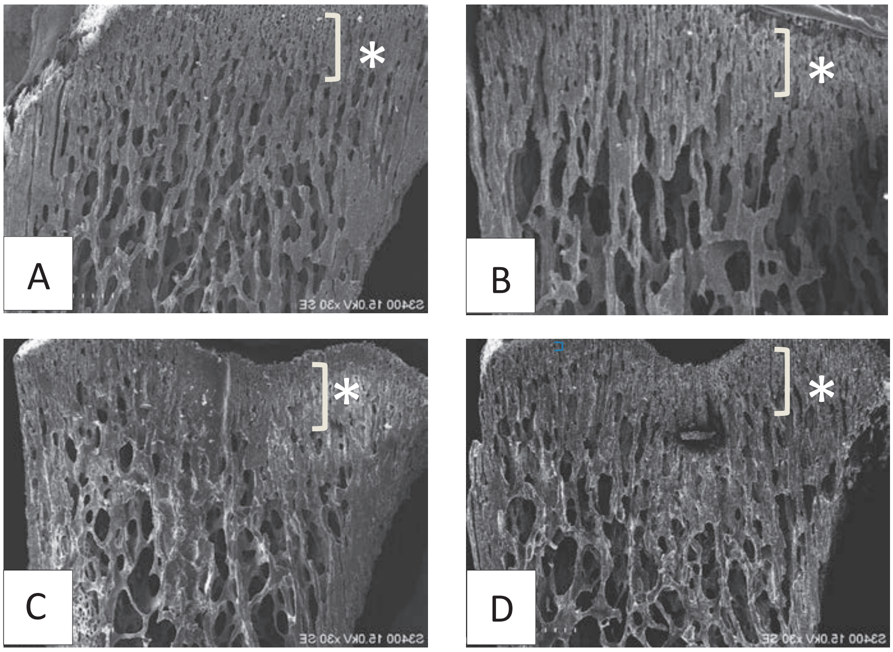

Fig. 4 Comparison of proximal cancellous bone in each group
Upper images: Experiment 1 (A: CO, B: IM)
Lower images: Experiment 2 (C: CO, D: IM)
＊：primary cancellous bone

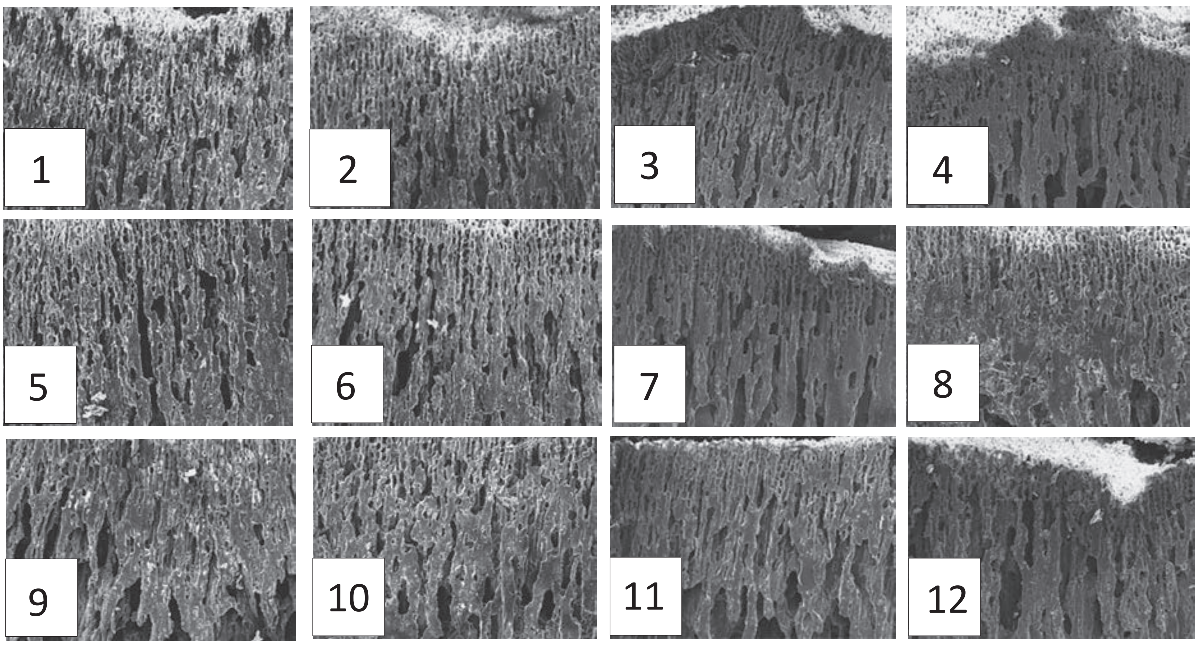

Fig. 5 SEM images of the primary cancellous bone in each group
5-1-4: 1-4W of CO,　5-5-8: 1-4W of EX
5-9-12: 1-4W of IM-EX

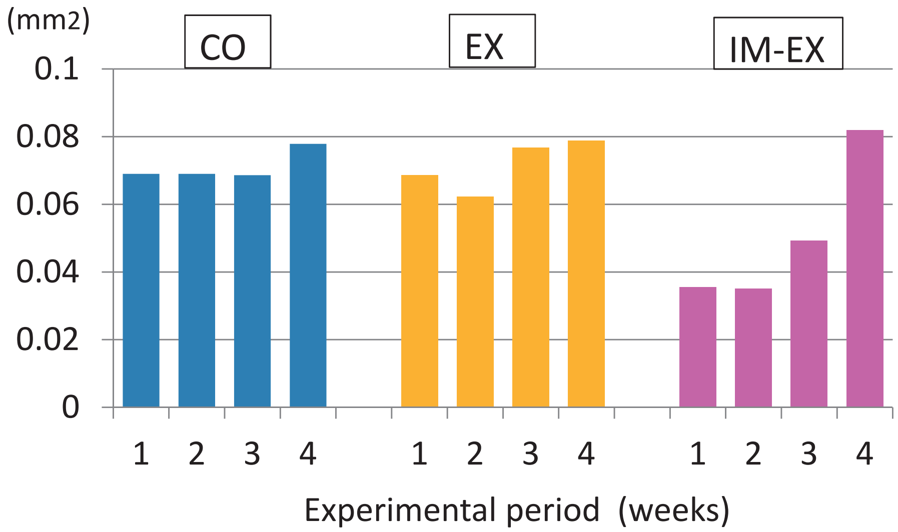

Fig. 6 Bone volume of secondary cancellous bone in each group

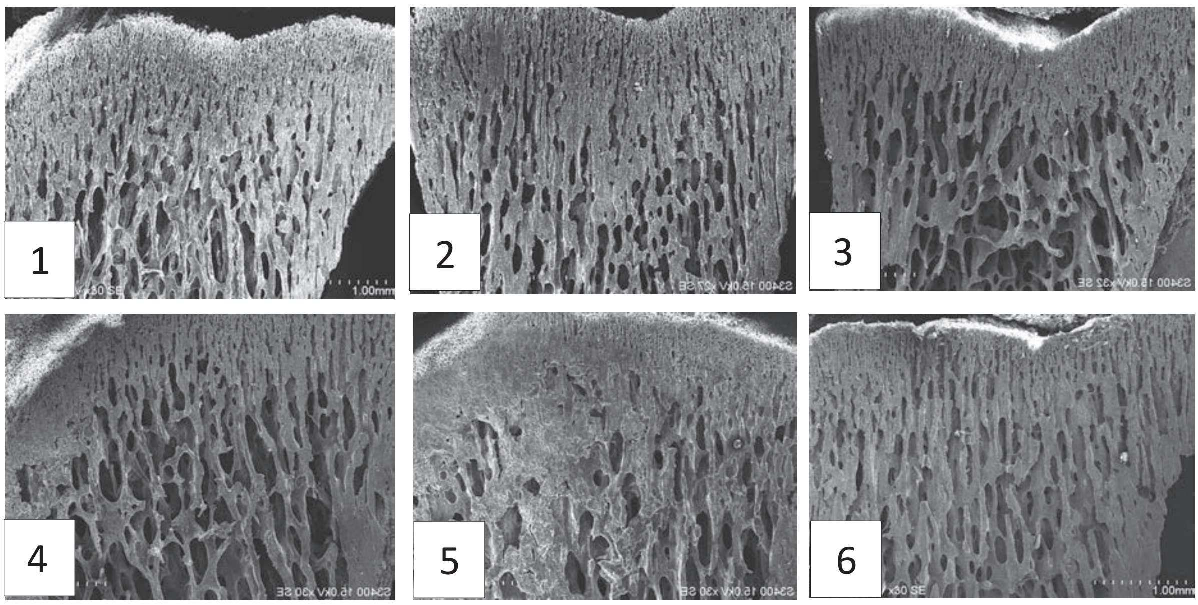

Fig. 7 SEM images of the secondary cancellous bone in each group
1: CO-1, 2: EX-1, 3: IM-EX-1
4: CO-4, 5: EX-4, 6: IM-EX-4

3.2. Experiment 2

As to ratio of width between the primary and secondary cancellous bone, little differences are recognized in IM and CO, when observing specimens treated by KOH, macroscopically. And, differences in thickness, density and arrangement direction of the bone trabeculae also couldn't be found, even observing them by SEM. (Fig. 4-C, D)

It was recognized, using SEM, that the primary cancellous bone composed of thin bone trabeculae and they arranged in superior and inferior directions, from starting of experiment until

7 days later. Thickness and density of the bone trabeculae of the primary cancellous bone already increased from two days later of the experiment in EX and especially IM-EX. (Fig. 8-1-9)

Bone volumes of CO-2D and EX-2D were 0.055±0.026 and 0885±0.036mm^2, respectively, and EX-2D indicated higher value（P＜0.01）than CO-2D. On the other hand, the value of IM-EX-2D was 0.0343±0.015mm^2, and was lower than CO-2D significantly（P＜0.05). The bone volume descended from EX-4D to EX-7D, but difference of them wasn't significant. That of IM-EX-4D was lower than CO-4D, but it was found that the value of IM-EX-4D ascended and resulted in higher value than CO. (Fig. 9)

The bone trabeculae of the secondary cancellous bone were thin and their arrangements were irregular in CO. Density of the bone trabeculae was low at the lower portion of the secondary cancellous bone in IM-EX-2D, and was going to be high slightly. However, arrangements of the bone trabeculae were irregular yet in IM-EX-7D. On the other hand, the density of the bone trabeculae was already higher, and they often indicated plate-like shape in EX-2D. Those bone trabeculae were remodeled, were going to be thinner slightly, and indicated regular arrangements. (Fig. 10)

Positive reactions of TGF-β were recognized in bone matrix of IM-EX immunohistologically. (Fig.

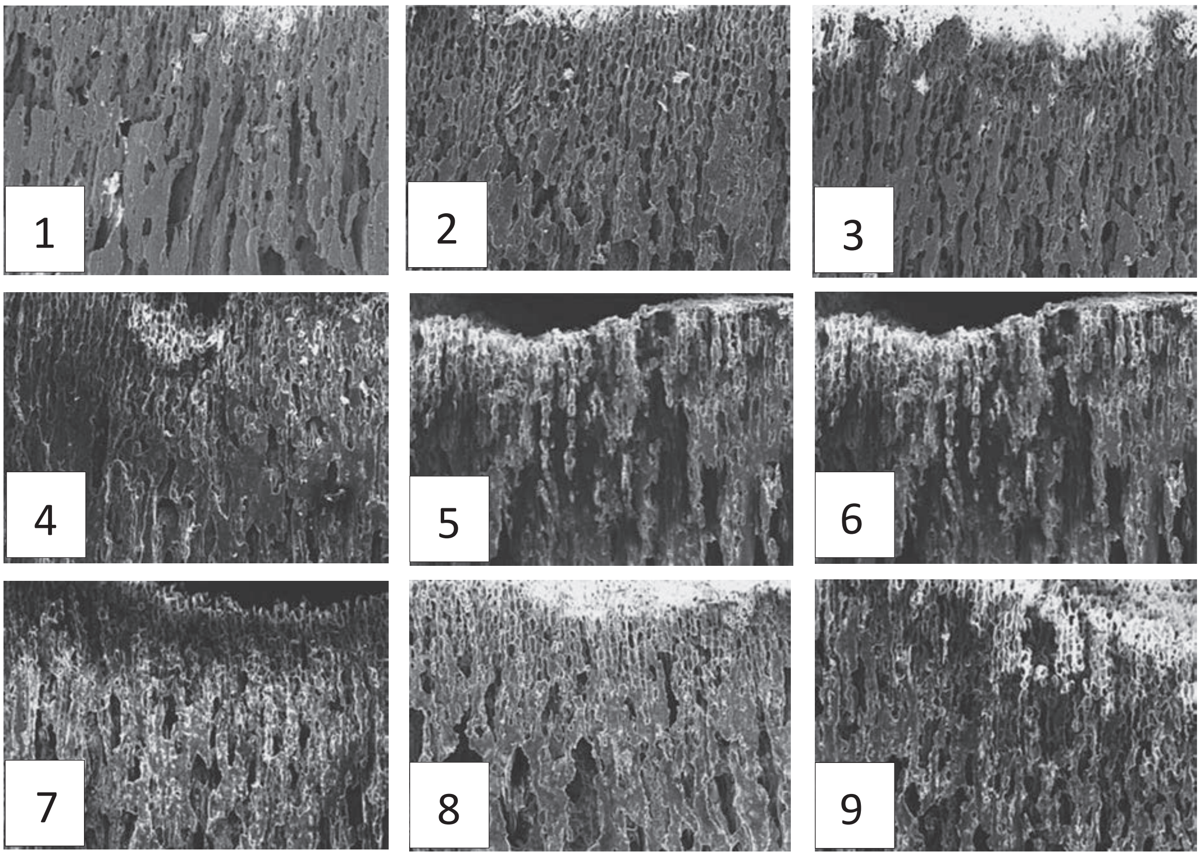

Fig. 8 SEM images of the primary cancellous bone in each group
8-1-3: 2-7 days of CO,　8-4-6: 2-7 days of EX,　8-7-9: 2-7 days of IM-EX

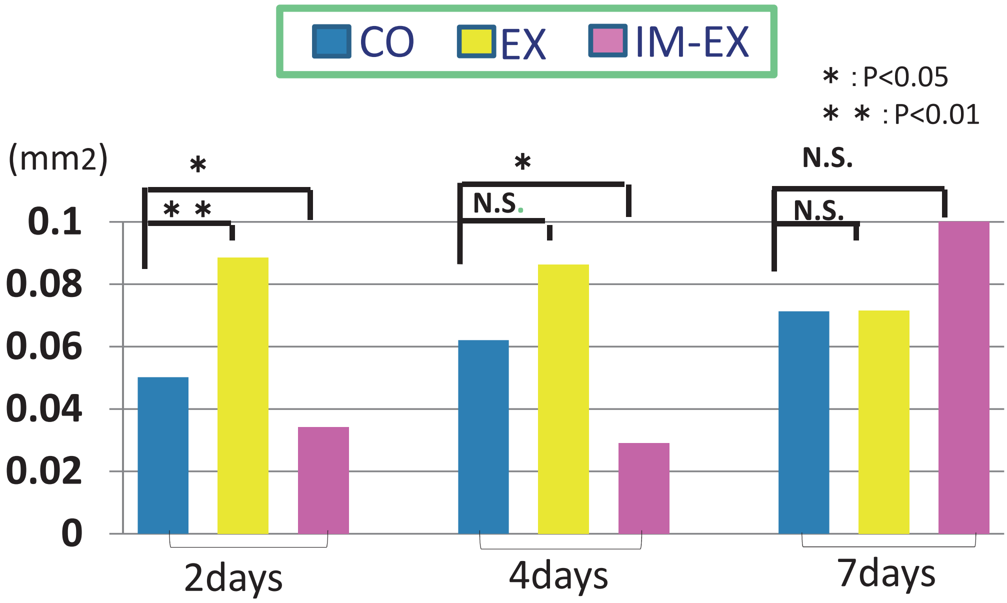

Fig. 9 Bone volume of the secondary cancellous bone in each group

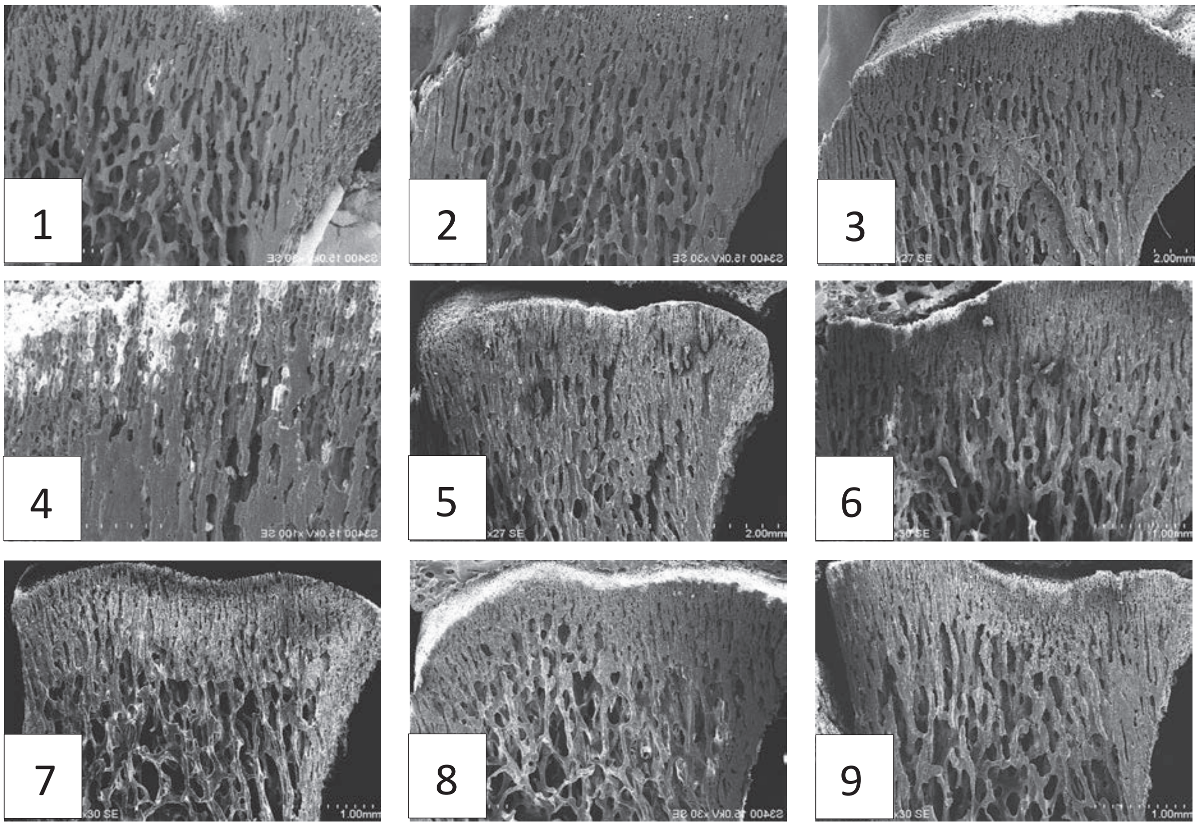

Fig. 10 SEM images of the secondary cancellous bone in each group
8-1-3: 2-7 days of CO, 8-4-6: 2-7 days of EX, 8-7-9: 2-7 days of IM-EX

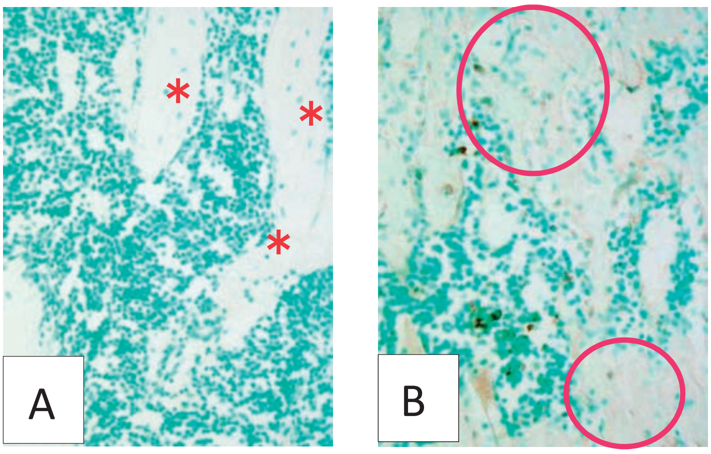

Fig. 11 Results of immunostaining by TGF-β
A: Negative Control, B: IM-EX
Image A: Bone matrix indicated negative reaction to immunostaining by TGF-β.
Image B: Bone matrix showed positive reaction to that.

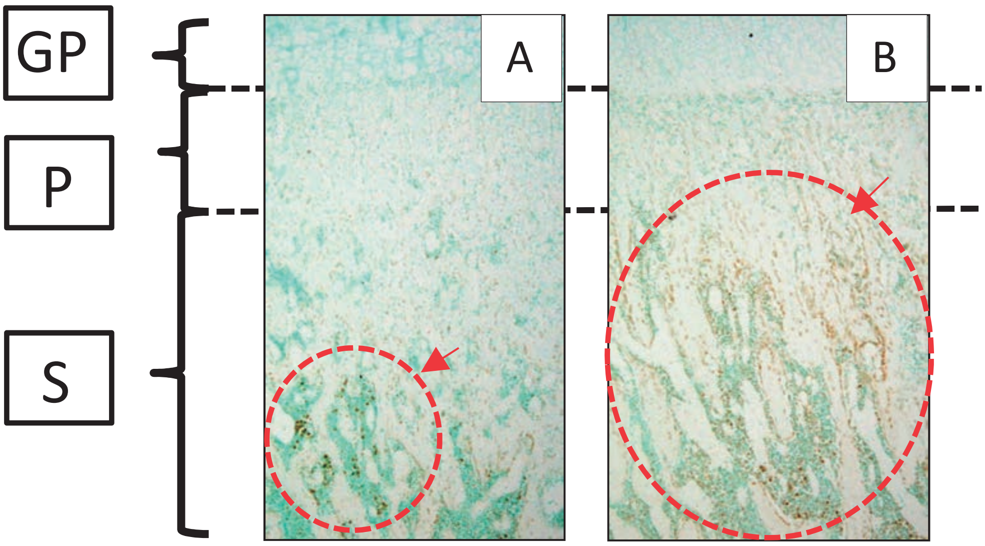

Fig. 12 Localization of the RANKL-positive cells in CO-2 and IM-EX-2
A: CO-2, B: IM-EX-2, GP: Growth Plate
P: Primary cancellous bone, S: Secondary cancellous bone
Many RANKL-positive cells were found in red circles.

11) It was found that many cells showing RANKL positive reactions in IM-EX-2D, compared to CO-2D, (Fig. 12) Many cells showing positive reactions to TRAP staining appeared in IM-EX-2D compared to CO-2D, but the number decreased in IM-EX-7D. (Fig. 13)

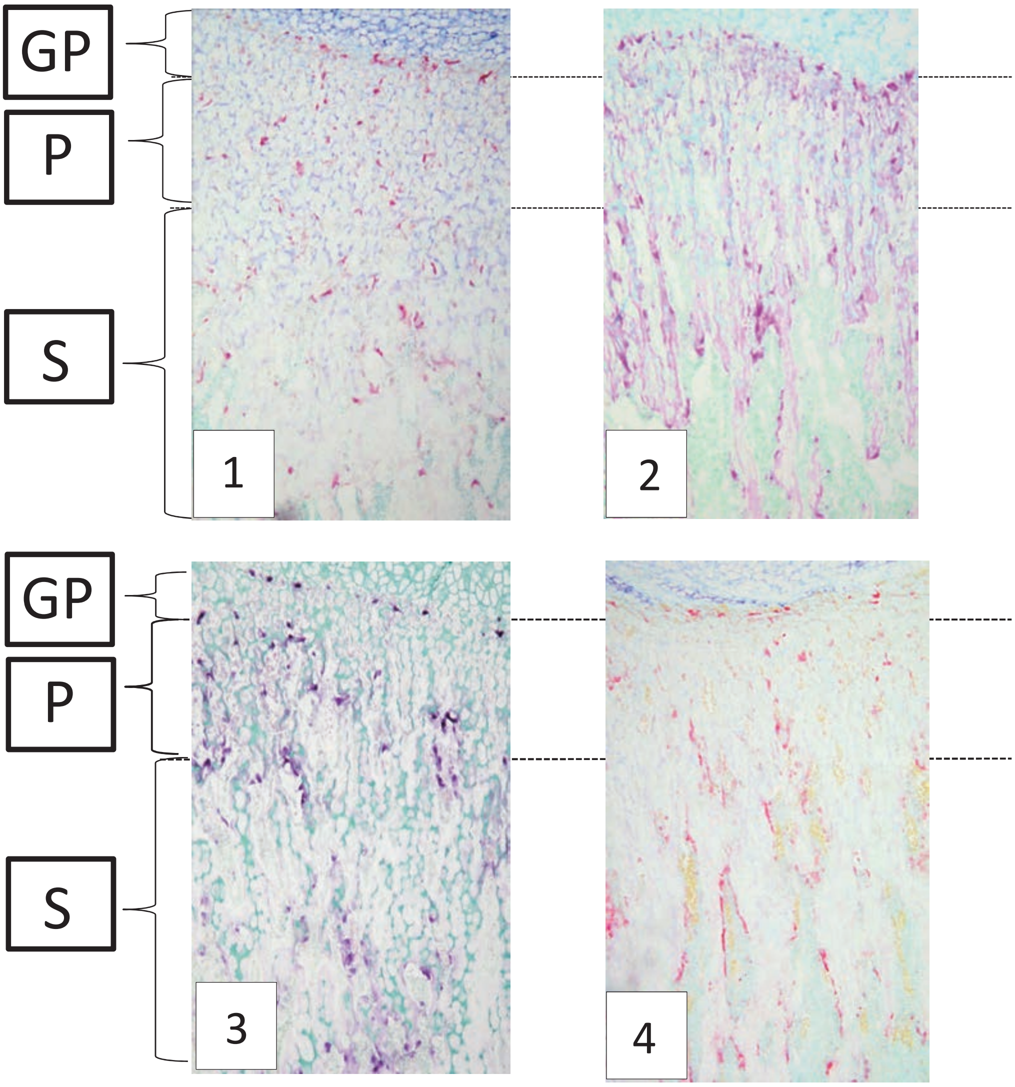

Fig. 13 Results of TRAP staining
1: CO-2, 2: IM-EX-2, 3: CO-7, 4: IM-EX-7
GP: Growth Plate, P: Primary cancellous bone
S: Secondary cancellous bone

4. Discussion

In this study, effects of immobilization treatment just before exercise period on tibial bone structures in rats were observed, and they were discussed below based on those data.

4. 1. Effects of immobilization

Many reports indicated that decrease in bone trabeculae by descended mechanical stress was started within short period[1 ~ 3]. And, it was shown that bone structures couldn't recover to the normal level by exercise for same period as immobilization period[13, 14]. Ju[15] reported, from reloading experiment using young rats, bone mineral density decreased 13.6% with tail suspension for two weeks, and it was necessary for five weeks to recovery to the normal level by reloading.

It was recognized, in this study, that short-term (four days) immobilization resulted in little changes of width of primary cancellous bone, but in remarkable decrease of that of secondary cancellous bone at mid portion. It was reported that bone mass decreased under no gravity environment in space and it recovered after return to the ground. Moreover, in this report, it was also indicated that exercise during space flight had protection effects of bone loss[16)]. Thus, it was understood that the mechanical stress related to maintenance of bone mass and the bone trabeculae reacted to decrease in the mechanical stress at earler stage.

The cancellous bone played a role of dispersion of the mechanical stress from neighbor bone to surrounding cortical bone. As corresponding with growing changes in body weight and physical activity, the width of the cancellous bone increased. To the contrary, alveolar bone was resorbed after tooth extraction[17)] . It was speculated that the mid portion bone played role of receipt of most large mechanical stress in the cancellous bone, because the fact that the bone trabeculae started in decrease from that portion.

Calcified cartilage trabeculae were formed at growth plate. They were used as core of the bone trabeculae and bone matrixes were added to surround them at the primary cancellous bone. Thickness of the bone trabeculae just under the growth plate increased, accompanied with growth. It was thought that thickness of the calcified cartilage trabeculae related to degree of hypertrophy of chondrocytes at the growth plate[18)] .

Sakai[3)] showed that the number and thickness of the bone trabeculae decreased at not only primary but also secondary cancellous bone due to experiment of 2-week-immobilization. It was supposed that difference between results of this study and reports of Sakai[3)] was related to their immobilization periods, because remarkable bone loss was observed only in the case of experimental 1 of this study.

It was thought that a given bone mass was needed in spite of degree of loading in the primary cancellous bone, because that bone existed just under the growth plate and always received from the femur. Tobita[8)] reported that shor-term immobilization of hind limbs caused increase in the number of osteoclasts and decrease of osteoblastic activity, and then, resulted in decrease of bone mass after four days of the experiment. Two-day-immobilization of hind limb of growing rats resulted in little changes in thickness, density and arrangement direction of bone trabeculae in this study. However, the bone trabeculae of the secondary cancellous bone decreased by four days immobilization. Thus, it was speculated that decrease of the bone trabeculae by that immobilization was due to synergistic effects of proliferation of the osteoclasts and inhibition of bone formation.

4. 2. Effects of Exercise

4. 2. 1. Primary Cancellous bone

The primary cancellous bone that was consisted of thin and short bone trabeculae changed to the secondary cancellous bone that was composed of thick and long bone trabeculae by bone remodeling at epiphysis of long bone in growing period. Width of the primary cancellous bone decreased gradually, according to growth. No differences were recognized as for thickness of the primary cancellous bone in each group. The calcified cartilage trabeculae composed core structures of bone trabeculae at the primary cancellous bone. Their morphologies related to width of cartilage matrix, erosion speed of septclasts from inferior edge, and division ability of chondrocytes, in growth plate. Thus, it was thought that the thickness of the growth plate was affected by genetic factor like division ability of chondrocytes. The bone trabeculae of EX-1W and IM-EX-1W were thicker than CO-1W at experiment 1, and the bone trabeculae of EX-2D and IM-EX-2D were also thicker than CO-2D at experiment 2. Therefore, it was hypothized that densities of the bone trabeculae were affected by genetic factors and those morphologies were decided by degree of mechanical stress, because the structure of the primary cancellous bone wasn't affected by duration of immobilization or exercise and thickness of bone trabeculae increased from the early stage of the exercise period.

4. 2. 2. Secondary Cancellous Bone

The secondary cancellous bone mainly played the role of scattering of mechanical loading from neighbor bones, to resist increase of body weight and activity accompanied with growth. The thickness and length of the bone trabeculae increased, and width of the cancellous bone also increased, with growing. Existence of the calcified cartilage trabeculae was essential to promote increase of thickness and length of the bone trabeculae.

It was reported that the bone mass increased owing to mechanical stress by exercise[4~7]. It was necessary for four weeks to recovery until normal bone mass level in experiment 1. It was thought that decrease of the bone trabeculae caused delay of recovery. By contrast, in experiment 2, the bone volume of IM-EX-7D was no significant but higher than EX-7D and CO-7D, and it recovered within one week of experiment. Tendency of decrease of the bone mass was recognized at both primary and secondary cancellous bones due to two-day-immobilization in IM-EX-7D. It was thought that little loss of the bone mass in IM-EX-7D was related to no decrease of the bone trabeculae after immobilization. It was considered that decrease of the mechanical stress just before the exercise affected an increase of bone mass by exercise, from data of the experiments 1 and 2.

It was shown that bone trabeculae formed a three dimensional network[19], and arrangement directions of them agreed with lines of force of mechanical loading[20], in order to scatter the loading to surrounding cortical bone. It was reported that the bone trabeculae increase their thickness and length at later stage of growing period[21]. And, it was also showed that the bone trabeculae arranged in direction from anterior superior to posterior inferior at that stage. However, they were thinner and shorter and arranged mainly in superior and inferior direction at early stage of that period[21]. It was recognized that the bone trabeculae decreased temporarily in EX-2W of experiment 1, but the bone mass increased remarkably in EX-1W (experiment 1) and IM-EX-7D (experiment 2). The bone trabeculae came to show typical structure that was arranged in direction from anterior superior to posterior inferior finally in EX-4W. Bone remodeling was proceeding gradually for long time in the case of normal growing process and such arrangement directions were given. The same arrangement were recognized in EX-4-7D, but before that, temporal increase of these was observed in both EX and IM-EX. Therefore, It was speculated that the bone mass increased first, the arrangement direction of the bone trabeculae were remodeled subseguently in agreed with the line of force of mechanical loading.

It was reporting that cells indicating positive reaction to tartrate-resistant acid phosphatase (TRAP) increased by exercise experiment over a long period[22, 23]. On the other hand, it was also shown that osteoblasts differentiated and had high activity, but osteoclasts didn't increase by the same experiment[24]. In this study, many TRAP reaction positive cells were observed in IM-EX-2D compared to CO-2D, but their number decreased in IM-EX-7D. And, many cells showing positive reaction to receptor activator of nuclear factor-κB ligand (RANKL) were recognized in IM-EX-2D. It was regarded that RANKL was secreted by osteoblasts, osteocytes, myeloid cells and/or various cells, and functioned on preosteoclasts and induced their differentiation[25, 26]. Then, it was supposed that RANKL was secreted by osteoblasts after two days immobilization, and therefore, this gave increase of the osteoclasts in IM-EX-2D and 4D.

It was reported that trance-forming growth factor-β (TGF-β) and bone morphorogenetic protein (BMP) related to the differentiation and activation of the osteoblasts, and was secreted by the osteoblasts during bone formation[27~30]. In fact, positive reactions of TGF-β were observed in bone matrix in this study. Thus, it was speculated that TGF-β and BMP were dispersed from bone matrix into bone marrow, the differentiation and activity of the osteoblasts were promoted, and this activated the bone formation in IM-EX-7D. It was supposed, as described above, that increase in the density and thickness of bone trabeculae at the primary cancellous bone in EX and IM-EX was related to the temporary bone resorption by shor-term immobilization.

Therefore, it was understood that temporary bone resorption by the immobilization just before the exercise increased in scatter of differentiation and activation factors the osteoblasts that were embedded in the bone matrix, and this promoted the active bone formation.

５．Conclusion

It was suggested that, accompanied with immobilization just before exercise, factors of the differentiation and activation of osteoblasts, like TGF-β, were dispersed into bone marrow by temporary bone resorption, and then, they could brought active bone formation.

Acknowledgment

This work was carried out thanks to the support of the graduate and undergraduate laboratory colleagues and their cooperation for guidance and encouragement.

Summary of this study was reported at Japanese Society of Physical Fitness and Sports Medicine Meeting 2013.

Ethical Investigation

This study was approved by Ethical Committee for the research of the Faculty of Human Life Design and by the Animal Care and Use Committee, Toyo University.

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Study on Structural Changes in Tibial Cancellous Bone with Mechanical Loading After Short-term Immobilization in Rats

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Abstract

This study aimed to investigate effects of exercise and immobilization just before exercise on bone remodeling processes. Rats were used as materials, structures of their tibiae were observed through experiment 1 and 2, and following data were obtained.

It was nessesory for four weeks that bone mass of the 4-week-exercise after 4-day-immobillization group recovered to the level that of the 4-week-exercise group. The bone mass of the 1-week-exercise after 2-day-immobilization group decreased at once, but reached higher level than 1-week-exercise group after one week of the experiment. Many RANKL and TRAP positive cells were observed after 2 days of the experiment in the 1-week-exercise after 2-day-immobillization group, compared to the control, and the bone matrixes indicated positive reactions to TGF-βimmunostaining.

Then, it was suggested that the bone resorption activated, differentiation and activation factors like TGF-β were released from the bone matrixes into bone marrow, and then, this could brought active bone formation, by the exercise following the immobilization.

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