# 発育期ラット脛骨の一次海綿骨における骨形成に及ぼす加重増加の影響

論　文

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# Effects of mechanical loading on bone formation in tibial primary cancellous bone of growing rats

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## Summary

A purpose of this study was to investigate histologically effects of jumping exercises performed with different intensities and durations on structures of a primary cancellous bone at a proximal metaphysis of tibia in growing rats.

Thirty-six wistar male rats aged 7 weeks were used as materials. They were divided into an exercise group and a control group (CO) randomly, and each group was subdivided into a 7-day group (7D), a 14-day group (14D) and a 21-day group (21D) for each experimental period. Furthermore, the exercise groups were subdivided into E30, E45 and E90 randomly. Rats in each group performed jumping exercises at 30, 45, or 90% of their maximum jumping heights measured, respectively, 100 times per day, 5 days per week, for each experimental period.

A density and a thickness of trabecular bones at the primary cancellous bone increased as the exercise intensity was enhanced. A bone addition to the surface of a calcified cartilage trabecula already started from the site next to the growth plate in E30 and E45. However, the bone addition of E90 was delayed at that site, compared to the others.

In conclusion, it was understood that a bone formation was promoted at the primary cancellous bone by exercise but could be suppressed at the area next to the growth plate by high intensity exercise.

Keywords: primary cancellous bone, exercise intensity, histological structure

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# 1. Introduction

When experiencing vascular invasion, a cartilage matrix in the hypertrophic zone of the growth plate is left as a scaffold of a bone formation, and then plays a role as a core of the trabecular bone after bone matrix deposition by osteoblasts. The trabeculae just after the new bone addition are called a primary cancellous bone. The primary cancellous bone becomes a secondary cancellous bone through a bone remodeling. A thickness of trabecular bone in primary and secondary cancellous bone is increased with growth[1]. The trabecular bone of the primary cancellous bone is covered with no or little bone matrix, so it has low strength structure.

Then, interface between growth plate and primary cancellous bone is a common site for epiphysiolysis in children. Epiphysiolysis is caused by overuse during sports activity. It accounts for 30% of pubertal fracture[2] and leads to growth disorder at the site because of inappropriate treatment, so it is considered as a critical disorder at clinical site. Thin trabecular bone in the growing period or a loss of bone mass caused by aging and inactivity is able to be risk factors of fracture. Then, appropriate mechanical loading is considered as a way of enhancement of the bone mass and a strength. In a cortical bone and the secondary trabecular bone, it has been proved that exercises such as running or jumping are effective for an increase in the bone mass by the experiments using animals[3, 4] and humans[5, 6]. Additionally, bone formation is promoted in an intensity-dependent manner at these sites[7, 8]. On the other hand, in primary cancellous bone, it is known that the bone formation was increased by exercise[9], but it is unclear that whether the bone formation was enhanced in an intensity-dependent manner. Therefore, the purpose of this study is to investigate histologically the effects of jumping exercises through various conditions of intensities and durations on structures of primary cancellous bone at a proximal metaphysis of tibia in growing rats.

# 2. Materials and Methods

## 2.1 Materials

Male wistar strain rats aged 7 weeks were used as materials in this study. They were divided into an exercise group and a control group randomly, and each group was subdivided into 7-day group (7D), 14-day group (14D) and 21-day group (21D) according to each experimental period.

## 2.2 Methods

### 2.2.1 Experiments

The exercise groups were accustomed to jumping exercise prior to the experimental period. By several electron stimulations on the floor of jumping apparatus, rats acquired jumping reflex within an acclimation period before the experiments. Therefore, rats were able to jump without

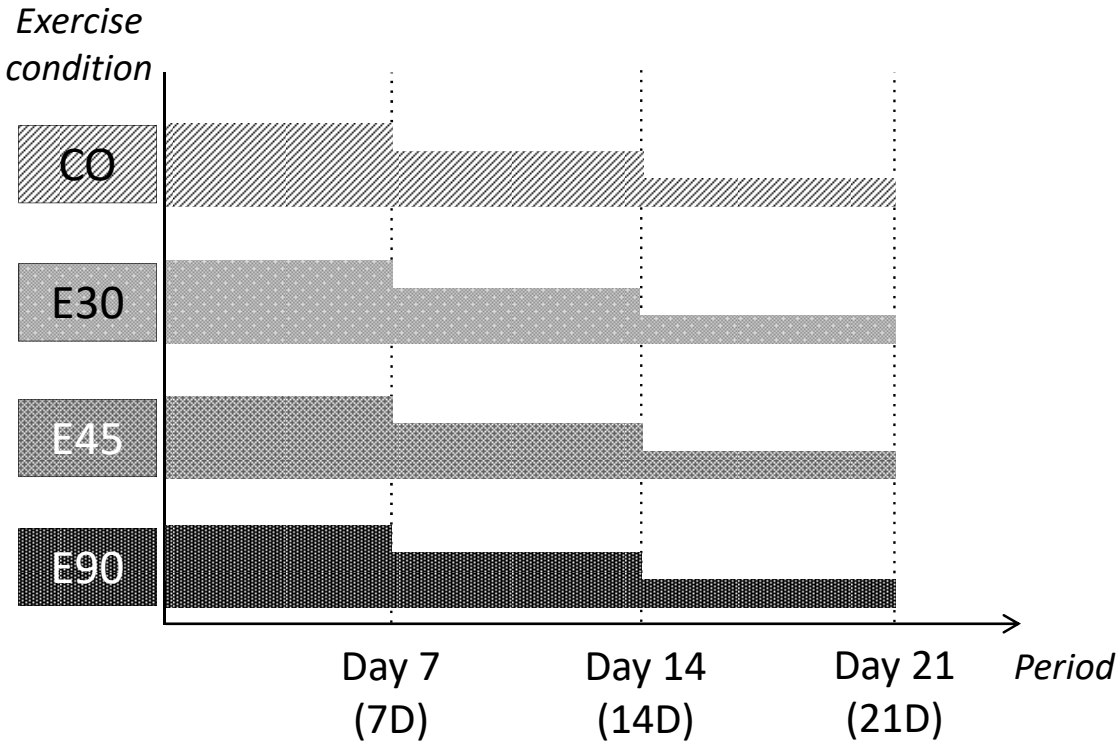


Fig.1: Experimental protocol
At the end of the each experimental period, rats were euthanized and their tibiae were sampled.

electron stimulation in the experiments. Maximum jumping height was measured in each rat of the exercise groups. The exercise groups were subdivided into E30, E45 and E90 randomly, and for the experimental periods, each group performed jumping exercise at 30, 45, or 90% of the maximum jumping height on the basis of the measurements, respectively. The jumping exercise was performed 100 times per day, 5 days per week for each period by every group. The experimental protocol was performed as shown in Fig.1. The maximum jumping height was also measured after the first and second week of the experiments and the jumping height of each rat was corrected.

### 2.2.2 Sampling

After the experimental periods, rats were euthanized by carbone dioxide inhalation, soft tissues were removed from hind limbs and tibiae were excised after confirming their death. Proximal portions of tibiae were divided in sagittal direction and were fixed in 4% paraformaldehyde. All samples were decalcified in 8% ethylene diamine tetraacetic acid (EDTA) at 4℃ .

### 2.2.3 Analyses

Specimens fixed in 4% paraformaldehyde were dehydrated, cleared, and embedded in paraffin wax. Sections of 4 $\mu$m thickness were cut by microtome, stained with Hematoxylin-Eosin staining

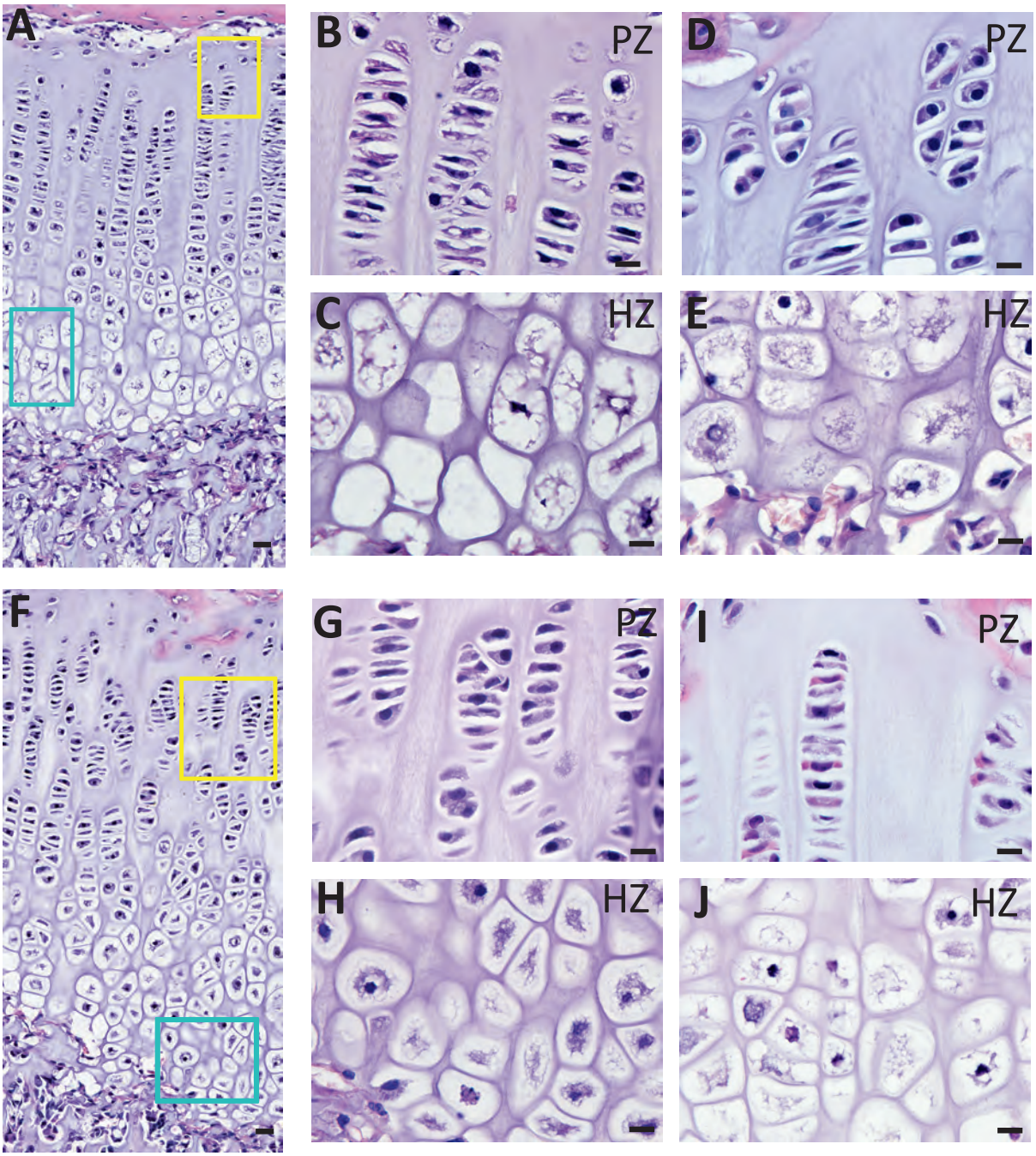


Fig.2: Images of growth plate chondrocytes.

Proliferative (PZ) and hypertrophic (HZ) chondrocytes in the exercise groups were smaller compared with those of control group and cartilage matrixes between columns were thicker in the exercise group than those in control group.

Yellow squares: proliferative zone

Blue squares: hypertrophic zone

A-C: CO-7D, D, E: CO-21D, F-H: E90-7D, I, J: E90-21D

Bar: A, F: 20μm, B-E, G-J: 10μm

methods and then observed through a light microscopy.

## 3. Results

### 3.1 Growth plate

In the growth plate of all groups, small chondrocytes of proliferative and hypertrophic zone were increased with growth. (Fig.2.A-E) However, chondrocytes were smaller in exercise groups than in control group. Thickness of the cartilage matrix between the chondrocytes was increased, accompanied with miniaturization of chondrocytes. (Fig.2.F-J)

### 3.2 Erosion zone on growth plate and primary cancellous bone

#### 3.2.1 Thickness of calcified cartilage trabeculae just under the growth plate

In CO-7D, purple-stained calcified cartilage existed densely in the erosion zone. (Fig.3.A) Most of the calcified cartilage trabeculae were extended to the bone marrow, and there were some cartilage trabeculae across the trabeculae running along the longitudinal axis of tibia. These images were not observed at the area far from the growth plate. (Fig4.A) In CO-14D, a density of the calcified cartilage trabeculae was decreased compared to those of CO-7D. (Fig.3.B) Those trabeculae linked to other trabeculae were decreased near the erosion zone. The trabeculae in CO-14D were thicker than in CO-7D. In CO-21D, the trabeculae linked across the other trabeculae were only a few, especially around the erosion zone. (Fig.3.C) Calcified cartilage in CO-21D was thicker than in CO-14D, but the density of the trabeculae was decreased. (Fig.4.B) Thus, the thickness of the trabeculae was increased, and the number of them decreased with growth in CO.

In E30-7D, the density of the trabeculae increased obviously, compared to CO-7D. (Fig.3.D) The thickness of the trabeculae of E30-7D was almost the same as CO-7D. (Fig.4.C) Many calcified cartilage trabeculae running across the longitudinal trabeculae were observed. Differences in the trabeculae observed were not different between E30-14D and E30-7D. (Fig.3.E) Compared to E30-14D, the number of longitudinal and transverse calcified cartilage trabeculae was increased in E30-21D. (Fig4.F)

In E45-7D, the density of the trabeculae was lower and horizontal links to the others were fewer compared to E30-7D. (Fig.3.G) However, thickness of the trabeculae were increased in E45-7D. (Fig.3.H) In E45-14D, thickness and density of the trabeculae were slightly increased in E45-14D compared to E30-14D. In E45-21D, the density of the trabeculae was decreased clearly. (Fig.3.I) Thickness was increased slightly, but this change was not obvious compared to day 14 groups.

In E90-7D, the density of the trabeculae was decreased compared with E45-7D. (Fig.3.J and Fig.4.E) The number of them was also reduced in E90-14D. (Fig.3.K) There was no clear difference between E90-21D and E45-21D. (Fig.3.L) However the trabeculae was thinner near the erosion zone than in the other site. (Fig.4.F)

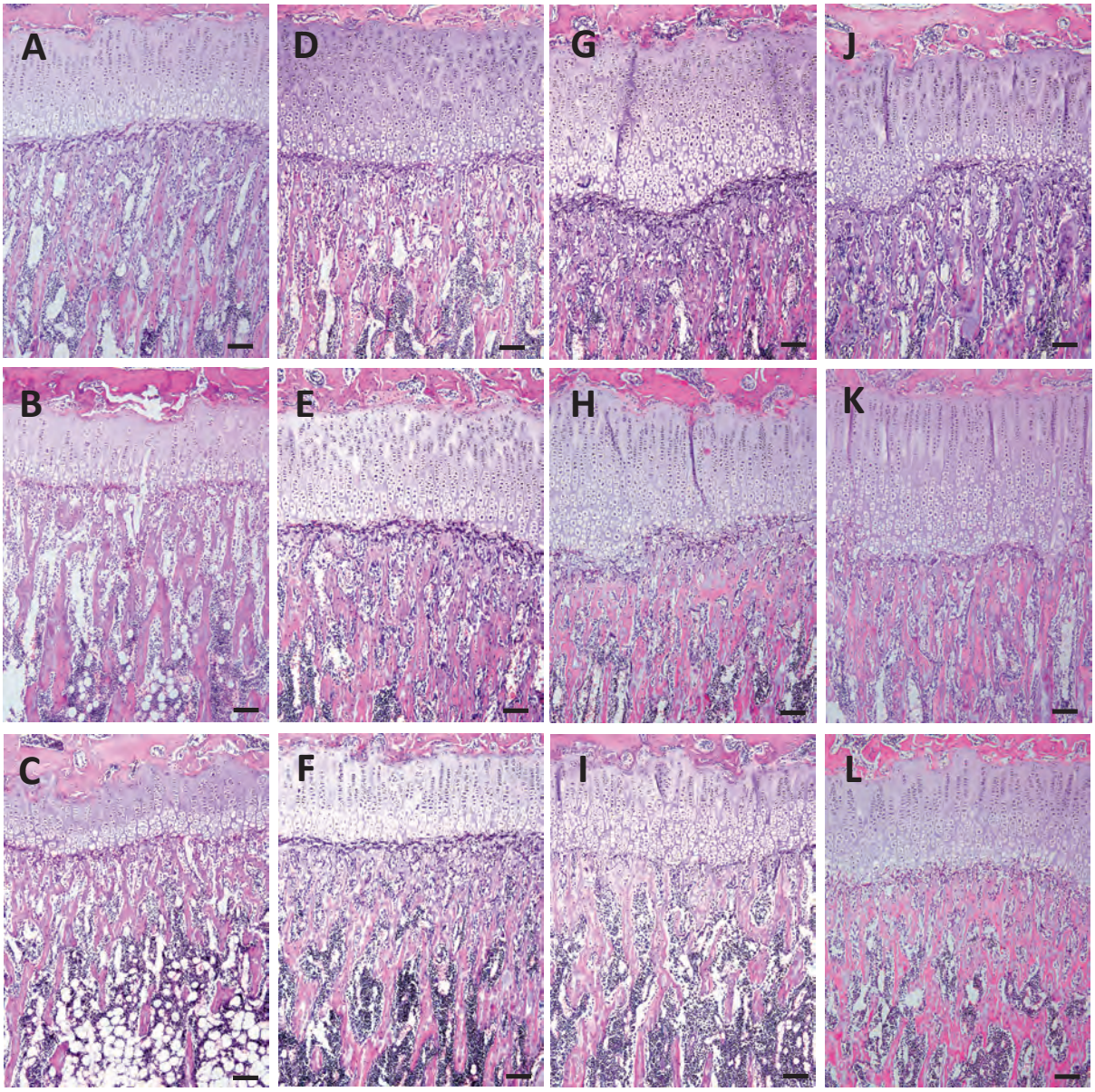


Fig.3: Low magnified images of each group.
Thickness of bone trabeculae was increased with growth in both control and exercise group.
A: CO-7D, B: CO-14D, C: CO-21D, D: E30-7D, E: E30-14D, F: E30-21D, G: E45-7D, H: E45-14D, I: E45-21D, J: E90-7D, K: E90-14D, L: E90-21D
bar: 100$\mu$m

### 3.2.2 Onset of bone formation and the cells around the sites

In a cancellous bone of CO-7D, the bone formation was started at a distance from the growth plate, and many calcified cartilages without addition of a new bone were observed near the erosion zone. These calcified cartilages were covered with many mono- or multi-nuclear cells stained dark pink. At a distance from the growth plate, purple-stained spherical cells whose cytoplasm was partly light appeared on the surface of the calcified cartilage trabeculae. Far from the growth plate, bone matrix stained pink was formed at the marrow space between the cells and the calcified cartilage matrix. (Fig.5.A) In CO-14D, the starting portion of the bone formation slightly

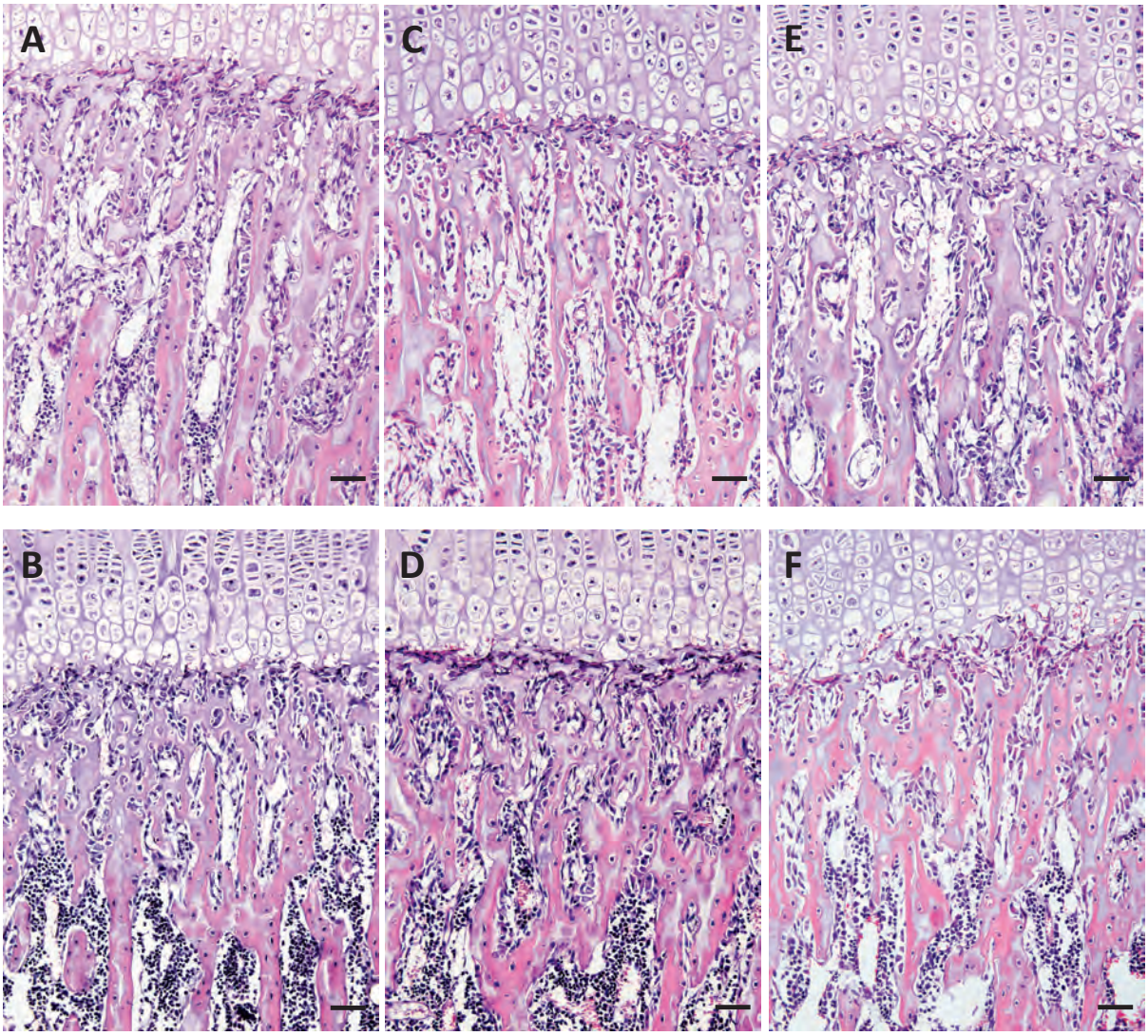


Fig.4: Images of primary cancellous bone.
There was no obvious difference in thickness of bone trabeculae between 7-day groups. However, at day 14 and 21, primary cancellous bone in both exercise groups was thicker than that in CO.
A: CO-7D, B: CO-21D, C: E30-7D, D: E30-21D, E: E90-7D, F: E90-21D
Bar: 50$\mu$m

approached to the erosion zone. (Fig.5.B) In CO-21D, that portion was closer to the growth plate than in CO-14D, and the bone addition was observed at the surface of the calcified cartilage around the erosion zone. (Fig.5.C and Fig.6.A) However, in both CO-14D and CO-21D, pink-colored mono- or multi-nuclear cells were observed in the interface between the growth plate and the primary cancellous bone. (Fig.6.B) Additionally, purple-stained mononuclear cells containing a larger light area in their cytoplasm were seen around these cells compared to CO, as bone matrix between the cells and the calcified cartilage became thicker. To sum up, in CO, the starting portion of the bone formation approached the growth plate, and bone matrix was added at this point and the light area in cytoplasm of purple-stained cells was increased.

In E30-7D, the calcified cartilage trabeculae that were not covered with the new bone were

observed the same as in CO-7D, however, the bone formation was started closer to the growth plate than in CO-7D, and the purple-stained spherical cells were seen on the surface. The bone matrix observed at this site was thicker than in CO-7D. (Fig.5.D) In E30-14D, the new bone matrix was recognized near the growth plate and was thicker than in E30-7D. Pink-colored mononuclear cells were reduced and the purple-stained cells appeared in the erosion zone. (Fig.5.E) In E30-21D, there is no obvious difference in the starting portion of bone formation, yet bone matrix added at the site was clearly thickened. There were many pink-colored mononuclear cells

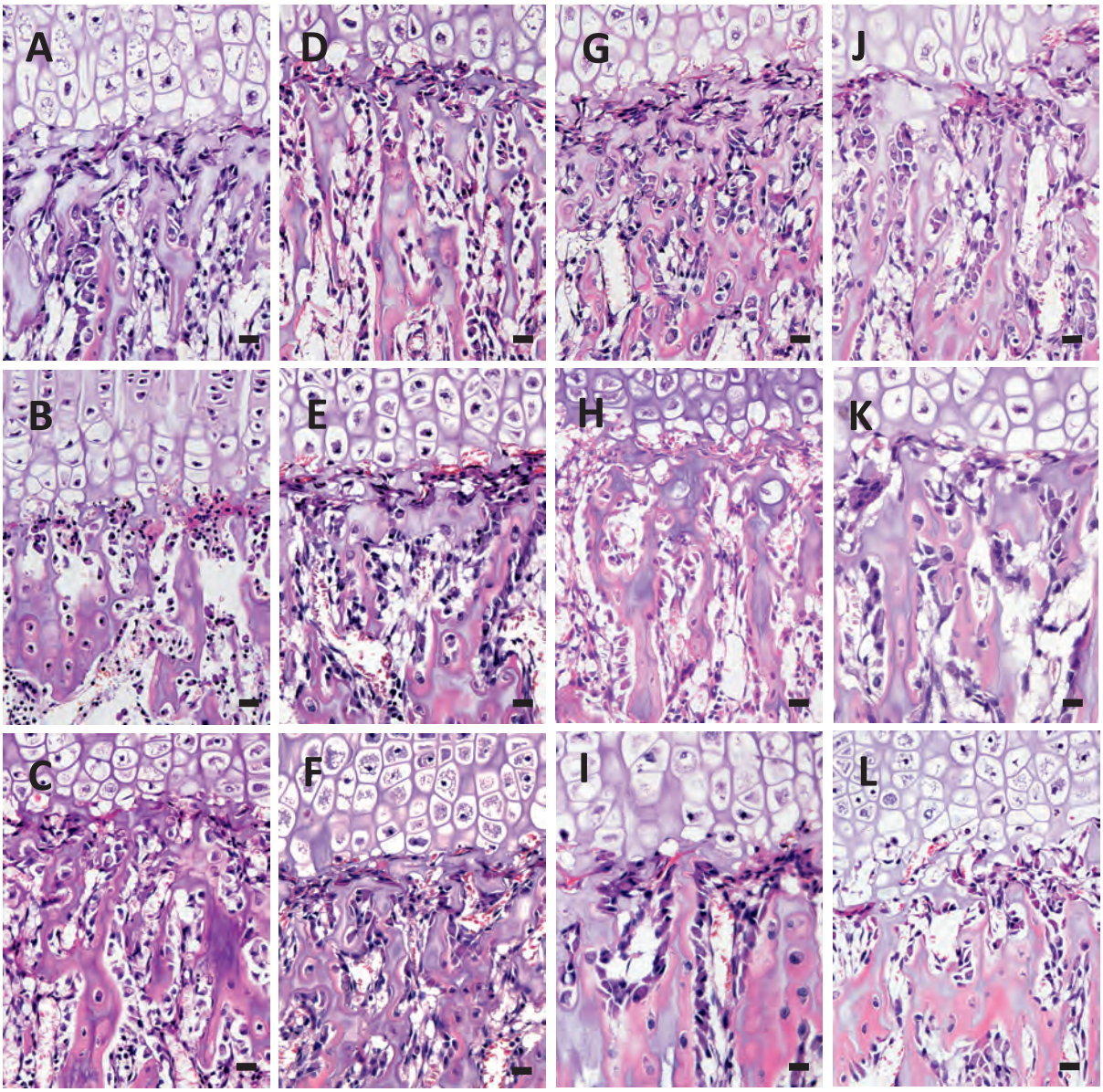


Fig.5: Magnified images of starting portion of the bone formation.
Bone formation was started closer to the growth plate and bone addition was increased as the experimental periods were proceeded.
A: CO-7D, B: CO-14D, C: CO-21D, D: E30-7D, E: E30-14D, F: E30-21D, G: E45-7D, H: E45-14D, I: E45-21D, J: E90-7D, K: E90-14D, L: E90-21D
bar: 20μm

in the erosion zone compared to E30-14D, and the purple-stained cells were observed around these cells. (Fig.5.F and Fig.6.C,D)

In E45-7D, there is also the calcified cartilage trabeculae covered with no bone matrix, and the number of these trabeculae was limited. The starting portion of bone formation slightly approached the growth plate and an increase of thickness in bone matrix at the site was obvious. Pink-stained cells in the erosion zone were bigger than in E30-7D and were already appeared with the purple-stained cells that had the light area in their cytoplasm. (Fig.5.G) In E45-14D, an obvious difference in the starting portion of bone formation was not observed. (Fig.5.H) In E45-21D, the bone addition to the calcified cartilage trabeculae in the erosion zone was increased and the bone matrix was thicker than in E30-21D. (Fig.5.I) With these changes, the purple-stained cells and the light area in their cytoplasm were bigger near the growth plate compared to E45-14D.

In E90-7D, the starting portion of bone formation was slightly far from the growth plate, and the bone addition to the surface of calcified cartilage had not occurred just under the growth plate. However, bone matrix at the starting portion of bone formation was thicker than in E45-7D. The pink-colored cells were localized in the erosion zone and the purple-stained cells were observed only far from the growth plate, so they were not co-localized in E90-7D. The pink-

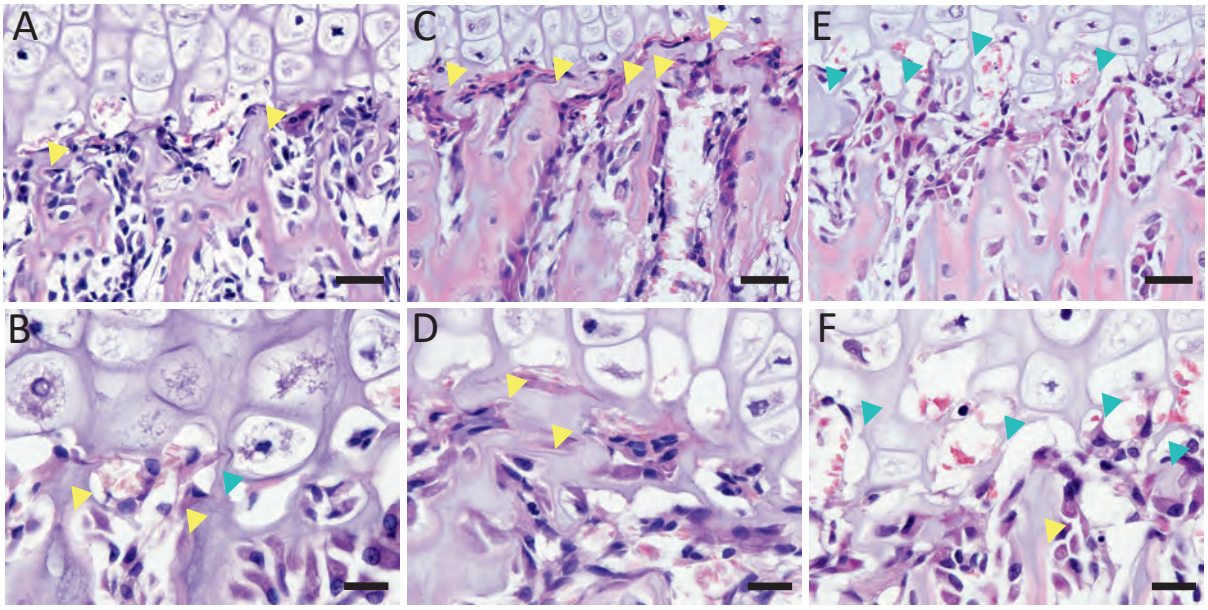


Fig.6: Delay of the bone formation in E90.

At day 21, in the primary spongiosa just under the growth plate in CO, little new bones were added around the calcified cartilage trabeculae. Bone formation was started near the cartilage lacunae in E45. However, bone formation was delayed in E90 and thin calcified cartilage trabeculae was not covered with the new bones.

Yellow arrow heads: starting portions of bone formation

Blue arrow heads: calcified cartilage trabeculare without addition of new bones

A, B: CO-21D, C, D: E45-21D, E, F : E90-21D

Bar: A, C, E: 20μm, B, D, F: 10μm

colored cells were often seen around the calcified cartilage without bone addition. (Fig.5.J) In E90-14D, the starting portion of bone formation was also far from the growth plate compared to E45, and the cartilage lacunae in which transverse cartilage trabeculae were removed were running longitudinally. (Fig.5.K) In E90-21D, the starting portion of bone formation was receded from the growth plate compared to E45-21D, and at this site, bone matrix became thicker than in E45. (Fig.5.L) In the erosion zone of E90-7D, the pink-colored cells were reduced, however, the new bone addition was not started near the growth plate. Many calcified cartilage trabeculae were not covered by any bone matrix and cell near the growth plate. Pink-stained cells appeared in the not inferior border of the growth plate but slightly far from this area. Cartilage lacunae lying longitudinally were surrounded with calcified cartilage trabeculae in which bone addition had not occurred. (Fig.6.E,F) In these lacunae, there were many erythrocytes and the cells that had extended their flattened process to the growth plate. At sites slightly far from the growth plate, these cells were not observed, but the pink-colored cells and the purple-stained cells had emerged.

## 4. Discussion

The aim of this study was to investigate effects of different height jumping exercises on structural changes of primary cancellous bone.

It has been known that differences of ways of mechanical loading lead to different changes of bone structure. In the experiment to investigate the effects of running and jumping on trabecular structure, it has been reported that increase of trabecular thickness distributes to increased bone volume in jumping exercise, on the other hand, in the running exercise, the number of trabecular bone was increased with bone gain[10]. Notomi et al.[11] have stated that in an anaerobic exercise such as jumping, bone formation was started more quickly than in an aerobic exercise. Also, not continuous but intermittent loading leads to quick response and formation of woven bone[12]. From the above, bone metabolic and structural changes are affected by loading manner. In these experiments, the thickness of primary cancellous bone in both control group and exercise groups is the same as the report by Takizawa et al.[1]. Especially, the primary cancellous bone was thicker in exercise groups than in control groups, and the differences between the 2 groups were remarkable as experimental periods proceeded. Additionally, increase in thickness was obvious with elevation of exercise intensity. The results showed increase of bone volume in an exercise intensity-dependent manner in primary cancellous bone, and this was concurred with previous study on secondary cancellous bone and cortical bone[7, 8]. Moreover, Obuchi et al.[13] have reported that the bone formation in tibial primary cancellous bone of rats was promoted as the number of jumps increased. From the above, it is thought that the bone formation in the primary cancellous bone is in proportion to intensity or work output of exercise.

However, when the mechanical loading exceeds a threshold, the anabolic effect is reduced. Many

reports that investigated effects of different load intensity, indicate that the level of effect by the loading which reaches the threshold of intensity is the same as by the loading which exceeds the threshold[14–16]. Moreover, excessive mechanical loading could cause breakdown of tissue. Actually, it has reported that mechanical loading over the physiological condition inhibits bone growth[17]. Besides, when the bone received a repetitive low-intense loading, stress fracture caused by accumulation of the small stress occurs[18]. To sum up, there is a threshold of effects on promotion of bone formation and inhibition of bone resorption by mechanical loading. In our observation, the bone formation on primary cancellous bone was promoted in all exercise groups. On the other hand, the bone addition to calcified cartilage trabeculae was started just under the growth plate in E30 and E45 but not in E90. Accordingly, osteoblast with mature goldi area clearly observed was increased. Elevation of the starting portion of bone formation and maturity of osteoblasts associated with the elevation were observed in growth[19] and mechanical loading[20]. In E90, the high intensity exercise group, there was delay of bone addition to calcified cartilage trabeculae just under the growth plate, therefore jumping exercise at 90% of the maximum jumping height has a negative effect at this site. Takahashi et al.[21] have investigated the bone formation of secondary cancellous bone using the same exercise conditions as our experiment. They have reported that the bone formation was increased as intensity went higher and was not inhibited. Their results at the secondary cancellous bone correspond to our observation at the primary cancellous bone from this study. It was also observed that the thickness of trabeculae bone increased in an intensity dependent manner. However, these changes were not observed just under the growth plate, and it is shown that there is a very different response to mechanical loading between the sites of cancellous bone.

In the bone remodeling of cancellous and cortical bone that responded to increase and decrease of mechanical stress, it is known that osteocyte, mechanosensor, embedded in bone matrix plays a key role as a regulator of osteoblast and osteoclast[22]. Factors involving in bone remodeling, receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) which are synthesized not by osteoblasts but rather by osteocytes are important for regulation of bone remodeling[23]. Various pathways that osteocyte controls bone remodeling have been reported[24–28]. It is known that by increase of mechanical loading, bone remodeling is promoted by activation of canonical Wnt signaling pathway in osteocyte[24], inhibition of transcription of sclerostin via expression of transforming growth factor beta (TGF-$\beta$) in osteocyte accelerates bone formation[25], and secretion of insulin-like growth factor-1 (IGF-1) enhances bone formation[26]. On the other hand, it has reported that osteoclastogenesis in the bone surface is promoted by a micro fracture induced by repetitive loading and death of osteocyte caused by unloading[27, 28]. However, these responses to mechanical stimulation occurred in mature bone matrix that contains osteocytes. The sites observed in this study were primary cancellous bone composed of immature bone matrix and the calcified cartilage trabeculae without new bone matrix just under the growth plate. The

microenvironment is greatly different between the secondary cancellous bone and the primary cancellous bone. Therefore, it could be thought that the effect of exercise is different between primary and secondary cancellous bone.

Chondrocyte, a mechanosensor in cartilage matrix, is embedded in extracellular matrix just like osteocyte. It has been also confirmed both protein and mRNA expressions of RANKL and OPG in chondrocyte[29, 30]. In fact, it has been reported that there could be an interaction between chondrocyte and osteoblast or osteoclast[31-33]. The previous study which carried out co-culture has suggested that chondrocyte and osteoblast regulate matrix synthesis of each other by paracrine[31]. In long bone specifically deleted beta catenin in hypertrophic chondrocytes, trabecular bone formation just under the growth plate was significantly inhibited[31]. This phenotype was accompanied with elevation of RANKL expression, so it has been indicated that changes of Wnt signaling in growth plate hypertrophic chondrocyte enhance activities of osteoclasts or chondroclasts near the erosion zone. Moreover, sternal hypertrophic chondrocyte from mouse and chick embryo co-cultured with osteoclast precursor controls osteoclastogenesis through RANKL expression via bone morphogenetic protein 2 (BMP2)[33]. From the above, there is a possibility that chondrocytes in tibial growth plate regulate the early trabecular bone formation. However, only a few studies investigate the effects of changes in growth plate on trabecular bone formation. Therefore, interaction between the growth plate chondrocyte and osteoblast or osteoclast around the trabeculae is still unclear.

Histomorphometric investigation on effects of mechanical stress on growth plate and trabecular bone has been performed by Nyska et al.[34]. They have stated that growth plate proliferative chondrocytes increased by swimming exercise, so metabolic acceleration in the growth plate leads to increment of trabecular bone. Nevertheless, whether there is a relationship between events of growth plate and trabecular bone or not is unknown. Besides, Takahashi et al.[20] have described the increase of osteocalcin-positive mature osteoblasts near the growth plate by short-term jumping exercise after short-term immobilization. This result supports our observation in this study, But it is unclear whether these changes were caused by loaded chondrocyte in the growth plate. From the above, there are many unclear points in effect of mechanical loading to the growth plate on bone formation. Like the report that examined effect of mechanical stress only in the growth plate, Tsutsumi et al[35] have reported that calcification of hypertrophic zone was accelerated with elevation of running speed. Katsuta[36] has noted increase of size in chondrocyte of rats with elevation of speed in treadmill running. However, miniaturization of chondrocytes instead of increasing in size was observed in this study. It is unclear whether the difference between these reports is dependent on exercise pattern or not.

Moreover, Reich et al.[37] have showed that expressions of matrix metalloproteinase (MMP) -9, MMP-13, and osteopontin in the growth plate were enhanced by continuous loading, and indicated that increase of these factors could induce appearance of osteoblast, osteoclast and their precursor

from bone marrow. In E90 showing the delay of bone formation, there were many erythrocytes and endothelial-like cells between calcified cartilage trabeculae without bone matrix or any cell on the surface, yet it is an unclear association between suppression of bone formation and appearance these cells. However, it has been previously reported that increase of vascular invasion to the growth plate with enhancement of mechanical loading[37]. the osteoblast and its precursor delivered with angiogenesis are contained in a part of bone formation cells at trabecular bone[38]. Increase in such bone formation cells could promote bone formation. From the experiment that treadmill running was performed on rats treated with anti-vascular endothelial growth factor (VEGF) antibody, it has been proved that angiogenesis in bone marrow is essential to gain of bone formation on trabeculae by exercise[39]. These reports suggest that angiogenesis is a critical event not only in bone formation of developmental stage, but also in that of growing stage. However, inhibition of bone formation just under the growth plate with high intensity exercise is not able to be explained by exercise-induced activation of angiogenesis. In fact, there has been a study performing the in vivo mechanical loading on growth plate that reported no relation between VEGF expression and vascular invasion[17]. Furthermore, there are some evidences for changes in these factors in the growth plate on trabecular bone formation and association between the changes and exercise intensity.

The site just under the growth plate is the interface of cartilage and bone, and is known as a common site of epiphysiolysis in children[2, 40]. Moreover, it has been described that extensively strained tibia had showed fracture in osteochondro-junction[41], and indicated that it is a low strength site. In this study, just under the growth plate in high intensity exercise groups, bone formation didn't start and remained bare of thin calcified cartilage trabeculae. In the osteochondro-junction, bone formation in E90 was more delayed than in CO. High intensity exercise does not enhance the bone structure applicable to mechanical loading. To the contrary, it could reduce the strength at the site.

In this study, jumping exercises with various exercise intensity conditions were performed and increase of bone volume in primary cancellous bone in an exercise intensity-dependent manner was observed. However, just under the growth plate, delay of bone addition was recognized only in the high intensity exercise group. So, it is indicated that response to mechanical loading differed between the sites. It is considered that bone formation and resorption on primary cancellous bone are controlled by the growth plate chondrocyte, or influenced by vascular invasion. However, effects of these factors corresponding to changes of mechanical loading on trabecular bone formation were not investigated. Further study is needed.

## 5. Conclusion

In conclusion, generation of calcified cartilage trabecular as a scaffold of bone formation were

promoted yet bone apposition was suppressed next to the growth plate in the case of high exercise intensity.

## Acknowledgement

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## Committee of Animal Experiment and Ethics

This study was approved by the 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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# 発育期ラット脛骨の一次海綿骨における骨形成に及ぼす加重増加の影響

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**要旨**

【目的】本研究は発育期ラットを用い、異なる高さ（運動強度）の跳躍運動が脛骨近位骨幹端における一次海綿骨の構造に及ぼす影響について組織学的に検討した。

【材料および方法】材料として７週齢のラット36匹を用い、それらを実験期間別に７日群、14日群、および21日群（7D、14D、21D）に分けた。これらの群をそれぞれ対照群（CO）と運動群に分け、運動群はさらに実験開始時に計測した最大跳躍高の30％跳躍群（E30）、45％跳躍群（E45）、90％跳躍群（E90）に無作為に分類し、それぞれの高さの跳躍を１日100回、週５日行わせた。

【結果】一次海綿骨における骨梁の密度および太さは運動強度の上昇に伴って増加した。骨端板直下における石灰化軟骨梁周囲への骨形成開始は、E30およびE45ではCOより骨端板に近い位置となっていたが、E90では逆にそこから離れた位置から開始された。

【結論】海綿骨形成の基礎となる骨形成は運動によって促進されるが、高強度運動の場合、骨端板直下での骨添加の遅れが見られることが理解された。

**キーワード**：一次海綿骨、運動強度、組織構造