Effect of transcutaneous electrical stimulation of the different intervention frequency on bone structure of femur in hindlimb-suspended rats

MINAMIZONO, Wataru*
ZENG, Xueqian*
SUITO, Hirai*
YASHIMA, Nao*
YANG, Chuwei*
OHSAKO, Masafumi**

Summary

〔**Purposes**〕 This study was aimed to morphologically investigate the effects of transcutaneous electrical stimulation(TE) of different frequency on a bone structural changes caused by a hindlimb-suspension (HS) in rats.

[Materials and methods] Forty-eight male rats (wistar strain, 7-week-old) were used as materials and were divided into a HS group: HS, a HS and TES group: TE and a control group: CO. Moreover, TE was subdivided into a once (TE1), three times (TE3) and five times (TE5) per week groups, due to differences in intervention frequency. HS and TE were hindlimb-suspended in the cage for two weeks. In TE, the Transcutaneous electrical stimulation (TE) using the carrier wave was performed 10min/day, 5days/week for two weeks. Femur was excised from each group, the bone strength was measured and the

* : Master's Program in Graduate School of Human Life Design, Health Care and Sports, Second Year Student, Toyo University.
* : Doctoral Program in Graduate School of Human Life Design, Course of Human Life Studies, Second Year Student, Toyo University.
* : Doctoral Program in Graduate School of Human Life Design, Course of Human Life Studies, First Year Student, Toyo University.
* Master's Program in Graduate School of Human Life Design, Course of Health Care and Sports, First Year Student, Toyo University.
* Master's Program in Graduate School of Human Life Design, Course of Health Care and Sports, First Year Student, Toyo University.
** Professor of the Faculty of Human Life Design, Toyo University.

histological structure was observed. Calcein were administered to each group 7 days before sampling.

〔**Result**〕The thickness of the cortical bone in the center of the metaphysis was reduced in HS compared to CO. The thickness of TE1 was close to HS, while TE3 and TE5 were close to CO. Longitudinal images of the cortical bone in the center of the metaphysis of each group were observed by light microscopy. In addition, the longitudinal section of the cortical bone at the center of the metaphysis of the upper cortical bone was observed under an optical microscope. In addition, different substrates were added to the endosteal side of the front surface of the cortical bone in the upper row and to the periosteal side of the thick cortical surface in the lower row. However, the HS had a rough surface on the periosteal side of the anterior surface and the periosteal side of the posterior surface. This is indicative of resorption fossae. In HS, the cortical bone thickness was thus reduced by the weight reduction associated with hindlimb suspension. In contrast, in TE1, the cortical bone on both surfaces had a smooth bone surface, and the amount of bone added increased with increasing frequency of intervention.

〔**Discattion**〕In deep cortical bone, toluidine blue staining showed metachromatic substrate, which was absorbed from the periosteal side of the anterior and posterior cortical bone by hindlimb suspension. The metachromatic substrate was not absorbed by percutaneous electric current, and new bone was added to the surface of the metachromatic substrate. This effect was more pronounced at the intervention frequency of 3 days/week than 1 day/week, but tended to decrease at 5 days/week, which is a subject for further study.

[Conclusion] Rat hindlimb suspension causes osteopenia in the cortical bone of the central femoral stem, which is suppressed by transcutaneous electrical stimulation for at least three days per week, suggesting that bone fragility may be prevented.

keywords： unloading , transcutaneous electrical stimulation , cortical bone

1. Introduction

An effect of a transcutaneous electrical stimulation (TES) on bone loss have been studied for a long time[1)] initially had investigated the effects of the electrical stimulation by inciting the skin and implanting electrodes invasively. However, it was not practical due to the heavy burden of invasive surgery although the method was effective in maintaining bone mass. For this reason, the electromagnetic field therapy device (PEMF) is currently

used for promotion bone formation after fracture[2] It is desired to develop a new method for promoting bone formation and suppressing bone resorption with a short treatment. In our laboratory, it has been confirmed that the TES suppressed bone loss under mechanical unloading[3], but the effect of different intervention frequency has not yet been investigated. Then, this study was to investigate the appropriate frequency of the intervention for the maintenance of bone mass by TES during bone loss by mechanical unloading.

2. *Materials and Methods*

2.1 Materials

Forty-eight 7-week-old Wistar rats were used as materials and classified into groups.

2.2 Methods

The experimental groups were classified into two groups: hindlimb-suspension (HS) and transcutaneous electrical stimulation during tail suspension. The transcutaneous electrical stimulation group was further classified into three groups according to the frequency of intervention: once a week (TE1), three times a week (TE3), and five times a week (TE5). The control group was kept normally in cages during the same period. After euthanasia was confirmed by carbon dioxide overdose at the end of the experiment, femurs were removed and immediately fixed in 4% paraformaldehyde solution. After that, specimens were prepared by various methods and histological analysis was performed.

2.2.1 hindlimb-suspended

In order to add height to the cage for tail suspension, a wooden box was placed on the cage to provide height for tail suspension. During the hindlimb suspension, we made sure that the animals had access to food and water.

2.2.2. Transcutaneous electrical stimulation

When percutaneous electric current was applied, a mixture of anesthetics (medetomidine hydrochloride 0.15 mg/kg, midazolam 2 mg/kg, and ptolphanol tartrate 2.5 mg/kg) was injected into the abdomen, and the current was applied under anesthesia. The hair on the thighs was removed as a pretreatment before the current was applied. A pad was placed on the anterior surface of the femur for percutaneous electrical stimulation. The stimulation conditions were (DC, voltage 60 V, frequency 31 Hz, 200 μsec, carrier wave of

frequency 80 kHz was used), 10 minutes/day for 3 weeks.

2.2.3. Bone labeling

In all groups, calcein (80 mg/g) was administered intraperitoneally five days before the end of the experiment and alizarin (40 mg/kg) was administered one day before the end of the experiment.

2.2.4. Sampling

The femur was removed from the rat after the experiment was completed. The femur was removed from the center of the diaphysis by a hand motor.

2.2.5. Non-Demineralized Resin Specimen Preparation

Using specimens fixed in 4% paraformaldehyde, the specimens were non-demineralized, embedded in Rigolac resin, and polished with a three-stage grinding wheel. After polishing, the specimens were further polished with polishing film to produce polished specimens with a thickness of 100 microns. The specimens were stained with toluidine blue (TB) staining solution and observed under an optical microscope.

3. Result

Cross-section of the central diaphysis femur bone

A transversal section of the cortical bone in the middle part the femoral diaphysis of CO was an oval shape with the long axis in the medial and lateral directions, when observing in low magnified image. When the cortical bone was divided into anterior and posterior parts, the former showed remarkable curvature more compared to the later.

However, the thickness of the cortical bone was similar throughout the entire circumference. About half of the periosteal side of the cortical bone was darker, while the endosteal side was slightly lighter, and this difference was observed throughout the entire circumference of the cortical bone. This difference was observed throughout the cortical bone. In the same cross-section, calcein labels in the anterior part of the cortical bone was clear only on the endosteal side. A clear labeling was observed on the outer face of periosteal and endosteum sides of the femur at the posterior part of the cortical bone (Fig.1)

There was a calcified cartilage matrix composed of a matrix that caused metachromagy in the deep part, observing the area on the periosteal side that was darkly

observed in the low-magnified image of the cross section of the cortical bone. The calcified cartilage matrix was surrounded by a low-staining bone matrix. The osteocytic lacunas that existed in the that bone matrix were smaller, denser, and irregularly arranged compared to those of the periosteal side. (Fig. 2)

CO was the thickest and HS was the thinnest at the both anterior and posterior parts of the cortical bone, as comparing the thickness of the cortical bone in the transversal section in each group. TE1 was thinner than CO but thicker than HS.

The cortical bone in TE3 and TE5 were thicker than TE1. The thickness of TE3 and TE5 were similar, and some of TE3 was thicker than TE5. (Figs. 3 and 4) This tendency was also observed in the longitudinal section of the cortical bone. (Fig. 6, Fig. 7)

Both faces of periosteum and endosteum were smooth in the anterior part of the cortical bone and this is similar to other group, as observing in detail the structures of transversal and longitudinal sections of the cortical bone in each group. Short Volkmann's canals were found on the endosteal side of T1, while that of T3 was longer, and a narrow Volkmann's canals were present, in the posterior part of the cortical bone in TE5. Thick bone trabeculae were found in the endosteal side of in the posterior part of the cortical bone in CO and TE, but those were not present in the HS. (Figs. 3, 4, 6 and 7)

As observing the labels of calcein in each group, linear responses were observed at the anterior part of the cortical bone in CO and TE, but the labels of CO was the most distinct.

On the other hands, observing the labels of calcein in HS , they seemed unclear. In the posterior part of the cortical bone, the label was found except for the periosteal side of HS, but even in this part, the CO was clear, and the labels in other group was thin and unclear. In the posterior part of the cortical bone. (Figs. 3 and 4)

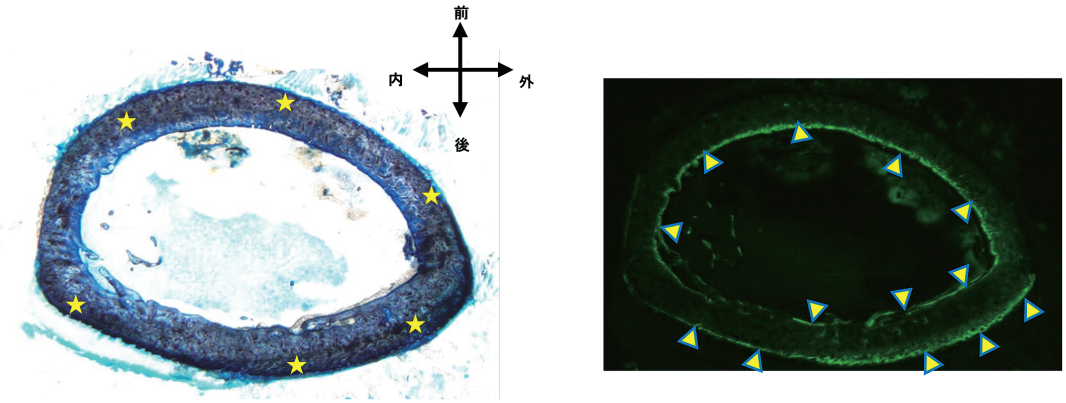

Fig. 1 Weakly expanded image of the transverse section of the central femur metaphysis in CO (non-demineralized resin specimen).

Left: toluidine blue staining, right: fluorescence microscopy image of calcein label

★: area darkened by TB staining, arrowhead: calcein label

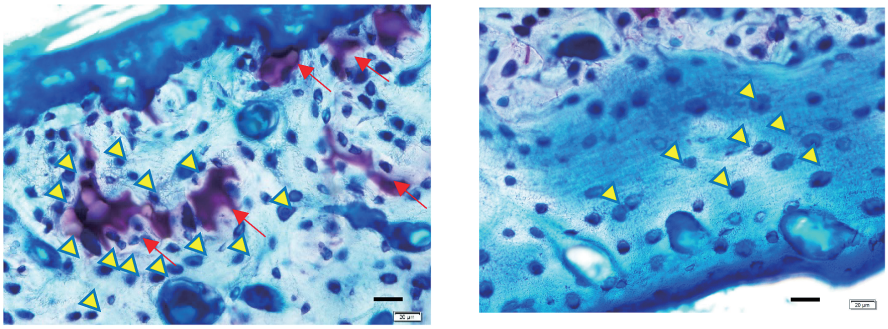

Fig. 2. Magnified image of the central transverse section of the femoral diaphysis in CO (non-demineralized resin specimen, bar=20 μm).

Left: Enlargement of dark areas on the periosteal side of the cortical bone,

Right: Enlargement of light areas on the endosteal side of the cortical bone

Sagittal seal: calcified cartilage matrix,　　sagittal head: bone cavity

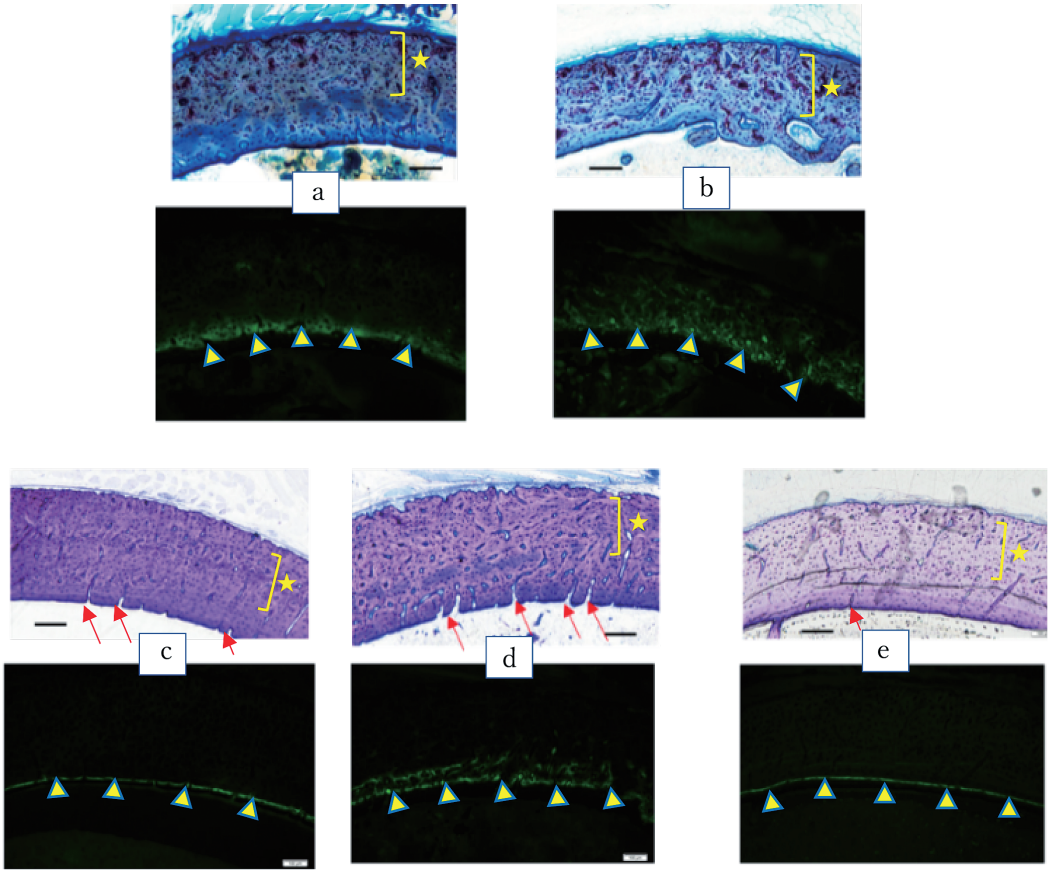

Fig. 3: Magnified image of the anterior section of cortical bone (non-demineralized resin-embedded polished specimen, bar=100μm)

a : CO, b: HS, c: TE1, d: TE3, e: TE5

Upper: TB-stained specimen, Lower: Fluorescent microscopy image of calcein label

★: metachromatic area caused by TB staining, arrow: Folkmann's canals

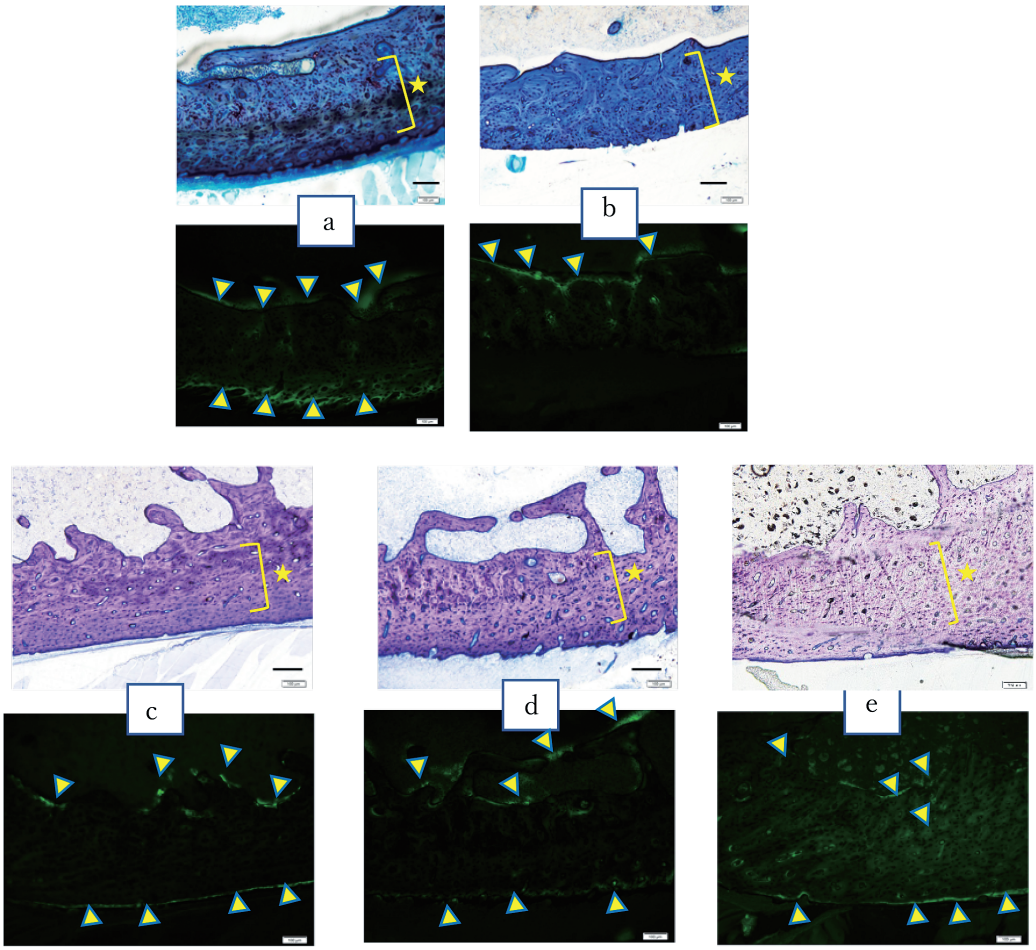

Fig. 4: Enlarged image of the transverse section of the posterior part of cortical bone (non-demineralized resin-embedded polished specimen, bar=100μm)

a : CO, b: HS, c: TE1, d: TE3, e: TE5

Upper: TB-stained specimen, Lower: Fluorescence microscopy image of calcein label

★: metachromatic area caused by TB staining, arrow: Folkmann's canals

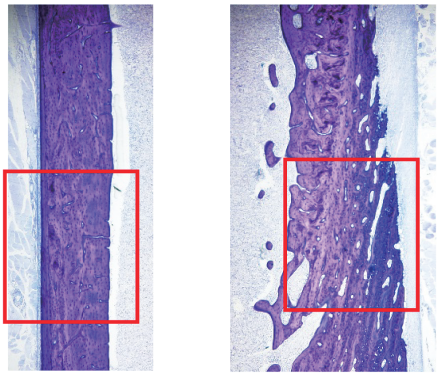

Fig. 5: Weakly expanded image of the longitudinal section of the median femur metaphysis (non-demineralized resin specimen, TB staining)
Left: anterior part of cortical bone,　right: posterior part of cortical bone

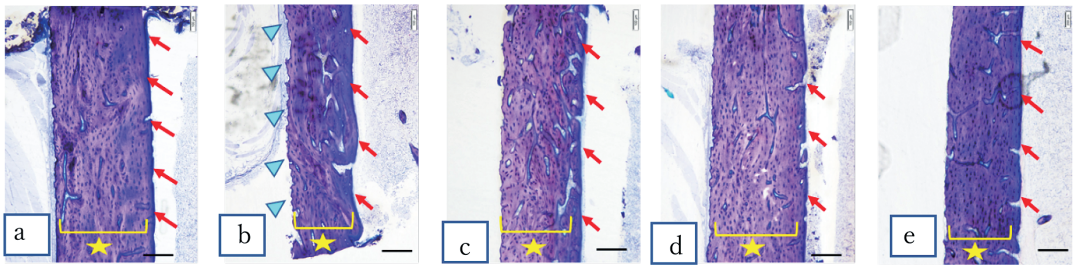

Fig. 6: Magnified image of longitudinal section of anterior part of cortical bone
(Undemineralized resin-embedded polished specimen, TB staining, bar=200μm)
a : CO,　b: HS,　c: TE1, d: TE3,　e: TE5
★: Dark areas in cortical bone , Arrow: Newly added substrate
Arrowhead: Absorption fossa

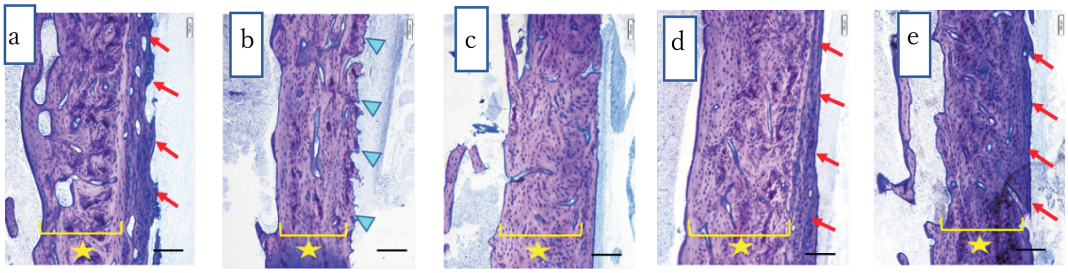

Fig. 7: Enlarged image of longitudinal section of posterior part of cortical bone
(Undemineralized resin-embedded polished specimen, TB staining, bar=200μm)
a : CO, b: HS, c: TE1, d: TE3, e: TE5
★: Dark areas in cortical bone , Arrow: Newly added substrate
Arrowhead: Absorption fossa

4. Discussion

The calcein label indicates the site of bone addition from the period when the calcein was administered[4)-6)]. In the horizontal section of the femur, bone is known to be added to the periosteal and endosteal surfaces[7)]. In the central part of the 9-week-old rat femoral diaphysis used in this experiment, bone was added to the entire endosteal surface side of the bone, and in the posterior direction of the cortical bone, bone was added to the periosteal surface side. In consideration of these characteristics of osteogenesis, this part of the bone seems to have narrowed the marrow cavity as it developed, and only the posterior part of the cortical bone has increased in thickness.

The third trochanter, the site of various muscle attachments, projects strongly outward from the top of the femur, but it expands downward as it develops. The increased thickness of the cortical bone in the outer center of the femoral diaphysis is presumably related to the vertical elongation of its third tubercle.

Moreover, focusing on the structure of the cortical bone, about half of the periosteal side of the cortical bone was dark, and the endosteal side was slightly light. When the dark areas were focused on, calcified cartilaginous matrix, which causes metachromasia, was found in the deep areas. In addition, a low-staining substrate enveloped the cortical bone above the endosteal surface, where a dense population of poorly differentiated osteocytes was present[7)]. The calcified cartilage matrix is originally formed at the

epiphyseal plate, and therefore the darker matrix in the deep cortical bone may have passed a longer time after formation than the clearer bone matrix on the endosteal side. In addition, as inorganic crystals grow over time, the dark bone matrix is harder than the clear bone matrix on the endosteal side, which is thought to play a role as the core of the cortical bone.

It is reported that hindlimb suspension inhibits osteogenesis and reduces the thickness of the cortical and endosteal surfaces of the central femoral metaphysis[8)-12)]. In this study, we compared the thickness of cortical bone in transverse and transverse longitudinal sections. CO was the thickest and HS was the thinnest in both anterior and posterior cortical bones. The results of the this study are consistent with previous studies, suggesting that the reduction of mechanical stress on bone due to load reduction accelerated bone resorption and reduced the thickness of cortical bone.

As in the case of the previous day, bone was added to the medial part of the bone anterior to the cortical bone because of the presence of calcein label in TE1, where a short Volkmann's canal was found, but in TE3 it was longer than in TE1, and in TE5 the Volkmann's canal was narrower. This suggests that in the anterior portion of the cortical bone, more bone was added to the medial side of the bone in TE3 than in TE1, and more bone was added in TE5, resulting in a narrower Volkmann's canal. Therefore, we speculate that active osteogenesis occurred in the medial side of the anterior cortical bone in TE3 and even TE5 more than TE1 during the experimental period.

Observing the labels of calcein in each group, a linear reaction was observed in the anterior part of the cortical bone of CO and TE on the cortical endosteal side, but the label of CO was the most clearly visible. On the other hand, the HS label appeared to be thicker, but there was no continuity to it and it was generally not clear.

In the posterior part of the cortical bone, labels were found except for the periosteal side of the HS, but even in this area, the CO label was clear, and the other labels were thin and indistinct.

These results suggest that transdermal electrical stimulation may have a suppressive effect on bone loss induced by weight reduction by transdermal electrical stimulation at a frequency of three or more times per week.

The labels of calcein on the transverse section of the cortical bone at the middle part of the femoral diaphysis in CO showed that the label was clear only at the endosteal side of the anterior cortical bone. On the posterior periosteal side of the femur, strong labeling was observed on the outer periosteal side of the femur, while weak responses were observed on the same area on the endosteal surface. Thus, the label was observed almost entirely on the endosteal side in the middlel part of the diaphysis, and on the periosteal side only in the posterior part of the cortical bone.

5. Conclusions

It is understood that bone loss due to load reduction by tail suspension can be suppressed by electrical stimulation for more than three days a week.

Animal Experiment Committee

This study was conducted with the approval of the Animal Experiment Committee of Toyo University.

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後肢加重低減ラット大腿骨の構造に及ぼす異なる頻度の経皮通電刺激の影響に関する研究

ライフデザイン学研究科健康スポーツ学専攻修士課程2年
南園　　航
ライフデザイン学研究科ヒューマンライフ学専攻博士後期課程2年
曾　　雪倩
ライフデザイン学研究科健康ヒューマンライフ学専攻博士後期課程1年
水藤　飛来
ライフデザイン学研究科健康スポーツ学専攻修士課程1年
楊　　薔薇
ライフデザイン学研究科健康スポーツ学専攻修士課程1年
八嶋　奈央
ライフデザイン学部健康スポーツ学科教授
大迫　正文

要旨

【背景】 本研究はラット後肢の加重低減によって骨量減少を図り、異なる介入頻度の経皮通電刺激が大腿骨における骨強度および骨構造の変化を形態学的に比較、検討することを目的とした。

【方法】 7週齢のwistar系雄性ラット48匹を用い，それらを後肢懸垂群（HS)、後肢懸垂・経皮通電刺激群（TE）および対照群（CO）に分類した。さらに，TEは介入頻度の違いから，週に1回（TE1)、3回（TE3）および5回（TE ５）の群に分類した。HSおよびTEはケージ内で2週間後肢懸垂し、TEには 大腿前面から経皮的に通電刺激（直流、電圧60V、周波数31Hz、200μsec、周波数80kHzの搬送波を使用）を10分／日，3週間行った。実験期間終了後，各群から大腿骨を摘出し、骨強度の測定および組織学的分析を行った。なお、サンプリング7日前にカルセインおよび、3日前にアリザリンレッドを各群に投与した

【結果】 各群の骨幹中央部の皮質骨横断像を光学顕微鏡にて観察すると、前方、後方共に皮質骨の厚さはCOとHSで比較するとHSで減少していた。

各群の骨幹中央部の皮質骨縦断像を光学顕微鏡にて観察すると、いずれの面においても皮質骨内には、トルイジンブルー染色にてメタクロマジーを強く起こす、基質が認められた。また、上段の皮質骨前面の骨内膜側、下段の皮質厚面の骨膜側では異なる基質が添加されていた。しかし、HSでは、前面の骨膜側および後面の骨膜側の表面が粗造な状態にあった。これは吸収窩を示すものであり、HSではこのように、後肢懸垂に伴う加重低減によって皮質骨の厚さが減少した。それに対して、TE 1ではいずれの面の皮質骨も、骨表面が滑沢な状態にあり、また、介入頻度の増加に伴って骨添加量は増加した。

【考察】 皮質骨深部にはトルイジンブルー染色でメタクロマジーを起こす基質が認められるが、それは後肢懸垂によって皮質骨前面および後面の骨膜側から吸収された。経皮通電によって、そのメタクロマジーを起こす基質は吸収されず、さらにその表面にも新たな骨添加が認められた。この効果は1日/週より3日/週の介入頻度で顕著であったが、逆に、5日/週では低減する傾向がみられ、このことについては今後の検討課題とする。

【結論】 ラット後肢懸垂によって大腿骨骨幹中央部の皮質骨には骨量減少が生じるが、それは1週間に3日以上の経皮通電刺激によって抑制され、骨の脆弱化が防止される可能性が示唆された

キーワード：加重低減、経皮通電刺激、皮質骨