論　文
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Effects of transcutaneous electrical stimulation of different voltages on structure of femur in hind-limb suspended rats

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Summary

This study aimed to investigate an effects of transcutaneous electrical stimulation of different voltages on the bone loss of rat's femur by decrease in mechanical stress.

Forty male rats（7-week-old）were used as materials, they were divided into three groups : hind-limb suspended group（HS）, hind-limb suspended and electrically stimulated group（TE）and control（CO）. Furthermore, TE was subdivided into TE20, TE40 and TE60, according to differences in stimulating voltages. HS and TE were hind-limb suspended for 2weeks and TE was transcutaneous-electrical-stimulated 10mins/day, 5days/week, under the conditions of electrical stimulation of a direct current of 31Hz and a carrier wave of 80kHz, 200μsec. After the experimental period, femur in each group was excised and analyzed histologically and morphometrically.

Concerned to a cortical bone, bone resorption in TE20 was similar to HS and that of TE40 and 60 was inhibited remarkably. As to a cancellous bone, bone densities of HS and TE20 were lower significantly than CO and there was not significant difference between TE40, 60 and CO.

It was understood that decrease in bone volume caused by decline of the mechanical stress could be inhibited by the transcutaneous electrical stimulation using over than 40V.

Keywords : Bone structure, Electrical stimulation, Various voltages

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1. Introduction

Recently, it is expected, in Japan, that a rate of bone fracture increases with a population aging and the tendency inclines in the future. A maintaining and an improving of bone health are also important in promoting "elongation of health expectancy". In women, the lack of estrogen associated with a menopause causes a decrease in bone mass[1)]. Development of drug for the maintenance and promotion of bone has been advanced so far and bisphosphonate is also one of them. When it was administered 1 time /week to mice for five weeks, a white line appeared in the soft X-ray image of the tibia corresponding to the position, which is shown to correspond to the site that the bone resorption was not undergo[2)]. Further research has been carried out since then, and drugs using RANKL antibodies related to osteoclast differentiation have been developed[3)]. However, it has been pointed out that there is a side effect of jawbone necrosis by a long-term administration of the drug[4)]. On the other hand, as bone-mass-maintaining method without side effects, there is exercise therapy[5)] and electrical stimulation[6)]. In the latter[6)] reported that bone formation was promoted by the acupuncture electrical stimulation to femur after hind-limb suspension in rats. Bone resorption was suppressed by the acupuncture electrical stimulation to femur during hind-limb immobility also seen in rats[6)]. Mochizuki et al.[7)] gave the acupuncture and the transcutaneous electrical stimulation to femur in hind-limb suspended rats, compared the inhibitory effect of bone mass reduction on the cortical bone between both stimulations, and then, cleared that those treatments showed a significant inhibitory effect on bone mass reduction. Nakai et al.[8)] compared the inhibitory effects to the bone loss by hind-limb immobilization between the acupuncture and the transcutaneous electrical stimulation using a low frequency therapy device of AC. As a result, a remarkable effect was observed in the acupuncture electrical stimulation. However, significant effect was not obtained in the transcutaneous electrical stimulation. Mochizuki et al.[7)] reported that DC using carrier wave had significant effect on the suppression of bone mass reduction.

High voltage electrical stimulation has a large effect on body. It may lead to sense of discomfort and pain if the voltage is excessively high. If the same effect can be obtained, lower voltage is safer. Mochizuki et al[7)]. observed the inhibitory effect of bone mass reduction by using DC of 60V. It is not known whether it is effective or not at lower voltage than that. In this study, the frequency of DC (31Hz) and carrier wave (80kHz 1,200μsec) and the stimulating time were the same conditions as Mochizuki et al[7)]. And then, the purpose of this study was to compare and investigate the effects of different voltages (20, 40 or 60V) of the transcutaneous electrical stimulation.

2. Materials and Methods

2.1. Materials

Forty rats (male, wistar strain) aged 7 weeks were used as materials in this study. They were classified into three groups of a hind-limb suspension group (HS), a hind-limb- suspended and a transcutaneous electrical stimulated group (TE) and a control (CO). Moreover, TE was divided into three groups by the difference in voltage of electrical stimulation : a 20V group (TE20), a 40V group (TE40) and a 60V group (TE60).

2.2. Methods

2.2.1 Hind-limb suspension experiment

Of the experimental group, HS, TE20, TE40 and TE60 were hind-limb- suspended for two weeks. In order to remove the sense of pain, discomfort and anxiety as much as possible, rat was anesthetized in advance pentobarbital Na (Somnopentyl, 40mg/kg body weight) in the treatment. During the experiment period, feeding and water drinking were free.

2.2.2 Transcutaneous electrical stimulation

Before the start of the experiment, femur was shaved in TE. TE was carried out the transcutaneous electrical stimulation using a low frequency treatment device (Manufactured by Oshima Seisakusho, Bio Trainer1). In order to suppress the sense of pain, discomfort and anxiety as much as possible, rat was anesthetized in advance pentobarbital Na in the treatment. Distal portion of the femur was electrical-stimulated. Conditions of the transcutaneous electrical stimulation : frequency DC was 31Hz, the frequency of carrier wave was 80kHz and 200μsec and they were as the common to the transcutaneous electrical stimulation groups. And then, the purpose of this study was to compare and investigate the effects of different voltages (20, 40 or 60V) of the transcutaneous electrical stimulation.

2.2.3 Sampling

After the end of the experimental period, rats were euthanized by carbon dioxide suction. After confirming the death, and femur was excised. The distal part of femur was cut, and further cut in the sagittal direction. Specimens were quickly immersed in 4% paraform-aldehyde solution (PFA) or Karnovsky solution (KAR) containing 4% paraform-aldehyde and 5% glutaraldehyde buffered by 0.1M cacodylate sodium (pH7.4), and were fixed

2.2.4 Analyses

Macroscopic observations : The specimens fixed with PFA were immersed in 30% sodium hypochlorite, to remove the organic substances from the bone and bone marrow, morphology and structure of them were observed macroscopically.

Histological observations : The specimens were the calcified by 8% EDTA solution, were embedded in paraffin wax after dehydration and clearance and were sectioned in 4μm thickness. They were stained by various methods and were observed by light microscope.

Other specimens were embedding in resin after dehydration and clearance without decalcification and were ground up to about 100μm thickness. Then, they were etched, were stained by toluidine blue (TB) dye and were observed. In addition, the other specimens were freeze-dried, carbon and platinum evaporated and were observed by scanning electron microscope (SEM).

Bone morphometry : Width and density of the bone trabeculas in the secondary cancellous bone in each group were measured. Using IBM SPSS Statistics 24, they were analyzed by Tukey method.

3. Results

3.1 Macroscopic observations

A bone surface of a femoral epiphysis was smooth in every groups, macroscopically. A distal epiphyseal cancellous bone existed in bone marrow side at the same portion. The width of the cancellous bone in proximal-distal direction was narrow in HS and that of TE20 was wider than HS. On the other hand, the width of that in TE40 and TE60 was maintained close to CO. (Fig.1.)

3.2. Histological observation

3.2.1. Decalcified Paraffin specimens

The bone surfaces of every groups were not smooth and these characteristics were remarkable in HS and TE20, especially. That of TE40 was likely to CO. That of TE60 was smoother than CO at not only anterior but also posterior face of the femur. (Fig.2.)

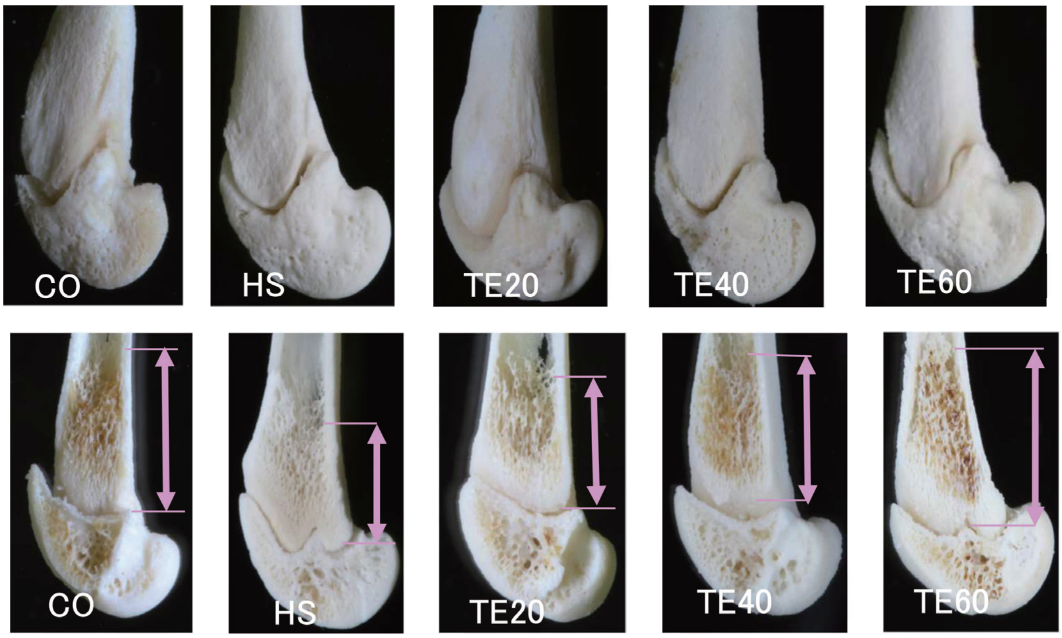

Fig.1. Macroscopic images of the surface and sagittal cross section of the femur
(Sodium hypochlorite treatment specimens)
Left side = forward, right = rear
Arrows : Proximal-distal width of the cancellous bone

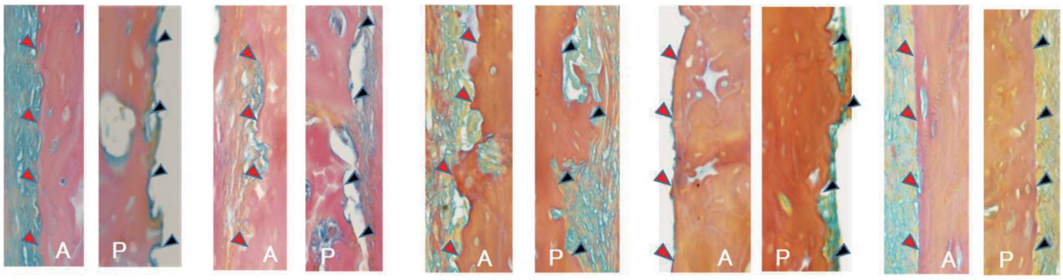

Fig.2. Structure of the anterior and posterior cortical bone surfaces
(Paraffin sections, Polychrome Staining) A = Anterior, P = Posterior

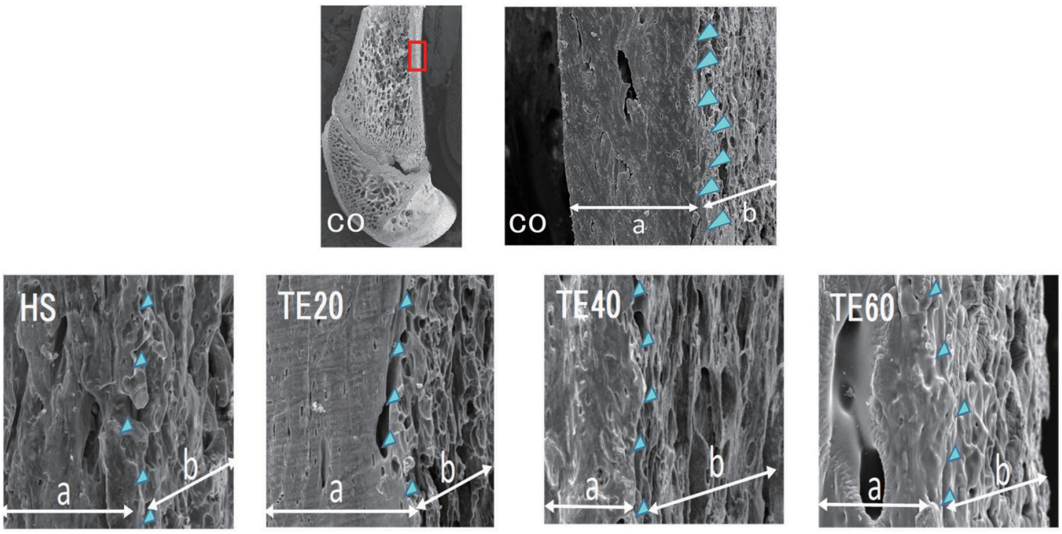

Fig.3. Structures of the cortical bone in the periosteum side
Upper left : Specimen sagittal-sectioned (sodium hypochlorite treated)
Others : Magnified images of the portions like a red square of CO
a : Sagittally cross-sectioned face of cortical bone b : Periosteum face
Arrowheads : Cross-sectional structure of periosteum surface of cortical bone

3.2.2. Scanning electron microscope (SEM)

The bone surface of CO was rough when observing the bone structure in the periosteum side, using specimen cross-sectioned in sagittal direction. (Fig.3.)

3.2.3. Undecalcified resin-embedded ground specimens

The bone trabeculas of primary and secondary cancellous bone were scarce in HS when observing the undecalcified resin-embedded ground specimens to verify the structures of those cancellous bones in detail. Moreover, little bone trabecula existed at the boundary between those cancellous bones. The bone trabeculas extend toward proximal-distal direction but they were thinner than CO. To the contrary, in TE40 and TE60, the thickness and density of the bone traveculas were same as those of CO. (Fig.4.)

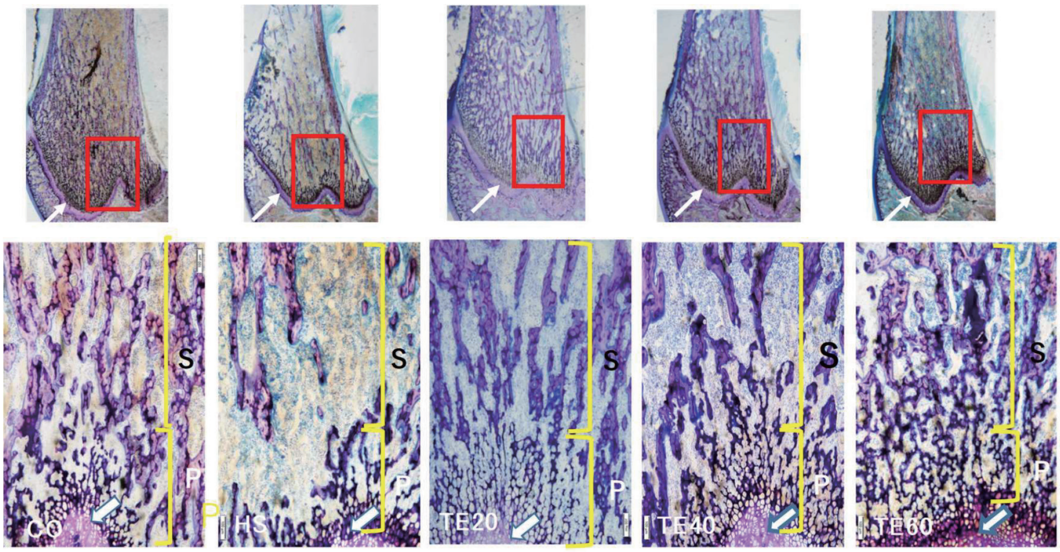

Fig.4. Structures of cancellous bone at the distal epiphysis of the femur
(Resin embedded ground specimen, toluidine blue staining)
The lower：Magnified images of the red frame of the upper.
Arrow：Growth plate, P：Primary cancellous bone, S：Secondary cancellous bone

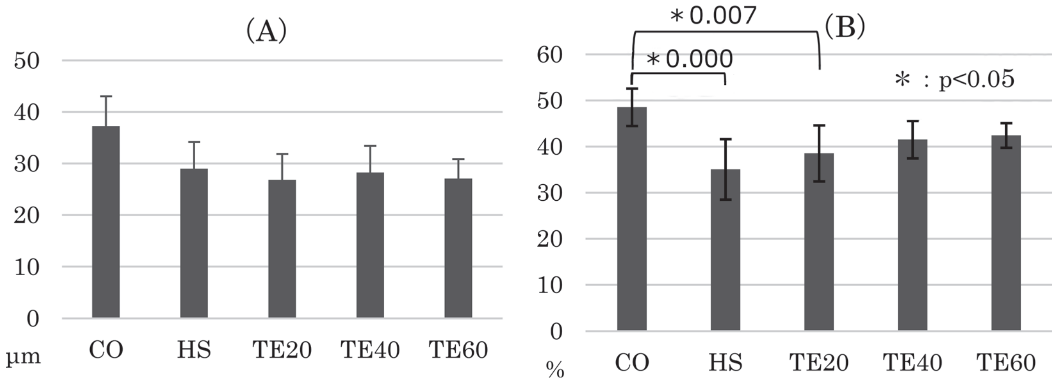

Fig.5. Thickness and density of bone trabeculas in secondary cancellous bone
A：Bone trabecular thickness, B：Bone trabecular density

3.3. Bone morphometry

As measuring thickness and density of the bone trabeculas in the secondary cancellous bone of each group bone morphometrically, no differences were found in the thickness of them. However, the bone density of HS and TE20 was lower significantly than CO. The bone densites of TE40 and TE60 were lower than CO but the difference was not significant.（Fig.5.）

4. Discussion

Previously, using hind-limb suspended rats, it had been reported that an acupuncture[6, 7] and a transcutaneous electrical stimulation[8, 9] resulted in the inhibitory effect on bone mass reduction by hind-limb suspension. Concerning to the transcutaneous electrical stimulation, it has also been reported that there is no effect in the low frequency therapy device of AC[9]. The stimulation reaches until the bone by the electrical stimulation of DC using the carrier wave and the effect has been already shown[7]. However, there are no reports as to the effect of different voltages of the electrical stimulation. Therefore, in this study, the effect of transcutaneous electrical stimulation was compared and investigated between different voltages, i.e. 20, 40 or 60V.

When observing the surface of the cortical bone macroscopically, it was smooth in any group. However, observing the cortical bone with a light microscope and SEM, the periosteum face of the cortical bone in HS and TE20 showed considerably rougt compared to CO. On the other hand, the periosteum face of TE40 and TE60 is close to that of CO. TE60 was remarkable smooth. Similar to this study, Mochizuki et al.[7] investigated the effects of the acupuncture and the transcutaneous electrical stimulation in rats that caused bone mass decrease by hind-limb suspension, and cleared the relationship between images of the cortical bone surface by SEM and light-microscopic images of TRAP-stained sections. According to this report, small fossas at the surface of the cortical bone were correspond to Howship's grooves that were observed in the sections stained by that staining method. Hancox[10] was showed same observations, too. On the other hand, there were also some smooth areas except for the areas consisted of many small fossas. This meant that the bone resorption was restricted by the electrical stimulation, compared to HS. Thus, as to the suppression of the bone mass reduction of the cortical bone mass, 60V is the most effective in the conditions of this experiment, 40V also showed substantially the same effect as it. However, 20V was close to HS.

It has been known that the bone trabeculas in the cancellous bone decrease due to aging and immobility[11]. The difference in voltage of the transcutaneous electrical stimulation already appeared in the results of macroscopic observation. As for the cancellous bone, the thickness of the bone trabculas in proximal-distal direction decreased in HS. Those findings of TE20 was similar to those of HS. The thickness of the bone trabecula increased as the voltage enhanced, and TE60 was almost the same as CO.

Mochizuki et al.[7] found that the acupuncture electrical stimulation is effective in maintaining bone mass not only in the cortical bone but also on the cancellous bone. It was reported that, in contrast, the transcutaneous electrical stimulation showed a remarkable effect on the cortical bone but such an effect was not observed much in the cancellous bone. In the acupuncture electrical stimulation was inserted directly into the periosteum. oppositely, in the transcutaneous electrical stimulation, it was necessary that an attenuation of the electrical stimulation. was considered because the that pads were attached to the body surface. However, in this study, it was confirmed that the transcutaneous electrical stimulation of high voltages suppressed the bone resorption of the cancellous bone. The bone density of HS and TE20 showed significantly lower values than CO when comparing with bone morphometric method. No significant differences were found between TE40/TE60 and CO. However, the electrical stimulation group could not suppress the decrease in thickness of the bone trabeculas, compared to control. When the bone trabeculas were resorbed, they became thinner, and disappeared finaly[12]. From the above, it was suggested that the transcutaneous electrical stimulation at the voltage above 40V had the suppressing effect of bone resorption of the cancellous bone.

The cancellous bone plays a role in dispersing a mechanical load from the adjacent bone to the surrounding cortical bone. Therefore, the main arranging direction of the bone trabeculas constituting the cancellous bone is corresponded to the force line brought to the bone[13)]. If the mechanical stress to the bone is reduced by immobility etc., the bone trabeculas became thin, so that the number thereafter is also reduced[14)]. On the other hand, the bone volume increases when the movement is resumed after immobility[15)]. Thus, the mechanical load and the cancellous bone is closely related. Considering the results of this study from this viewpoint, it seems that the thickness of the bone trabeculas decreased due to hind-limb suspension in HS. Further more, if the voltage of the transcutaneous electrical stimulation is lower like TE20, the bone mass reduction of the cancellous bone cannot be suppressed. The cancellous bone was maintained with the increase in the voltage of the transcutaneous electrical stimulation.

Because the transcutaneous electrical stimulation contracts the muscle, the traction force from the muscle acts on the bone, and then, there is a possibility that bone volume was maintained by the force. It is possible that, mechanical stress associated with muscle contraction causes inactivation of osteoclasts, this point is not understood.

As described above, in fact, the higher voltage of the transcutaneous electrical stimulation suppressed to the bone mass reduction. In this study, only 20V is hardly effective, significant suppression effect was obtained in 40V and especially 60V.

5. Conclusion

In the voltage of the transcutaneous electrical stimulation used in this study, it was understood that the stimulation of 20V had little inhibitory effect on the bone mass reduction associated with hind-limb suspension but a remarkable effect was obtained in 40V and especially 60V.

Committee of Animal Experiment and Ethics

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

Acknowledgments

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異なる電圧の経皮通電刺激が加重低減中のラット大腿骨の構造に及ぼす影響

Effects of transcutaneous electrical stimulation of different voltages on structure of femur in hind-limb suspended rats

小林　宗弘　　望月　将希　　大迫　正文
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要旨

本研究は、異なる電圧による経皮的通電刺激が、加重低減されたラット大腿骨の骨量減少に及ぼす影響について組織学的・形態計測学的に検討した。

材料として、7週齢のウィスター系雄性ラット40匹を用いた。それらを3群（後肢懸垂群HS：8匹、後肢懸垂・通電刺激群TE：24匹、対照群CO：8匹）に分類し、さらにTEは刺激電圧の違いによりTE20、TE40、TE60に分類した。HSおよびTEは2週間尾部をケージの天井から吊し、さらにTEは経皮的に20、40または60V（基本周波数31Hz、搬送波周波数80kHz 200μsec）の通電刺激を10分/日、5日/週、2週間実施した。実験期間終了後、各群のラットから大腿骨を摘出し、組織学的・形態計測学的に分析した。

皮質骨においては後肢懸垂群に比べて通電刺激群は骨吸収が抑制され、TE60が最も効果があり、TE40もそれとほぼ同等の効果を示した。一方、TE20はHSに近い状況にあった。一次および二次海綿骨の構造においては、HSでは一次、二次海綿骨のいずれにおいても骨梁が乏しく、TE20では骨梁が近遠心方向に直線的にのびているが、COにくらべてかなり細かった。それに対して、TE40と、特にTE60では骨梁の太さと密度がCOに近い状態にあった。二次海綿骨の骨梁幅はCOが最も高値を示したが、群間に有意（P＜0.05）な差は認められなかった。二次海綿骨の骨密度は、HSおよびTE20がCOより有意（P＝0.000、P＝0.007）に低値を示したが、TE40および60とCOとの差は有意（P＜0.05）ではなかった。

これらのことから、加重低減によってもたらされる骨量減少は40V以上の経皮通電刺激で抑制されるであろうことが理解された。

キーワード：骨構造　通電刺激　種々の電圧