Study in effects of transcutaneous electrical stimulation of different intervention frequency on bone structures in hindlimb-suspended rats

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Summary

[Purposes] It was aimed to investigate histologically the effects of different frequencies of percutaneous electrical stimulation on changes of bone structure accompanied with hindlimb-suspension in rats. As experimental materials, forty-eight male rats (7-week-old, wistar strain) were e randomly classified into a tail suspension group (TS) and a control group (CO), and TS was subjected to tail suspension for 14 days by the following method. The TE group performed once a week (TE1), 3 times (TE3), and 5 times (TE5) due to the difference in intervention frequency.

[Materials and Methods] The transdermal stimulations (DC, voltage: 60 V, basic frequency: 50 Hz, 200 μsec, frequency of carrier wave: 80 kHz) was given to TE using a low-frequency electrical stimulator from the anterior surface of the femur, at the condition of 10minutes / day, 5days/week, for 2 weeks. The electrical stimulation was performed under anesthesia, after removing the hair from the femur. Rats in each group were euthanized and femurs were excised at the end of the experimental period. Those femurs were analyzed histologically.

[Results] Numberless fine fossulae on the outer surface of the femur decreased, from T1 to T5. Regarding the cancellous bone at the distal metaphysis of the femur, many thick trabeculae were found in CO, but the trabeculae of HS and TE1 were thin. The density of

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the trabecular bone increased from TE1 to TE3, and the trabecular density increased from TE3 to TE5. The arrangement direction of the trabecula also became regular from T3 to T5, and showed a structure similar to CO.

[Conclusion] It was suggested that bone loss in the cancellous bone in the distal femur caused by rat hindlimb suspension may be suppressed by percutaneous electrical stimulation three or more times a week, and bone weakening may be prevented.

Keywards: Hindlimb-suspension, transcutaneous electrical stimulation

1. Introducction

It was reported that costs incurred during activities of daily living and while in a facility account for most of the total cost from age 70 to death[1]. Mechanical stress is important for maintaining bone mass, and mechanical unloading such as long-term bedridden causes osteoporosis [2]. Osteoporosis can be treated with a drug or an exercise therapy, and Bisphosphonates are often used for their treatment[3]. However, it has also been shown that long-term administration of that drug has side effects such as mandibular necrosis[3]. It is considered that the exercise therapy is desirable because the drug treatment has such side effects.

It has been reported that an appropriate walking exercise under proper calcium intake maintains the bone mineral density of a lumbar spine in postmenopausal osteoporosis patients[4]. It was also shown that the prevalence of both osteoporosis and osteoarthritis increases with age[5]. In this way, patients with osteoporosis often have other locomotorium diseases at the same time, and it is difficult to prescribe exercise therapy. For this reason, the author focused on the effect of mechanical stimulation by electrical stimulation on bone as a method other than joint movement. It has reported, from previous study[6] using a transcutaneous electrical stimulation, that the bone formation is promoted by that stimulation after the hindlimb-suspension in rats. However, there are no reports of differences in the effects of cost-on stimuli with different intervention frequencies.

In this study, it was aimed to investigate histologically the effects of different frequencies of percutaneous electrical stimulation on changes of bone structure accompanied with hindlimb-suspension in rats.

2. Materials and methods

2.1. Materials

As experimental materials, forty-eight male wistar rats (7-week-old, wistar strain) were randomly classified into a tail suspension group (TS) and a control group (CO), and TS was subjected to tail suspension for 14 days by the following method. The TE group performed once a week (TE1), 3 times (TE3), and 5 times (TE5) due to the difference in intervention frequency.

2.2. Experiments

2.2.1. Hind-limb−suspension

As for the tail suspension, we prepared a cage with a wire mesh 20 cm higher than the normal cage, and the hindlimbs of HS and TE suspended from the ceiling of the cages for 2 weeks. It was considered so that water could be fed and watered while the hindlimbs were suspended.

2.2.2. Transcutaneous electrical stimulation

The transdermal stimulations (DC, voltage: 60 V, basic frequency: 50 Hz, 200 μsec, frequency of carrier wave: 80 kHz) was given to TE using a low-frequency electrical stimulator from the anterior surface of the femur, at the condition of 10minutes /day, 5days/week, for 2 weeks. The electrical stimulation was performed under anesthesia, after removing the hair from the femur.

2.2.3. Sampling and fixation

Rats in each group were euthanized by carbon dioxide inhalation at the end of the experimental period. The femurs were excised after confirming death, and the distal part of them were sagittally cut with a hand motor. Those specimens were immersed in 4% paraformaldehyde solution (PFA) or Karnovsky solution immediately after excision and fixed.

2.2.4. Analyses and observations

An outer face and an inner face of the cortical bone were observed macroscopically and furthermore observed using a scanning electron microscope (SEM), after the femurs in each group were treated by a sodium hypochlorite. Non-decalcified resin-embedded polished specimens were prepared using femur in each group, were stained with toluidine blue, and were observed with an optical microscope.

3. Results

When observing macroscopically the cortical bone on the outer surface of the distal femur treated with sodium hypochlorite revealed, it was smooth in both groups. When this area was enlarged and observed by SEM, the surfaces of all groups were rough. However, when the bone surface of each group is observed in detail, there are many relatively smooth sites in CO, and such areas were accompanied by an increase in intervention frequency from TE1 to TE5. (Fig. 1)

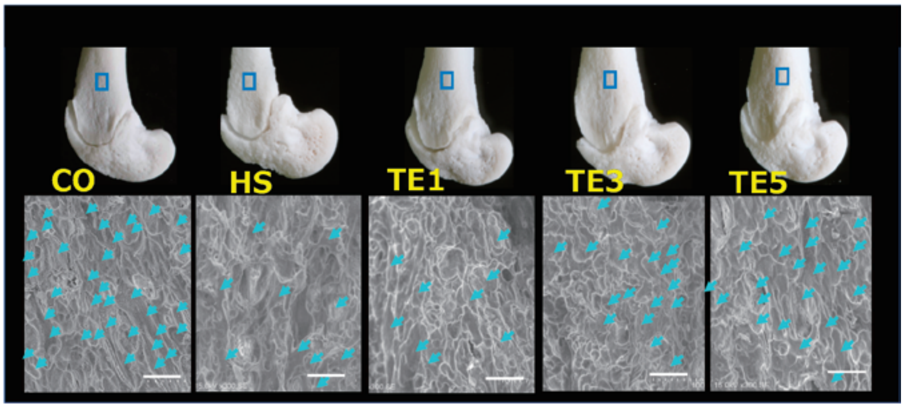

Fig.1. Structure of the cortical bone on the outer surface of the distal femur

Bar=500μm

Sodium-hypochlorite treatment specimens, Arrows shows relatively smooth areas.

Upper: macroscopic images, Lower: SEM images magnified square part of Fig.1

Many small fossulae were observed, and a site without such fossulae was also observed between them, as magnifying and observing by SEM the outer surface of the femur in TE5. (Fig. 2-a) When the area was further enlarged, the surface of the small fossulae were smooth, and the surface of the area without those fossulae was in a rough state. (Fig. 2-b)

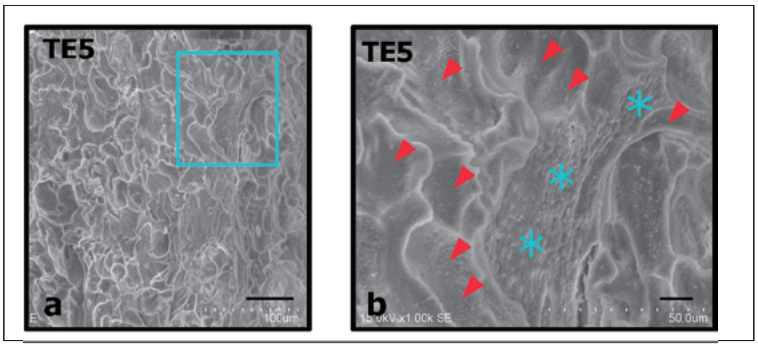

Fig.2. Magnified images of the outer surface by SEM in TE5

Bar of " a" :50µm　Bar of " b" :10µm

Sodium-hypochlorite treatment specimens,

b: magnified image of square part in "a"

Arrows shows: fossulae.　＊: Arrows shows relatively smooth areas.

Macroscopic observation of the inner surface of the femur of each group treated with sodium hypochlorite revealed cancellous bone at the metaphysis, and no significant difference was observed between the groups in the structure of the trabecula there. When they were observed by SEM, many thick trabeculae were observed in CO, but the HS and TE1 trabeculae were thin.　In addition, the density of the trabecular bone increased from TE1 to TE3, and the trabecular density furthermore increased from TE3 to TE5. In this way, the bone mass increased in a manner depended on intervention frequency, and moreover, the trabecular bone alignment direction also became regular from T3 to T5, showing a structure likely to CO. (Fig. 3)

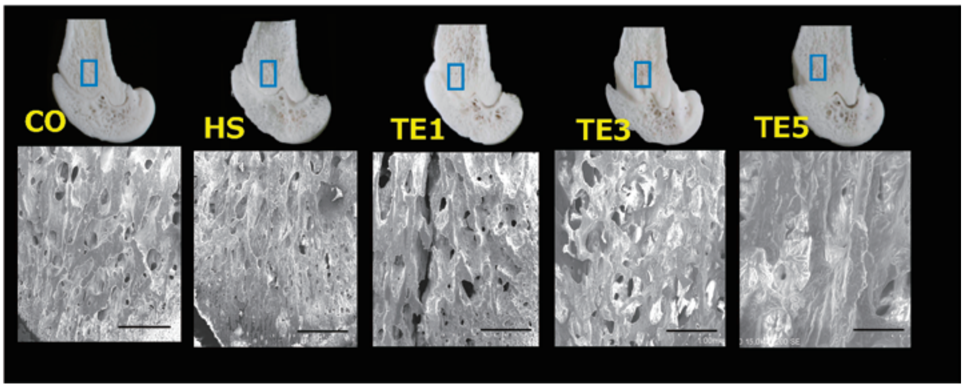

Fig.3. Structure of the cortical bone on the inner surface of the distal femur

Bar=10µm

Sodium-hypochlorite treatment specimens, Arrows shows relatively smooth areas.

Upper: macroscopic images, Lower: SEM images magnified square part of Fig.1

Toluidine blue stain was applied to the non-decalcified resin-embedded polished specimen to clarify the difference between the trabecular structures, and the trabecular bone at the end of the bone was enlarged and observed. The arrangements of the trabecular bones were irregular fairly in HS, but otherwise arranged vertically. The trabecular bones of both groups were deeply dyed, and there were different stained bones on the surface side. There was almost no difference between the groups concerned to the deep dark area. However, the bone on the surface side had the thickest CO and the entire trabecular bone was also thick. On the contrary, in HS, the bone on the surface side was thin and the trabecula was thin. There was not much change in the thickness of the trabecular bone from TE1 to TE5, but many connections between adjacent trabecular bones were observed in TE3 and TE5. (Fig. 4)

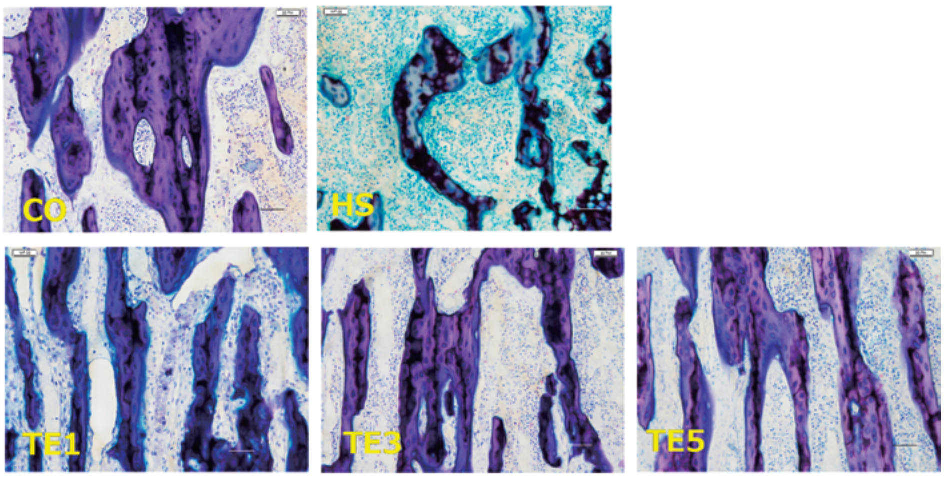

Fig.4. Light microscopic image in each group
Bar=50μm
Undecalcified resin-embedded ground section, Toluidineblue stain.

4. Discussion

In this study, it was histologically investigated how different frequencies of transcutaneous electrical stimulation affect trabecular bone loss in rat hindlimbs caused by a reduction of mechanical-loading. It has been reported that acupuncture[6] and trsnscutaneous[7] electrical stimulations function suppressively on the bone loss in the cortical and cancellous bone accompanied with a reduction of mechanical-loading. A study using a rat fracture model have also shown that an acupuncture stimulation promotes recovery from injuries[8, 9]. As described above, it is shown that the effect of the electrical stimulation inhibits a bone resorption due to a reduction of mechanical-loading and promotes a bone formation after fracture.

First, regarding the cortical bone in this study, the outer surface of the cortical bone of CO was relatively smooth, but the site of HS was rather rough. However, the smooth area increased as the intervention frequency increased from TE1 to TE5. Moreover, when the outer surface of the cortical bone was magnified and observed by SEM, the surface of the small fosses was in a smooth state. Nakai, et al.[10] reported that there are more cells positive for a tartrate-resistant acid phosphatase (TRAP) reaction on the surface of the femur of rats with hindlimb immobilization than CO, but this is significantly reduced by the acupuncture stimulation. First, regarding the cortical bone in this study, the outer surface of the cortical bone of CO was relatively smooth, but the site of HS was rather rough. However, the smooth area increased as the intervention frequency increased from TE1 to TE5. Moreover, when the outer surface of the cortical bone was magnified and observed by SEM, the surface of the small fosses was in a smooth state. Nakai, et al.[10] reported that there are more cells positive for a tartrate-resistant acid phosphatase (TRAP) reaction on the surface of the femur of rats with hindlimb immobilization than CO, but this is significantly reduced by the acupuncture stimulation.

From this, it seems that the small fosses existing on the surface of the cortical bone correspond to the sites absorbed by TRAP-positive cells, that is, osteoclasts. It is thought that they increase with the reduction of mechanical-loading and decreases with the electrical stimulation. Furthermore, the condition is such that the cortical surface becomes smooth depending on the intervention frequency, and it is considered that a remarkable inhibitory effect on bone resorption is obtained at 5 days / week on the outer surface of the cortical bone. The cortical surface became smooth depending on the intervention frequency, and it seems that a remarkable inhibitory effect on bone resorption was obtained at 5 days / week on the outer surface of the cortical bone.

It has been reported that the cancellous bone in the distal part of the femur is decreased by reducing the mechanical-loading of the rat hind limbs, but is maintained by electrical stimulation[6, 7, 10]. In this study, many thick trabeculae were found in the cancellous bone at the distal metaphysis of the femur of CO, and they showed a regular arrangement in the vertical direction. On the other hand, the trabeculae of HS were thin and loose, and the arrangement was irregular. The trabeculae of TE1 was also thin, but TE3 and TE5 became thicker, and they were observed to be regularly arranged in the vertical direction in all groups of TE. The trabecula of the cancellous bone at the distal metaphysis of the femur is responsible for receiving the mechanical-loading from the tibial side and transmitting it to the diaphyseal cortical bone. It is considered that the trabeculae were resorbed by the decrease in the mechanical-loading, because the trabeculae of HS were thin, loose, and irregularly arranged. On the contrary, they approached the situation of CO as it changed from TE1 to TE5. This is similar to the change that appears due to the increase in weight due to growth and exercise, and it seems that the electrical stimulation has a similar effect.

When observing the undecalcified specimen that were embedded in the resin-embedded resin and were stained with toluidine blue and the trabecular bones of all groups were darkly stained, and no difference was observed in the thickness between the groups. Bone matrix that causes metachromasy by that stain was added to the surface of the trabecular bones. The trabecular bone of the cancellous bone at the metaphysis is formed by adding bone around the calcified cartilage trabeculae extended from the growth plate. The bone on the surface of the bone trabeculae had the thickest in CO and the bone trabeculae was also thick. On the contrary, in HS, the bone on the surface side was thin and the bone trabeculae was thin. These facts suggest that CO promotes bone addition around the calcified cartilage beam, and conversely, HS does not perform bone resorption and bone resorption occurs. These facts suggest that CO promotes bone addition around the calcified cartilage trabeculae, and conversely, HS does not perform bone resorption and bone resorption occurs.

The bone on the superficial side was clearly purple in CO, TE3 and TE5 by metachromasy, but was stained in light blue in HS and weak in metachromasy in TE1. In this way, the bone on the surface side of the trabecular beam strongly caused metachromasy in CO, whereas the metachromasy was weak in HS and TE1. Since toluidine blue causes metachromasy when a substance having a sulfate group such as cartilage is contained in a large amount in the substrate, the stainability of the CO and HS substrates may mean a change in the substrate component due to immobilization. In addition, all of TE groups that were decreased in the mechanical-loading showed intermediate stain ability between CO and HS, which is also presumed to be related to the state of the substrate component.

As regarding these trabecular structures, changes in the trabecular arrangement direction and stainability depend on the intervention frequency, and in this way, energization stimulation is effective for bone loss due to weight reduction, and its effect. It seems to be intervention frequency dependent, as is the outer surface of the cortical bone. It has been reported that the structure of the cortical bone differs between the proximal metaphysis and the central part of the diaphysis in the developing rat tibia[11], and that the structure and mechanical strength differ with development even at the same site[12, 13]. Similar findings have been reported from studies observed in rat femur[14]. In this study, specimens were sampled from the distal femur in both groups, so the central part of the diaphysis is unclear. In addition, no mechanical test has been conducted, and it is a future task to examine the effect of electrical stimulation on the structure and mechanical strength of not only the distal femur but also the central diaphysis.

5. Conclusion

It was suggested that bone loss in the cancellous bone in the distal femur caused by rat hindlimb suspension may be suppressed by percutaneous electrical stimulation three or more times a week, and bone weakening may be prevented.

Acknowledgement

We thank staffs of laboratory for support in performing the experiment.

Committee of Animal Experiment and Ethics

This study was approved by committee of Animal Experiment of Toyo University.

References

1)James L., et al.: Health, life expectancy, and health care spending among the elderly: Engl. J. Med.: 349：1048-1055,2003.

2) Keiko E., et al.: Disrupted bone metabolism in long-term bedridden patients. PLoS ONE 11（6）：e0156991,2016.

3) Michael McClung, et al.: Bisphosphonate therapy for osteoporosis: Benefits, risks, and drug holiday. Am. J. Med. 126: 13-20, 2013.

4) Satoshi Y., et al.: Effect of walking exercise on bone metabolism in postmenopausal. J. Bone Miner. Metabol. 22:500–508,2004.

5) Yun H. S., et al.: Exercise self-efficacy, exercise benefits and barriers, and commitment to a plan for exercise among Korean women with osteoporosis and osteoarthritis. Int. J. Nursing Studies 43:3-10, 2006.

6) Mochizuki M., et al.: Study in effects of acupuncture and transcutaneous electrical stimulations on structure of tibial articular cartilage in tail-suspended rats. J.Human Life design 15:195-203,2020.

7) Kobayashi M., et al.: Effects oftranscutaneous electrical stimulation of different voltage

on structure of femur in hind-limb suspended rats. J. Human Life design 15: 131－140, 2020.

8) Nakajima M., et al. : The effect of electroacupuncture on the healing process of a tibia fracture in a rat model. J. Japan Socie Acupunc Moxibustion 59 : 477－485, 2009.

9) Lam H., Qin Y.-X.: The effects of frequency-dependent dynamic muscle stimulation on inhibition of trabecular bone loss in a disuse model. Bone 43 : 1093－1100, 2008.

10) Nakai S., et al. : Effects of electro-acupuncture stimulation on femoral structure and strength during immobilization in rats. Bull. Grad. Sch., Toyo univ. 52 : 255－271, 2015.

11) Inoue S., et al. : Studies on differences of structure and strength of tibial cortical bone in rats. Bull. Grad. Sch., Toyo univ. 49 : 305－320, 2012

12) Inoue S., Ohsako M.: Study on changes of structure and strength of tibial cortical bone in growing rats. Bull. Grad. Sch., Toyo univ. 50 : 253－276, 2014.

13) Inoue S., Ohsako M. : Site differences of mechanical characteristics and structures of tibiae in growing rats. J. judo therapy 23 : 105－111, 2015.

14) Nakai S., et al. : Study on site differences in structures of femoral bone matrix and periosteum in rats. Bull. Grad. Sch., Toyo univ. 51 : 203－217, 2014.

異なる介入頻度の経皮通電刺激が後肢懸垂ラットの骨構造に及ぼす影響

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要　旨

【目的】本研究はラット後肢の加重低減によって骨梁減少を図り、そのような構造変化に対して、異なる頻度の経皮通電刺激がどのような影響をもたらすかについて組織学的に比較、検討することを目的とした。

【方法】7週齢のウィスター系雄性ラット48匹を用い、それらを後枝懸垂群（HS)、後枝懸垂・経皮通電群（TE)、対照群（CO）に分類した。TE群は介入頻度の違いから、週に1回（TE1)、3回（TE3)、5回（TE5）行った。テールサスペンションは、通常のケージより20cm高い金網のケージを用意し、HSとTEの後肢をケージの天井から2週間吊り下げ、TEは低周波電気刺激装置を用いて、大腿前面から経皮的に10分／日の通電刺激（直流、電圧60V、周波数50Hz、200μsec、周波数80kHzの搬送波を使用）を5日／週、2週間行った。実験期間終了後、各群のラットを安楽死させ、大腿骨遠位部を摘出し、その後、速やかに固定液に浸漬した。それらを用いて、肉眼的および組織学的観察用標本を作製した。後者の標本は、非脱灰樹脂包埋研磨標本を作製して光学顕微鏡により観察するものと、走査電子顕微鏡によって観察するものを用いた。

【結果】大腿骨遠位部外面は基本的に小窩が多数存在するが、その中に滑沢な部位があり、それはT1からT5になるに従って増加した。大腿骨内面の海綿骨ではCOでは、太い骨梁が多く認められるが、HSおよびTE1の骨梁は細かった。また、TE１からTE3になるのに伴って

骨梁の密度が増し、TE３からTE5になるのにしたがって骨梁密度が増した。骨梁の配列方向もT3からT5になるのにしたがって規則的となり、COに類似した構造を示すようになった。非脱灰樹脂包埋研磨標本で骨梁の構造をみると、いずれの群の骨梁も深部が濃く染まり、その表面側に異なる染色性の骨が存在した。深部の濃い部分の面積は群間にほとんど違いが見られなかった。しかし、表面側の骨は、COが最も厚く、骨梁全体も太かった。逆に、HSでは表面側の骨が薄く、骨梁が細かった。隣接する骨梁間の連結がTE3やTE5では多く認められた。

【結論】ラット後肢懸垂によって生じる大腿骨遠位部の海綿骨には骨量減少は、それは１週間に３回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：後肢懸垂、経皮通電、骨梁構造