論　文
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Study in effects of acupuncture and transcutaneous electrical stimulations on structure of tibial articular cartilage in tail-suspended rats

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Summary

This study aimed to investigate the effect of acupuncture and transcutaneous electrical stimulation on structural changes in a tibia articular cartilage caused by hind-limb suspension in rats.

Fortyeight male rats（wistar strain, 7-week-old）were used as materials and they divided into four groups：a hind-limb suspended group（HS）, a hind-limb suspended and acupuncture electrical stimulated group（EA）, a hind-limb suspended and a transcutaneous electrical stimulated group（TE）and a control（CO）. Hind-limbs of three groups except CO were suspended from the ceiling of a cage.

In EA, Stainless steel acupuncture needles were inserted until a periosteum of the anterior face of a femur and were stimulated eletrically（Lasper-A, Sankei Co., 0.24mA, 50Hz, 250μsec, 10 minutes / day, and 6 days / week）. Rats of TE were electrical-stimulated transcutaneously, using pads that were attached to faces of the femur. This stimulation was performed under the following conditions：the condition of a direct current（60V, 31Hz）using the carrier wave of 80kHz, 200μsec, 10 minutes / day, and 6 days / week）. After the experiment period, femurs were excised from each group and were analyzed histologically.

Ratio that a calcified layer occupied in the articular cartilage in HS was lower than CO. In HS, the cartilage lacunas decreased and their arrangements was also irregular, compared to CO. Concerned to the stainability of Safranin O dye, the intermediate layer was stained darkly but the deep layer was stained palely in CO. In HS, not only the deep layer but also the intermediate layer was stained palely. The stainability of TE was as same as CO but that of EA was low.

Therefore, it was suggested that the electrical stimulation gave the inhibiting effects to the structural changes of the articular cartilage due to the loss of the mechanical stress by the hind-limb suspension. Moreover, it was understood that both the acupuncture and the transcutaneous electrical stimulation have same effects.

Keywords：Hind-limb suspension, Electrical stimulation, Tissue structure

1. Introduction

Prolonged bed rest and immobilization are possible to cause complications, such as a loss of muscle strength, an osteoporosis, and an osteoarthritis[1]. The osteoarthritis, that is a degeneration of an articular cartilage, has been one of the most common causes of pain and disorder in middle-aged and older people. A prevalence of the osteoarthritis shows higher correlations to an aging and a decline of the matrix synthesis of a chondrocyte accompanied with it.[2,3]

It is thought that differentiations in cells and tissues of the cartilage with growth[4] and an increase and an decrease in a mechanical stress to the cartilage[5] cause those changes. On the other hand, as to a bone, Nakai et al[6]. had reported that a decrease in a bone mass with hind-limb suspension was inhibited by a transcutaneous or acupuncture electrical stimulation from results of an animal experiment. However, effects of the electrical stimulation on an articular cartilage haven't been reported.

In this study, it was examined how the acupuncture and transcutaneous electrical stimulation affect to structural changes in the articular cartilage caused by hind-limb suspension in rat's tibia.

2. Materials and methods

2.1. Materials

Fortyeight male rats (wistar strain, 7-week-old) were used as materials and they were divided into four groups : a hind-limb suspended group (HS), a hind-limb suspended and acupuncture electrical stimulated group (EA), a hind-limb suspended and transcutaneous electrical stimulated group (TE) and a control (CO).

2.2. Methods

2.2.1. Hind-limb suspension

In HS, EA and TE, i.e. three groups except CO, their tails were suspended from a ceiling of a cage for two weeks.

2.2.2. Acupuncture electrical stimulation

In EA, stainless acupuncture needles were inserted until a periosteum of the anterior face of a femur after the anesthesia and an epilation of femurs. EA was stimulated 10 minutes / day, 6 days / week by a low frequency stimulator of an alternating current (Lasper-A, Sankei Co.) under the condition of 0.24mA, 50Hz, 250μsec.

2.2.3. Transcutaneous electrical stimulation

In TE, pads were attached to the medial and lateral faces of distal part of the femur after the anesthesia and an epilation of femurs. TE was stimulated electrically 10 minutes / day, 6 days / week, using a direct current and low frequency stimulator (Bio-trainer I, Oshima industry Co.), under the condition of a direct current of 60V, 31Hz, and the carrier wave（80kHz, 200μsec）.

2.2.4. Sampling of specimens and fixation

After the experiment period, rats in each group were euthanized by a carbon dioxide absorption. After confirming their death, their skin of hind-limb was exfoliated, femurs were excised. They were divided in a sagittal direction and immersed rapidly in 4% paraformaldehyde（PFA）or Karnovsky fixation fluid（KAR）. These fixation fluids were buffered by 0.1M cacodylate buffer（pH7.4）.

2.2.5. Histological observations

Specimens fixed by PFA were decalcified by 8% EDTA, and were embedded in a paraffin wax after dehydration and clearance. Serial sections of 4μm-thickness were cut, were stained by a polychrome staining method and were observed by light microscope. Other specimens were dehydrated, were cleared and embedded in rigolac resin without decalcification and were polymerized. They were trimmed and ground up to 100μm-thickness. Furthermore, they were etched by 1% hydrochloric acid, were stained by toluidine-blue dye and were observed with the light-microscope. Moreover, specimens fixed by KAR were immersed in 1% Osmium tetraoxide, were freeze-dried after immersing in t-butyl fluid and were performed a carbon and platina evaporaion. And then, they were observed by Scanning electron microscope（SEM：S-3400N, Hitachi CO.）.

3. Results

3.1. Changes in the calcification layer

The bone trabeculas existed densely at the cancellous bone of proximal epiphyses of tibias in CO were thick and dense but they were thin and low dense in three groups that performed hind-limb suspension. Anterior and posterior portions of the articular cartilage was thin and its intermediate portion was thick.（Fig.1,2）

3.2. Thickness of calcified layer in the articular cartilage

No differences were recognized in cross-sectional areas of the articular cartilage of each group cut in the sagittal direction.（Fig.3, Left）Ratio that a calcified layer occupied in the articular cartilage was lowest in CO and that of HS was higher significantly than CO. The ratios of TE and EA weren't low significantly compared to CO.（Fig.3, Right）

3.3. Shape of tidemark

A shape of tidemark, that is a boundary face between uncalcified and calcified layers of the articular cartilage, was linear in HS. On the other hand, in CO, TE and EA, the tidemark wasn't

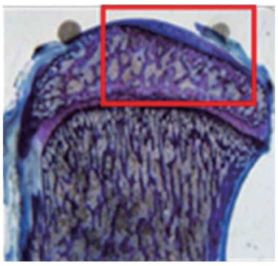

Fig.1 Low magnified image of an articular cartilage

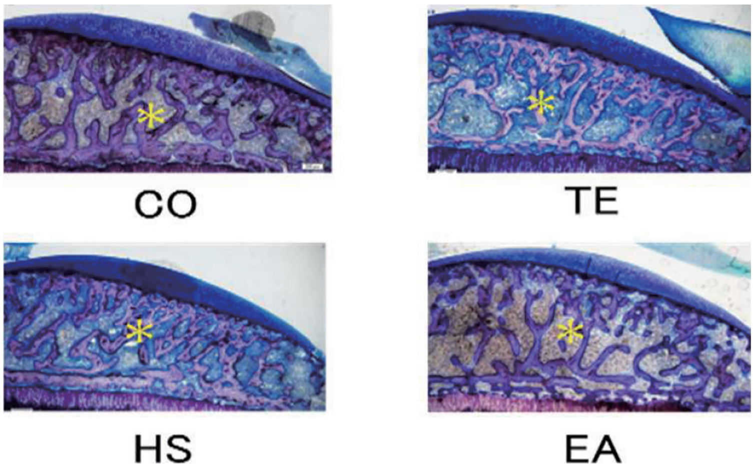

Fig.2 Structures of tibial epiphyses in each group.
Magnified images of area like a square in fig.1
＊：cancellous bone of tibial epiphyses

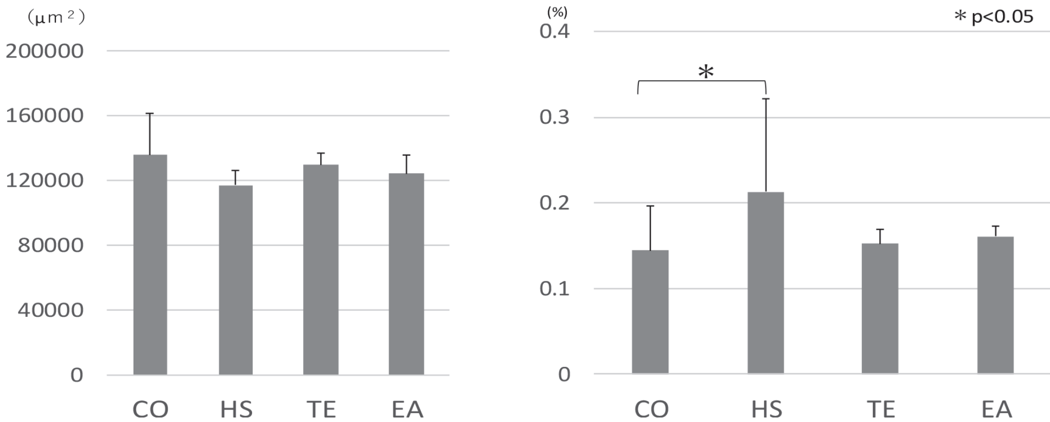

Fig.3 Area of articular cartilage and ratio of calcified layer in the articular cartilage
Left：Cross-sectional area of articular cartilage, Right：ratio of calcified layer in the articular cartilage
No differences were found in cross-sectioned area between groups.
Ratio of calcified layer in the articular cartilage of HS was significantly higher than CO.

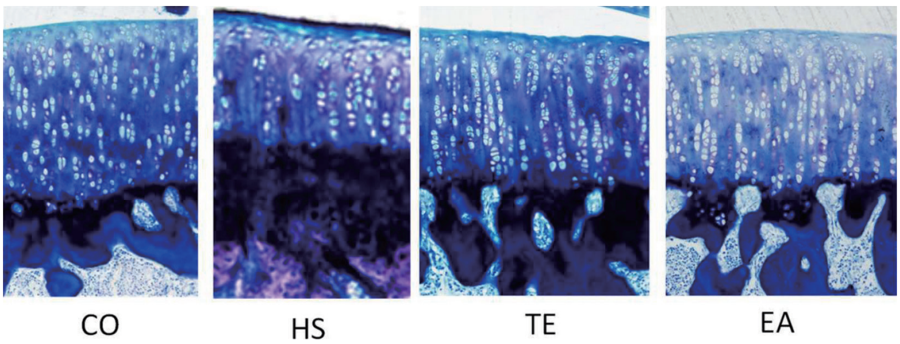

Fig.3 Area of articular cartilage and ratio of calcified layer in the articular cartilage
Left：Cross-sectional area of articular cartilage, Right：ratio of calcified layer in the articular cartilage
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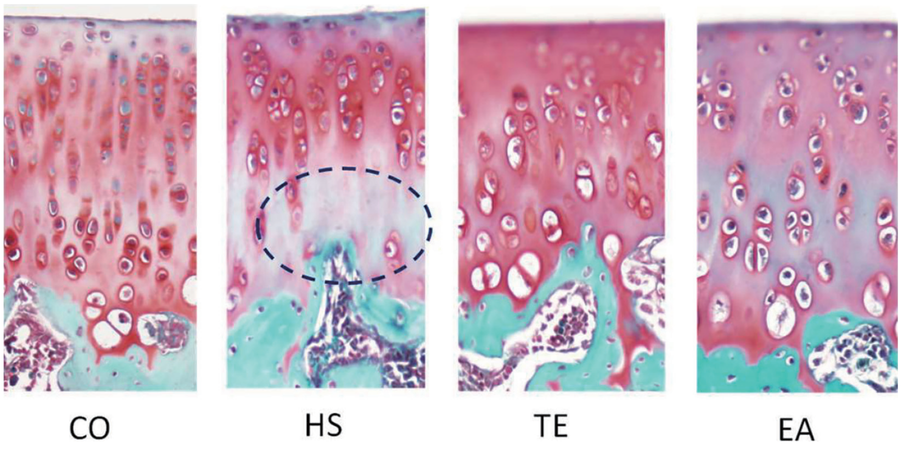

Fig.5 Stainability of safranin O in each group
Decalcified paraffin sections, safranin O staining
Intermediate layers were stained darkly in every groups but deep layer of CO was stained palely. The stainabities of TE and EA were almost same as CO. On the other hand,the deep layer of HS wasn't stained at all. (Dotted circle line)

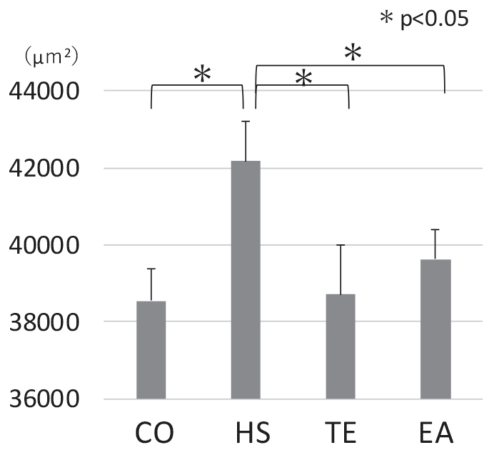

Fig.6 Cross-sectioned area of cartilage matrix except cartilage lacunas
Cross-sectional area of cartilage matrix except the cartilage lacunas in HS was significantly wide in every groups.

smooth and the cartilage lacunas existed at the portions that the calcified layer partially projected in the uncalcified layer. (Fig.4)

3.4. Stainability of articular cartilage

An intermediate layer was stained red darkly in every groups when observing the articular cartilage in each group that was stained by safranin dye. A remarkable decrease in the stainability was found in deep layer. (Fig.5) Moreover, in HS, the cross-sectional area of cartilage matrix except the cartilage lacunas was larger significantly ($p<0.05$) compared to TE, EA and CO.(Fig.6)

3.5. Sizes and densities of cartilage lacunas

The density of the cartilage lacunas was highest at the superficial layer and was going to be lower as going ahead to the deep layer. Hypertrophy of the cartilage lacunas at the deep layer of the articular cartilage in TE, EA and CO and the density of the cartilage lacunas decreased in HS. Arrangements of the cartilage lacunas were irregular in HS but they arranged regularly in the groups that were electrically stimulated. (Fig.4)

4. discussion

It had been already known that a bone weakened accompanied with an immobilization[7)]. In this study, thick bone trabeculas existed densely in the cancellous bone at the proximal epiphysis in CO but a thickness and a density of the bone trabeculas in the other three groups were lower compared to CO. Therefore, it was thought that a hind-limb suspension gave effective decrease in mechanical load to not only the bone but also the articular cartilage.

4.1. Thickness of articular cartilage

It has been showed that the thickness of the articular cartilage decreased by the hind-limb suspension[8-11)] . Kubo et al.[3)] obtained a same result, too and mentioned that a decline of matrix synthesis ability caused the change in the thickness of the cartilage. On the other hand, there were the reports[5, 12)] that the thickness of that didn't decrease by the decline of the mechanical stress. In this study, no differences in the thickness of the articular cartilage were recognized between four groups and this was corresponded to those reports[5, 12)] .

4.2.Matrix of the articular cartilage

Collagen fibers gave resistance to the mechanical stress. Proteogrican (aggrecan) gave an elasticity in the articular cartilage and the content is different in each zone of the articular cartilage[13)] . Remarkable decline in a stainability of Safranin O dye was observed in the deep layer of HS in this study. Then, it was supposed that proteogrican decreased by the decline of the mechanical load. To the contrary, not only the intermediate but also deep layers in TE and EA were stained darkly by that dye like stainability of CO, and it was thought that the electrical stimulation affected to this point, too.

It has been showed that Type X collagen fibers were mainly contained at the deep layer in the articular cartilage and they arranged in perpendicular direction to a surface of the articular cartilage[14)] . Type X collagen fibers weren't observed in this study. However, it is thought that, from the fact that the cartilage lacunas arrange regularly in same direction as the type X collagen fibers, the arrangements of the cartilage lacunas reflecte the arrangements of those fibers. On the other hand, it is supposed that the cartilage lacunas arrange irregularly, for the reason why the matrix fibers were loose. Thus, it is thought that the electrical stimulation inhibits the decline in the synthesis ability of the cartilage matrix, including proteoglycan of the above.

4.3.Tidemark

The articular cartilage consists of a superficial uncalcified layer and a deep calcified layer, and a tidemark that is formed at the boundary between those layers plays an important role as a calcifying front[15]. In this study, as to a ratio of the calcified layer that occupied in the articular cartilage, CO was lowest and HS was higher than CO. The ratios of TE and EA was not lower than CO.

It has been reported that the thickness of the calcified layer of the articular cartilage increased by the immobilization[16]. In this study, it was found that, the tidemark of HS was smooth considerably and linear, but that of the other groups weren't smooth and rugged. It was reported, from the study about the changes in tidemark accompanied with growth[17, 18] and immobilization, that the shape of the tidemark was linear in the infancy but it became rugged with growth, and furthermore, the shape became smooth by the immobilization. It is supposed that the ruggedness of the boundary between the uncalcifed and the calcified layer formed by growth and daily activity contributes to the stabilization of both layers by increasing the contact areas of both layers.

From the above, it was suggested that the electrical stimulation had inhibiting effects to the structural changes like the increase in the thickness of the calcified layer and the irregular arrangement of the cartilage lacunas that was caused by the decline of the mechanical stress with hind-limb suspension. Furthermore, it was understood that these effects were similar in both of the acupuncture and the transcutaneous electrical stimulation.

5. Conclusion

It was suggested that both the acupuncture and transcutaneous electrical stimulation gave the inhibiting effects to the structural changes of the articular cartilage due to the loss of the mechanical stress by the hind-limb suspension.

Acknowledgement

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Ethic examination

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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後肢懸垂ラットにおける脛骨関節軟骨の構造に及ぼす鍼および経皮通電刺激の効果に関する研究

Study in effects of acupuncture and transcutaneous electrical stimulations on structure of tibial articular cartilage in tail-suspended rats

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要旨

本研究は、低周波刺激装置を用いた経皮通電刺激（直流）と、従来の低周波装置（交流）による鍼通電刺激が、加重低減中の骨吸収に及ぼす抑制効果について比較検討することを目的とした。

７週齢のウィスター系雄性ラット32匹を用い、それらを後肢懸垂群HS、後肢懸垂・鍼通電刺激群EA、後肢懸垂・経皮通電群TEおよび対照群COの４群に分類した。CO以外の３群は、２週間尾部懸垂を行った。EAは大腿前面に鍼を刺入し、通電（条件：交流、幅250μsec、50Hz、0.24mArms）した。TEは大腿前面にパッドを貼り、経皮的に通電した（条件：直流、60V）。これらの通電刺激は10分／回、１回／日、６日／週とし、実験期間終了後、各群から大腿骨を摘出し、組織学的に分析した。

関節軟骨の中で石灰化層が占める割合は、HSがCOより有意に高かった。COに比べてHSでは軟骨小腔が減少し、他の群よりも配列が不規則であった。COでは中間層がサフラニンOで濃く染色されるが、深層でやや淡く染まった。HSでは深層に加え、中間層の下半分にまで染色性の低い領域が広がった。TEの染色性はCOに類似し、EAの染色性は低かった。

通電刺激は後肢懸垂による加重低減が引き起こす関節軟骨の構造変化に対して抑制的な効果をもたらすことが示唆された。さらに、この効果は鍼および経皮通電のいずれでも同様であることが理解された。

キーワード：後肢懸垂、通電刺激、組織構造