Study in effects of different periods of interventions with electrical stimulation on structural changes of tibial articular cartilage induced by hind-limb suspension in rats

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Summary

The purpose of this study was to histologically compare the effects of energization stimulation on structural changes in knee articular cartilage using rats with suspended hind limbs. As materials, 108 7-week-old Wister male rats were used and classified into a non-contact electrical stimulation group (VP), a hindlimb suspension group (HS), and a control group (CO). In the non-CO group, hindlimb suspension was performed during the experimental period of 1, 2 and 3 weeks. The energization stimulation conditions for VP were (AC 67V, 20kHz, 0.13mA), 30 minutes / day, and 5 days / week. After the end of the experimental period, the tibia of each group was excised and histologically observed. The total thickness of the articular cartilage was maintained in all groups, but was thin only in the HS group. Immunostaining for MMP-3 showed a positive reaction only in the HS

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group, and HS3 observed its localization in the superficial to intermediate layers, but these reactions were observed for CO and VP. It wasn't done. From this, it is considered that this phenomenon, in which the thickness of the cartilage is reduced after the substrate of the articular cartilage is destroyed by the hindlimb suspension, is suppressed by VP.

Keywords: tibia articular cartilage, hind-limb suspension, electrical stimulation

1. Introduction

Osteoarthritis (OA) is a chronic disease of joints, in which the cartilage tissue of the joints is degenerated and damaged, and the bones in the joints are deformed or proliferated, resulting in joint pain and dysfunction. Osteoarthritis (OA) is recognized as a serious problem worldwide and it affects the elderly.In modern Japan, the population over 100 years old as an index of a super-aging society is 72,000 as of June 2019 (male: 9,000, female: 62,000)[1)].

The etiology of knee osteoarthritis can be divided into primary and secondary. Primary is aging, exercise, obesity, genetic abnormality, secondary is ① biomechanical abnormality (trauma: fracture, meniscus injury, ligament injury), ② inflammatory disease (rheumatoid arthritis, purulent knee arthritis, etc.), It can be divided into (3) metabolic disorders (inflammatory diseases, bone system diseases, etc.) and (4) others (bone necrosis, osteochondritis dissecans, tumors, etc.).Statistics show that the incidence of osteoarthritis tends to increase with age and occurs more frequently in people over the age of 50.It is a degenerative and secondary disease that progresses to the joints.Lesions of cartilage peripheral tissue structure are a major pathological feature and one of the leading causes of joint pain and disability in middle-aged and elderly people.

Recently, there is no unified standard for diagnosis and prognosis of OA.
Clinical signs and imaging lack the ability for effective early diagnosis and lack sensitivity to early diagnosis and treatment of patients.During the course of the disease, various biomarkers change, the extracellular matrix is out of dynamic balance, articular chondrocyte apoptosis occurs, and articular cartilage structure and function change. Biomarkers can predict the onset and onset of disease and may be targets for OA treatment.

Therefore, articular cartilage is a special connective tissue that covers the surface that supports the weight of joints, and is composed of chondrocytes and extracellular matrix. Articular cartilage has no supply of blood vessels, nerves, or lymph, and its response to trauma and inflammation is mediated by cytokines secreted by chondrocytes and synovial

tissue.Chondrocytes are the only cells of adult hyaline cartilage and are capable of synthesizing and secreting highly specific cartilage matrix articular cartilage.It plays an important regulatory role in promoting chondrocyte proliferation, differentiation, apoptosis, and inducing matrix degradation.

2. Objective

Various exercise therapies for knee osteoarthritis are performed, but in addition, therapies using electrical stimulation are performed in clinical practice.Recently, it has been reported that acupuncture or percutaneous electrical stimulation is effective for bone loss caused by weight reduction.

However, there are few reports on the effect of percutaneous energization stimulation on the structural changes of articular cartilage associated with immobilization and weight reduction.On the other hand, it has already been confirmed by animal experiments that the energization stimulation using the recently developed vector potential generator (VP) is effective in maintaining the bone even for a short time of one day.But, it has not been clarified whether stimulation by VP is effective in maintaining the structure of articular cartilage.

The purpose of this study was, using hindlimb-suspended rats, to histologically investigate the effects of electrical stimulation with VP on structural changes of the tibial articular cartilage caused by mechanical unloading.

3. Materials and methods

3.1. Animals

One hundred and eight male rats (wistar strain, 7-week-old) were used as materials and they were divided into HS, VP and CO, and moreover, each group subdivided, by experiment periods, into HS1, HS2, HS3, VP1, VP2, VP3, CO1, CO2, and CO3.CO1, CO2 and CO3 were normally bred in the cages for 1, 2 or 3 weeks, respectively. Tails of HS1, HS2, HS3, VP1, VP2 and VP3 were suspended in cages for 1, 2 and 3 weeks, respectively.

Each rat was allowed to feed and water freely in the cage.All rats were preliminary bred for 3 days after delivery to acclimated to the breeding environment and their exercises in advance to the experiment.

After the experimental period, knee Joint in each group was excised and was analyzed histologically.

3.2. Methods

3.2.1. Hindlimb suspension

Fig. 1　tail suspension experiment

In the hindlimb suspension experiment, the rat hindlimb does not touch the ground because we want to reduce the weight on the rat tibial articular cartilage. First, the tail of the rat is wrapped with three octopus threads and the tape is fixed according to the method of Oishi[2]. After that, a wooden frame with a height of 20 cm was prepared, placed on a cage, and hung from a wire mesh ceiling. Then, it becomes as shown in Fig. 1.

3.2.2 Vector potential generator

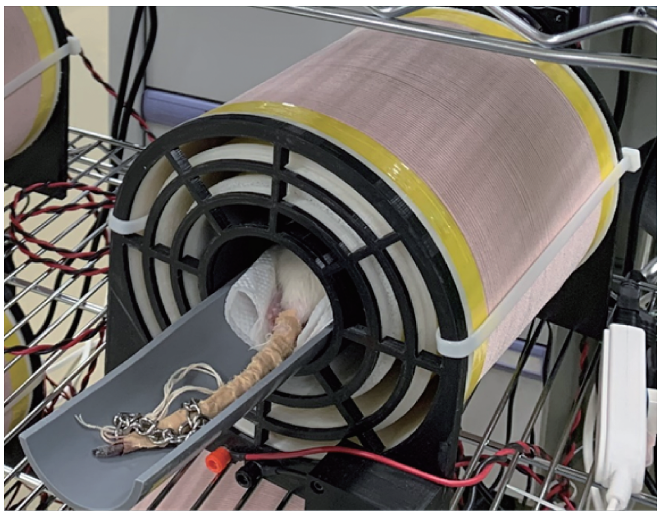

Fig. 2 Vector potential generator

The experiment was conducted under anesthesia using a VP generator (Sumida Denki Co., Ltd.).

The conditions of electrical stimulation of VP were 67V, 20kHz, 0.13mA AC, and were

set to 30 minutes / day, 5 days / week.

3.2.3. Sampling and fixation

Both groups were anesthetized and euthanized in rats after the end of the experimental period. After confirming the death of the rat, the soft tissue was removed and the tibia was removed. The tibia was sagittally truncated and fixed by immersion in 4% paraformaldehyde aqueous solution (4PFA) or Karnobsky (KAR).

3.2.4. Resin-embedded specimen

With 4PFA were dehydrated and permeable, and then rigolac2004: rigolac 70F (9: 1) and rigolac: BPO (1 g/ 100 ml resin) are stirred in a beaker and then embedded in the completed rigolac resin, weere polymerized at 38℃ , 45℃ , 55℃ and 60℃ for 24hr in a constant temperature container. The finished specimen was polished with a grindstone and then carefully polished with a film until the surface of the specimen was free of scratches. Then, after etching (1 minute) with 0.1N hydrochloric acid, toluidine blue (TB) staining was performed, and the observation was performed with an optical microscope.

3.2.5. Paraffin section

Specimens fixed with PFA were decalcified with EDTA, washed with water, and dehydrated. In the preparation of paraffin-embedded sliced specimens, the dehydrated sample was immersed in methyl benzoate, permeated through benzene, and then impregnated in paraffin at 60℃ and embedded in paraffin. Using the completed paraffin block, continuous sections with a thickness of 4 microns were prepared by microtome. The sections were stained with hematoxylin eosin (HE), safranin O fast green staining and immune tissue staining (IH) and observed with an optical microscope.

4. Results

4.1 Spongy bone structure

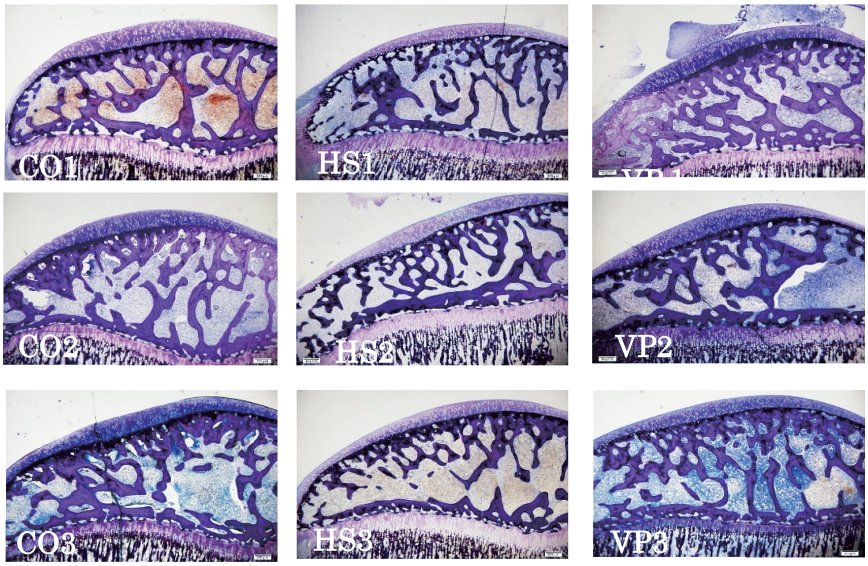

Fig.3 Low mfgnified image of knee joint in each group（Non-decalcified resin-embedded ground section, toluidine blue staining） Bar=1mm

Since the left side is anterior and the right side is posterior in each group, the trabeculae of the cancellous bone at the metaphysis of each group are basically arranged in the mesio-distal direction. (Fig. 3)

When observing the epiphyseal cancellous bone, the thickness of the cancellous bone for one week in the experimental period is close to that of the CO group in the HS group and the VP group, but the trabecula of HS3 is thin and sparse, and it is close to the CO group in VP3. It became a state. Observing the density of trabecular bones, VP1 is similar to that of the CO group, and many of them are connected. The density of VP2 and VP3 is less dense than that of VP1, but the thickness is the same as that of CO.

When HS1 and HS2 were observed in the anterior part of the epiphysis, the thickness of the trabecular bone was close to CO, but few were connected. In particular, the anterior trabeculae of HS3 were fairly sparse and none were connected.In VP2 and VP3, the trabecula was sparser than VP1 at the anterior part of the epiphysis, but the thickness was similar to that of CO (Fig. 3).

4.2 Structures of articiular cartilage

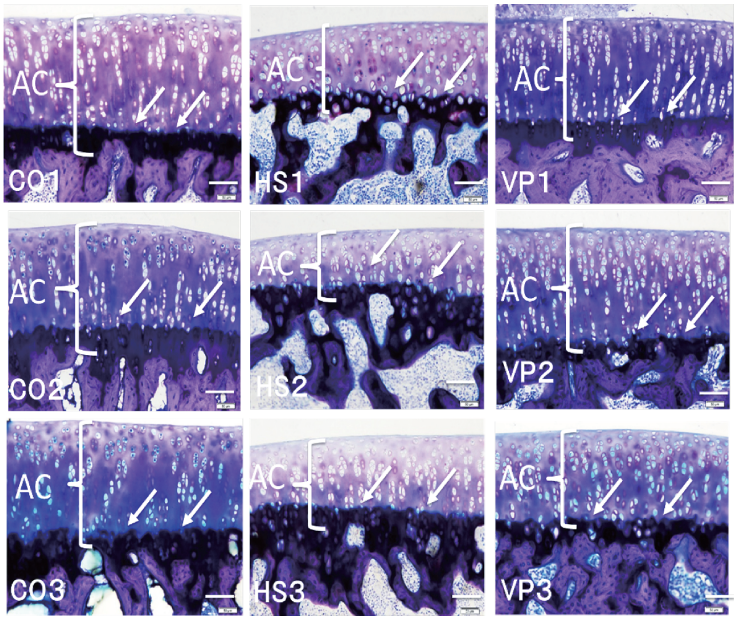

Fig.4 Comparison of thickness of whole articular cartilage and calcified layer in each group. (Non-decalcified resin-embedded ground section, toluidine blue staining)
Arrow: the position of the tide mark. AC: thickness whole of articular cartilage

When the articular cartilage is magnified and observed in the samples of the CO group and the VP group stained with toluidine blue, small and flat chondrocytes are densely present on the cartilage surface layer.The middle layer is slightly thicker than the surface layer, but the chondrocytes here are slightly larger than the surface layer and are spherical.The deep layer is considerably thicker than the previous layers, where the cells are columnar. The HS group has an irregular arrangement of chondrocytes.There was no difference in the density of the chondrocytes between the groups, but the size of chondrocytes was the largest in HS, followed by CO and VP were the smallest.(Fig.4)

When observing the stainability of the extracellular matrix of the CO group and VP group, it was dyed dark blue (dark blue) between the intermediate layer and the deepest layer of the articular cartilage, but the surface side of the articular cartilage caused metachromagy. , It was dyed reddish purple overall.HS1 was dyed slightly purplish red overall, but HS2 and HS3 were less dyeable. In addition, the stainability of HS decreased as the period became clearer.

Comparing the thickness of the calcified layer in each group, the HS group was considerably thicker than CO.The thickness of the calcified layer in the VP group was thinner than that in the HS group, which was similar to that in the CO group.Moreover,

in the CO group, metachromazy was strongly caused from the surface side to the deep layer of the articular cartilage, but when the HS group was observed, the metachromage was not caused in the articular cartilage. In particular, the substrate was clear on the surface side and deep part of the nodal cartilage of the HS group (Fig. 4).

4.3 Safranin 0 staining

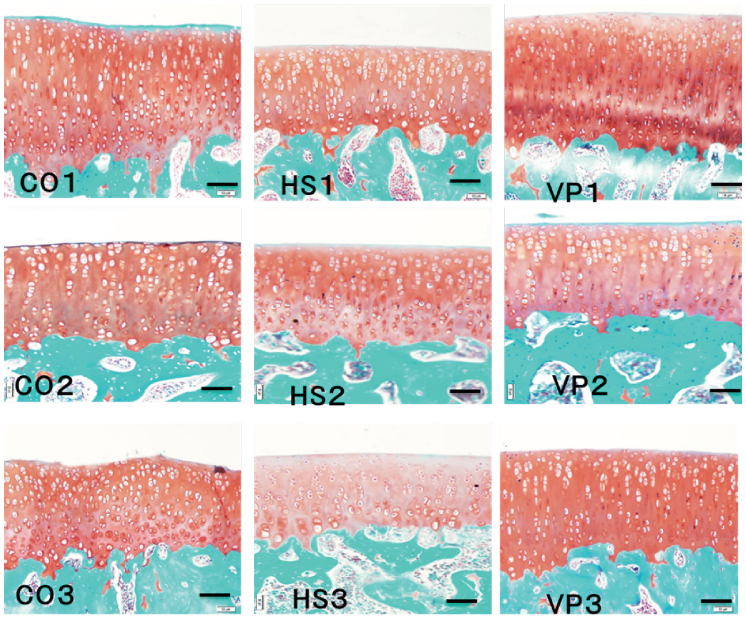

Fig.5 Comparison of the stainability of the articular cartilage in each group by Safranin 0 staining (Bar = 50 μm)

When observing the decalcified safranin 0-stained specimens, which comparing the total cartilage thickness of each group, it was thin only in the HS group.A layered structure of cartilage was observed from the morphology of chondrocytes in the CO group and VP group.

That is, it was possible to roughly classify cell layers such as the intermediate layer, the deep layer, and the calcified layer from the surface layer.The HS chondrocytes were irregularly arranged and the number of hypertrophied chondrocytes was high.In addition, safranin 0 stains cartilage matrix or proteoglycan.Compared with the staining power of the entire articular cartilage, CO stained the entire articular cartilage red, VP1 was close to CO1, VP2 stained the entire cartilage light red, and VP3 had a strong staining power. The HS1 intermediate layer was not dyed, or the dyeing power decreased from the HS2 intermediate layer to the deep layer, and the overall dyeing power of HS3 was weak. HS became less stainable as the period became clearer.

4.4 immunostaining of MMP-3

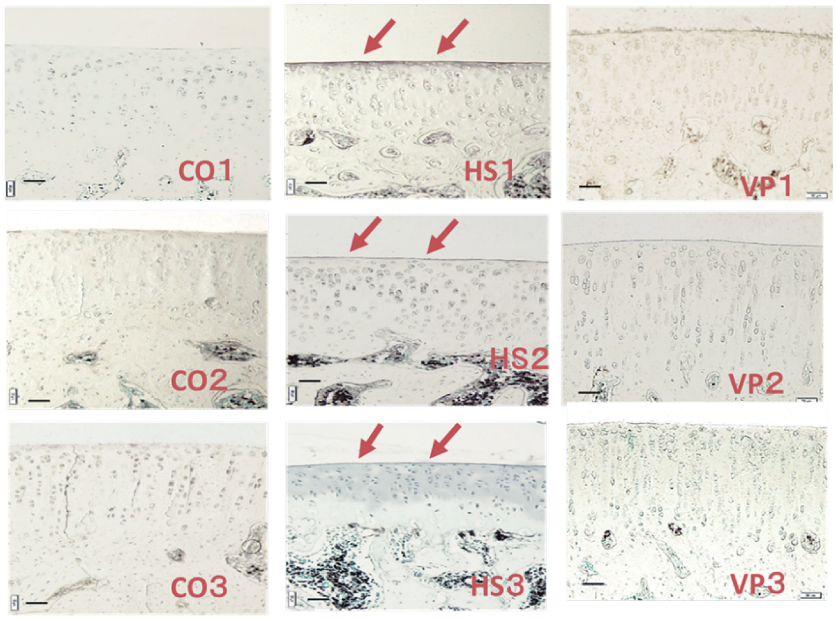

Fig.6 Comparison of the articular cartilage matrix immunostained with metalloproteinase-3 in each group (Bar = 50 μm)Arrow: Indicates where the MMP-3 reaction occurs

The results of immunostaining of MMP-3 showed positive reactions only in the HS group, and those reactions localized from superficial to intermediate layers of HS3. However, for CO and VP, those reactions were no longer observed from the CO1 and VP1 stages.

5. Test

In this study, we use rats with suspended hind limbs to verify whether non-contact energization stimulation has an effect of suppressing cartilage degeneration on the accompanying degeneration of articular cartilage.

First, changes in the trabecula of the cancellous bone at the epiphysis of the tibia were examined for the effect of weight reduction, and structural changes in tibial articular cartilage were observed. Below, we will consider based on the findings of the above.

5.1 Effect on spongy bone

Previous studies have shown that immobilization of the hind limbs of rats reduces the trabeculae of the epiphyseal cancellous bone [3].We also compared the effects of hindlimb immobilization and epiphyseal cancellous trabecular reduction of hindlimb suspension in

rats.As a result, it has been reported that the trabecula of the epiphyseal cancellous bone of the hind limb suspension is often reduced due to immobilization. [4)]

In this study, when observing the epiphyseal cancellous bone in each group, thick trabeculae were densely present in the CO group.When HS1 and HS2 were observed, the thickness of the trabecular bone was close to CO, but there were few connected ones.The trabecula of HS3 was thin and sparse. (fig.3)

This means that the longer the hindlimb suspension, the less weight was applied to the tibia.

However, when observing the epiphyseal cancellous bone of the VP group, VP1 was similar to that of the CO group, and many of them were linked. In VP2 and VP3, the trabecula was sparser than VP1 in the anterior part of the epiphysis, but the thickness became the same as CO.

In the VP group, the hind limbs were suspended as in the HS outside the energization stimulation time. It is considered that the reason why the thickness of the cancellous bone mass was maintained at the epiphysis of the VP group was related to the energization stimulation.

5.2 Effect on cartilage

Regarding articular cartilage, in articular cartilage, superficial cells secrete type I collagen fibers, which are thought to bring about smooth movement by arranging them almost parallel to the surface of articular cartilage.

In addition, cells in the middle layer are said to secrete type II collagen fibers and aggrecan, which are very important structures in articular cartilage [6)] .On the other hand, according to a study in which joint degeneration was observed using a knee joint model due to immobility due to knee joint fixation and immobility due to tail suspension, the surface layer of articular cartilage was exfoliated, and middle and deep layers of necrosis and emptying appeared. Articular cartilage table Spindle-shaped cell proliferation and non-thinning in the layer, loss of chondrocytes in articular cartilage and cartilage matrixReported a defect[7)8)].

The chondrocytes of the HS group observed in this study were spherical and irregularly arranged, but the CO chondrocytes were flattened into a spindle-shaped shape and arranged in columns.VP group became almost similar to CO.From these facts, it is inferred that the amount of fibers in the extracellular matrix of the HS group is small, and that the cells are regularly arranged in the CO group and the VP group due to the dense

presence of fibers.On the other hand, it has been reported that the calcified layer rises in both tail suspension and hindlimb immobilization. [9,10,11] The rise of this calcified layer is a reduction in the weight on the tibia.In this study, comparing the thickness of the calcified layer of articular cartilage in each group, the thickness of the calcified layer was thicker only in the HS group. The results regarding the thickness of the calcified layer in this study are the same as those in the previous studies [9,10,11].

In addition, the thickness of the calcified layer of the VP group was thinner than that of HS, which was almost the same as that of CO.With respect to the thickness of this calcified layer, an increase in the thickness of the calcification is consistent with an increase in the tide mark, which means a decrease in the uncalcified layer above the articular cartilage.The situation observed in the HS of this experiment was not observed in the VP group.It is considered that this is related to the suppression of the excessive increase in the thickness of the calcified layer by the energization stimulus by the non-contact energization stimulus.

On the other hand, when observing the trabeculae of the epiphyseal cancellous bones of each group, thick trabecular bones were densely present in the cancellous bones of the CO group. The trabecular bones of HS were thin and sparse, and the density of the trabecular bones decreased, especially in the anterior part of the epiphysis.This result was the same as that of KIRA [12]et al.In VP2 and VP3, the trabeculae were sparse at the anterior epiphysis, but many of them had a thickness close to CO.

Articular cartilage has no blood vessels or nerves, so the synovial fluid secreted from the synovium nourishes the articular cartilage and is pumped by joint movement, allowing the synovial fluid to move from the articular cartilage surface into the cartilage matrix. Promotes the movement of nutrients [13].It has been reported that HS has a reduced compression force from the femur and a reduced pumping effect on articular cartilage[14].

Therefore, it is speculated that the rats in HS state lacked the amount of nutrients to penetrate, which would have caused the loss of bone mass in the anterior epiphysis. Articular cartilage has already been shown to decrease in thickness with growth and aging [15) 16)] It has also been reported that such changes are promoted by immobilization of limbs [17)18)].

Observed in this study, in the central part of the articular cartilage where the femur and tibia are in strong contact with each other in the knee joint, when comparing the total thickness of the cartilage in each group, it was thin only in the HS group.Looking at the stainability of the extracellular matrix in the toluidine blue-stained resin-embedded

polished specimen, the entire articular cartilage was deeply dyed in toluidine blue in the CO group. In HS1, the superficial and intermediate layers of articular cartilage were lightly stained, and in HS2 and HS3, the stainability was low.In the VP group, it gradually approached the state of CO.

Regarding the mechanism of thinning of cartilage, Nakama et al.[19] cites a decrease in the ability of chondrocytes to divide, and Kubo et al.[20] argues that the cause is a decrease in the ability of chondrocytes to synthesize substrates.Chondrocytes synthesize and secrete proteoglycans and collagen fibers. Collagen fibers are involved in the strength and elasticity of articular cartilage.Proteoglycans bind to collagen fibers to retain water abundantly and have elastic properties for sliding and shock absorption [21]From this, it was confirmed that the cartilage thickness was reduced and the proteoglycan was also reduced in this study.As with ZENG et al[22], The thickness of articular cartilage is deeply related to the amount of extracellular matrix such as collagen fibers and proteoglycans in the cartilage, which may be influenced by the activity of chondrocytes.

In this study, the articular cartilage of each group was observed with safranin O staining.Articular cartilage is divided into superficial, intermediate, deep and calcified layers according to differences in cell size, shape and arrangement.[23] Superficial chondrocytes resembled small, flat fibroblasts.Chondrocytes in this layer secrete lubricinno PRG4, a glycoprotein rich in free-like structures, enabling lubrication of joint movements.The cells in the middle layer are elliptical and spherical, often in columns.It has also been reported that cells in this layer secrete type II collagen fibers and atypical substrates, especially chondroitin sulfate, and are involved in the formation of basic and important components of cartilage.[24] There are spherical cells in the deep layer, which are thought to secrete type X collagen fibers and keratan sulfate, especially among amorphous substrates.[24]

In the observations in this study, the layered structure of cartilage was observed from the morphology of CO and VP chondrocytes.That is, although it was possible to roughly classify the cell layers such as the intermediate layer, the deep layer, and the calcified layer from the surface layer, the HS chondrocytes were irregularly arranged and the number of hypertrophied chondrocytes was large.This may mean that HS had less extracellular matrix than the other groups[25].

In this study, compared to the overall staining power of articular cartilage, CO dyed the entire articular cartilage red, VP1 was close to CO1, VP2 dyed the entire cartilage light red, and VP3 had strong dyeing power.The HS1 intermediate layer was not dyed, the

dyeing power decreased from the HS2 intermediate layer to the deep layer, and the overall dyeing power was weak for HS3.HS became less stainable as the period became clearer.In the HS group, the decrease in stainability in the middle and deep layers is considered to be due to the decrease in proteoglycan.The amount of proteoglycan secreted into articular cartilage is low in the superficial and calcified layers and high near the cells in the middle and deep layers. It is also reported to be strongly affected by aging and the degree of cartilage degeneration.[24)]

Proteoglycans have the ability to respond to changes in cartilage load and friction from external forces. In this study, the proteoglycan of rats with suspended hind limbs was significantly affected, suggesting that the elasticity of articular cartilage may be reduced by reducing the weight.The VP group had the same hind limb suspension as the HS group, but there was no such situation.From this, it is considered that the VP group suppresses the decrease of proteoglycan.Also, CO1, CO2, VP1 and VP2 are dyed red, while the deep layers are dyed pale purple.It is considered that type X collagen fibers are densely present in the deep layer.

On the other hand, it has been reported that degrading enzymes are involved in the destruction of cartilage matrix [26)].Many facts have been revealed about a group of enzymes involved in articular cartilage matrix destruction, namely matrix metalloproteinase (MMP) in joint diseases, and these groups of enzymes play an important role in joint destruction. It was shown to be fulfilling [27)].Among MMPs, matrix metalloproteinase-3 (MMP-3; stromelysin-1) is typical of cartilage destruction.[28)] MMP-3 secreted from synovial tissue and chondrocytes (mainly cells on the surface of synovial tissue) destroys the cartilage matrix proteoglycan (aglican) [29)] Compared with MMP-3 immunostaining of articular cartilage in this study, HS1 and HS2 caused a reaction of MMP-3 on the cartilage surface layer, and when it became HS3, MMP-3 was stained up to the intermediate layer.

As mentioned above, articular cartilage is avascular and insensitive. Therefore, the main sources of nutrients, humoral factors, and oxygen to cartilage are only infiltration from synovial fluid secreted by synovial cells and present in small amounts in the joint cavity.[30)] It has also been reported that MMP-3 is mainly produced in the synovial joint.[31)] The synovial fluid secreted from the synovium acts as a lubricant, moisturizing the surface of the joint and smoothing the movement, but when inflammation occurs, the synovium proliferates and erodes bone and cartilage.From this, it is inferred that MMP-3, which contains synovial fluid, erodes from the cartilage surface because MMP-3 also

extends from the superficial layer to the intermediate layer as the age of the HS group increases.On the other hand, there was no change in the CO group and the VP group.

6. Conclusion

It was suggested that the destruction of cartilage matrix and the increase in the thickness of the calcified layer of the articular cartilage induced by mechanical unloading could be suppressed from the initial stage of the electrical stimulation by VP.

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異なる非接触性通電刺激の時間および期間が後肢懸垂ラットの骨および関節軟骨の構造に及ぼす影響

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要旨

本研究は、ラットを用いて後肢懸垂したラットを用い、非接触通電刺激を行う。それに伴う膝関節軟骨の構造変化に対する、通電刺激の影響について組織学的に比較、検討することを目的とした。材料として、７週齢のウィスター系雄性ラット108匹を用い、それらを非接触通電刺激群（VP)、後肢懸垂群（HS）および対照群（CO）に分類した。VPでは、ラットはベクトルポテンシャル（VP）発生装置の中に入れ、通電刺激以外の時間で両後肢懸垂した。HSはケージ内に後肢懸垂状態におき、COは正常飼育した。いずれの群の実験期間も1，2および3週間とした。VPの通電刺激条件は（交流67V、20kHz、0.13mA）で、30分/日、5日/週とした。実験期間終了後、各群の脛骨を摘出して、組織学的に観察した。

関節軟骨全体の厚さは、いずれの群では軟骨の厚さを維持されてるが、HS群のみ薄かった。各群の骨端海綿骨を観察すると、いずれCO群の海綿骨では太い骨梁が密に存在した。HS ３の骨梁は細く疎でが、HS １とHS2の骨梁はCOに近い状態になった。VP1とVP2は骨梁が骨端前方部で疎となっていたが、太さはCOに近いものが多く存在した、VP3はCOとほぼ同様になった。サフラニンOの染色性染色の結果は、HSの関節軟骨その染色性が低い傾向にあり、特にHSではHS3の関節軟骨浅層および中間層ではその染色性がほとんど消失していた。MMP-3の免疫染色では、HSの群にのみ陽性反応が示されており、HS3では浅層か

ら中間層にその局在が観察されていた。しかし、COおよびVPに関しては、それらの反応はCO1およびVP1の段階からすでに認められていなかった。このことから、後肢懸垂によって、関節軟骨の基質破壊が行われた後、軟骨の厚さを薄くなる、この現象はVPによって抑制されたと考えられる。

キーワード：脛骨関節軟骨、後肢懸垂、非接触性通電刺激