Effects of different forms of exercise on structures of tibial articular cartilage in rats

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Summary

In this study, we investigated the effects of a treadmill walking and a passive movement with unloading on the structures in articular cartilage. Thirty-two male wistar rats (7-week-old) were used as materials, and they were randomly divided into a tail-suspended group (HS), tail-suspended and a treadmill walking exercise group (WE), a tail-suspended and passive exercise group (PE), and a control group (CO). In HS, WE and PE, their tail were suspended in the cage for 3 weeks. Moreover, WE and PE performed the treadmill walking and PE performing passive exercise, 1 hour / day, 5 days /week, for 3 weeks, respectively. After the end of the experimental period, tibias were excised and analyzed.

A size of the chondrocyte was largest in TS, followed by WE, and CO and PE were smaller. Clear metachromasia was found at from an intermediate and a deep layer of the articular cartilage of the matrix in CO, but a same stainability was observed at only narrow area in TS. When observing a surface of the articular cartilage divided by a scanning electron microscope, the intermediate layer of CO was smooth and WE and PE showed a similar state, but the surface of that was rough and the matrix fibers existed at there in TS. From these facts, it was suggested that the structural changes of the articular cartilage caused by a loss of the mechanical stress could be suppressed by the treadmill walking and the passive movement.

Keywords: tibia articular cartilage, tail suspension, walking, passive movement

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1. Introduction

An exercise therapy is generally used to delay the progression of symptoms as much as possible and to improve the symptoms of a knee osteoarthritis. The exercise therapy is classified into a spontaneous exercise (resistance exercise, walking, bicycle ergometer exercise, etc.) and a passive exercise (continuous passive exercise, pendulum exercise, etc.).

There are many studies [1-7] concerned to the continuous passive movement, and it has shown that the movement is useful to promote a synovial circulation and improve the symptoms of arthrogryposis[8]. However, the studies of the passive motion focus mainly on the study by X-ray images, and the histological changes in cartilage by that motion has not fully cleared. The purpose of this study was to compare and investigate the effects of the passive and spontaneous movements on the structural changes in the articular cartilage of the knee of the hindlimbs due to tail-suspension in rats.

2. Materials and methods

2.1. Materials

Thirty-two male rats (wistar strain, 7-week-old, 252～370g) were used as materials, they were divided randomly into three groups (a hindlimb-suspended group: HS, an exercise group: EX and a control group: CO).

Tails of HS, WE and PE were suspended from the ceiling of cage for three weeks. Furthermore, WE performed a treadmill-running and PE performed a passive-exercise. Rats in CO were fed normally in the cage during the experimental period. All rats had a preliminary breeding period of 3 days after being carried, and were acclimated to the breeding environment and each exercise in advance.

2.2. Methods

2.2.1. Hindlimb suspension

In the tail-suspension experiment, first, a 20cm-height wooden frame was prepared and placed on the cage, and a wire mesh ceiling was set on it, in order to hang the tail from the ceiling of the cage, in TS. The rat's tail was wrapped with three tachymeter wires, was attached a chain, and then, was hung on the ceiling of the cage so that the hind limbs did not touch the floor of the cage. As hind-limb movement restricted due to tail suspension in HS, WE, and PE, the positions of the feed box and water bottle was adjusted to drink water and eat food freely.

2.2.2. Passive movement experiment

Power-driven fly-wheel

We have designed a device that allows rats to flex and extend their knees without loading. A rotary motion of a power-driven fly-wheel is converted motion of the calf into the anterior and posterior, and as a result, the rat was able to perform a flexion and extension motion of a knee joint. (Fig.1)

An arm of a passive-exercise device is fixed on the ankle of the hindlimb of the rat and a passive-exercise was performed under anesthesia. The passive-exercise was performed under the following conditions: 80 times/minute every day, 1 hour/day, 5 days/week, for 3 weeks.

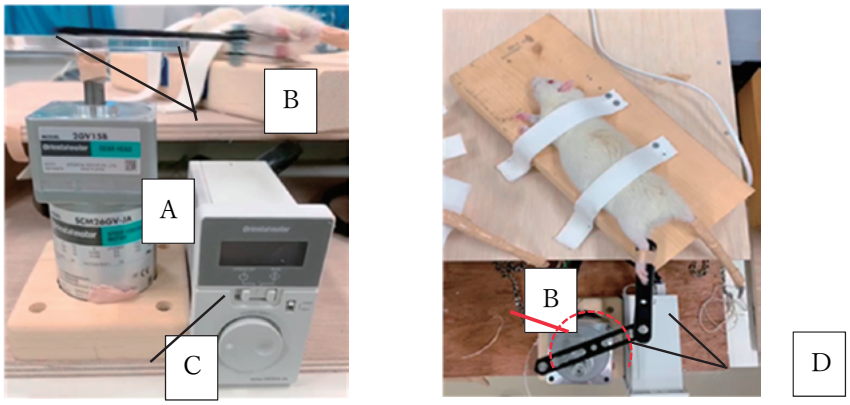

Fig.1. Composition of a passive-exercise device

Left: A passive-exercise device, Right: a practice using that device

A. a rectifier and voltage converter　　B.　a fly-wheel (transparent acrylic board)

C. a speed controller　　D. arms of a passive-exercise device

2.2.3. Treadmil running

WE performed the treadmill walking of an inclination angle of 10° and a speed of 10 m/min for 1 hour/day, 5 days/week for three weeks.

2.2.4. Sampling and fixation

After the experimental period, the rats were deeply anesthetized and euthanized. After the death was confirmed, the soft tissues other than the joint capsule were removed from the knee joint, and the hind limbs were removed and Divided in the sagittal direction. Then, immediately immerse it in a 4% paraformaldehyde aqueous solution (PFA) or Karnovski (KAR) and fix it. The PFA-fixed specimens are used as rigolac embedding in resin sample,

and the KAR-fixed specimens are used to prepare scanning electron microscope (SEM) .

2.3. Preparation and observation of histological specimens

The sample fixed with PFA is dehydrated and penetrated, and then immersed in resin. After imbedding and heated to the polymerization, use the block to carefully polish. The specimen was etched with 0.1N hydrochloric acid (1 min), stained with toluidine blue (TB), and observed with an optical microscope. The sagittal cleavage specimens fixed with KAR were grind until the articular capsule and articular cartilage were exposed on the surface. Then the specimen was treated with sodium hypochlorite, dehydrated, freeze-dried and vacuum deposited with carbon and platinum to vacuum deposition, and then observed with SEM.

3. Results

The meniscuses were recognized in the anterior and posterior parts of the joint, as observing a specimen cut in the sagittal direction at the central part between medial and lateral sides of proximal part of tibia. The meniscus was thinner in the central part between the anterior and posterior parts of the epiphysis of the tibia, and the surfaces of the epiphysis the femur and the tibia bone are very close at there. The articular cartilage is the thickest in the anterior-posterior central part, and becomes thinner toward the front or rear part, but is thicker in the posterior part than the front part. On the other hand, the articular cartilage was thickest in that part. But, the thickness of that cartilage became thinner toward the anterior or the posterior parts, and a decrease in that thickness especially in the anterior part. (Fig.2)

When magnifying and observing the sections of the articular cartilage that was stained by toluidine-blue dye, the chondrocytes in each group arranged in the perpendicular direction approximately toward the surface of the articular cartilage. There was no difference in the density of the chondrocytes between the groups, but the size of chondrocytes was the largest in TS, followed by WE, and CO and PE were the smallest. (Fig. 3)

When observing the stainability of the intercellular matrix, the deepest layer of the articular cartilage (here in after referred to as the calcified layer) was stained in dark blue, but the surface side of that cartilage (here in after referred to as uncalcified) caused a metacromasy and was stained color of a reddish purple as a whole.

However, when comparing the ratio of the thickness of the calcified layer to the whole thickness of the articular cartilage between groups, TS is significantly higher than CO. In

addition, the calcified layers of WE and PE were thinner than TS, and they were close to CO. Regarding to the shape of the boundary between the calcified and the uncalcified layer, many small irregularities were observed in each group. In CO, obvious metachromasy is recognized from an intermediate layer to a deep layer of the articular cartilage, but in TS, that width was narrow, and the stainability of the matrix in superficial and deep layers were light (Fig.3).

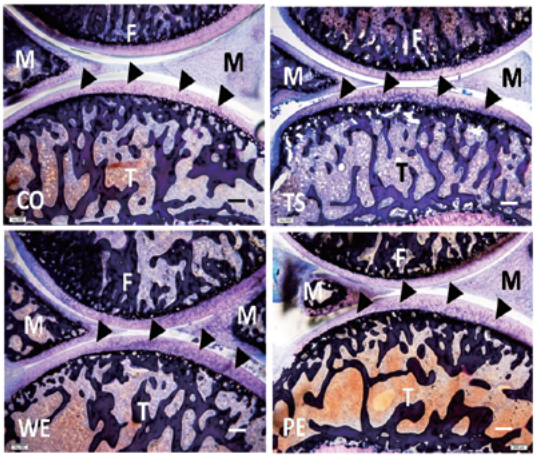

Fig.2. Low magnified image of the knee joint in each group
Undecalcified、embedded in resin and ground sections,. Stained by toluidine-blue dye,
F: Femur, T: Tibia, M: meniscus, Arrow heads: articular cartilage of tibia, Left and Right sides in each image is anterior and posterior direction, respectively

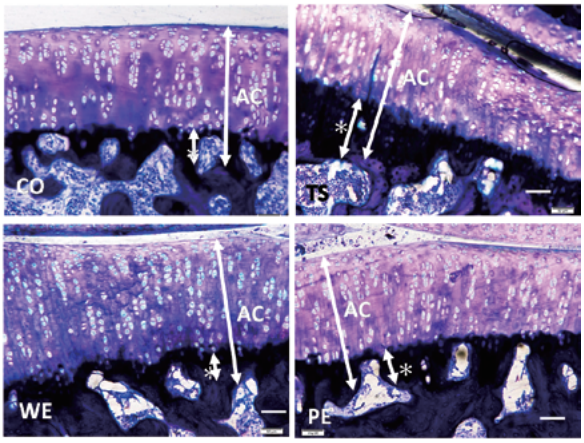

Fig.3. Ratio of calcified layer in the articular cartilage
Undecalcified and ground sections, staining with toluidine-blue dye, Bar=50μm
AC: Whole layer of the articular cartilage, ＊：Calcified layer of the articular cartilage

The polished surface of the sagittal fracture articular cartilage was treated with hypochlorite, and the surface was observed with a scanning electron microscope.

In CO, the surface of the unmineralized articular cartilage layer was dissolved by hypochlorite, and a depressed state was observed, but the calcified layer was not dissolved, and the interface between the two layers was clearly visible.

Comparing the length from the surface of the articular cartilage to the interface between the two layers in other groups of specimens treated similarly, WE and PE are similar to CO, but TS is significantly shorter. (Fig.4)

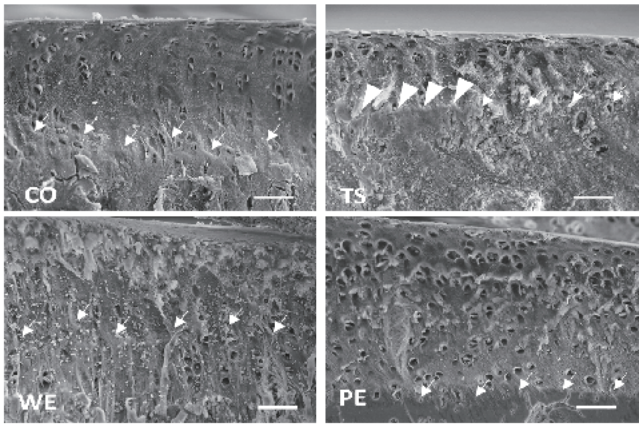

Fig.4. Structure of each layer of the articular cartilage

SEM images of the specimen that were divided in sagittal direction and were treated by a sodium hypochlorite. Bar=50μm Arrows: Uneven boundary between uncalcified and calcified layers. Arrow heads: Less uneven boundary between uncalcified and calcified layers.

When the specimen treated with hypochlorite by the same method as in Fig. 4 is magnified and observed by SEM, first, in the case of the intermediate layer, a cartilage cavity is observed on the CO surface of the specimen, but is in phase with the intercellular matrix It is flatter than it. On the other hand, in TS, a bundle of thick matrix fibers extending in the vertical direction is exposed in the articular cartilage, and fine fibers arranged in a network are observed between them. In WE and PE, fine fibers arranged in a network on the intercellular matrix can also be seen, but compared with TS, they are few. Especially in PE, smooth parts such as CO are widely present. (Fig. 5)

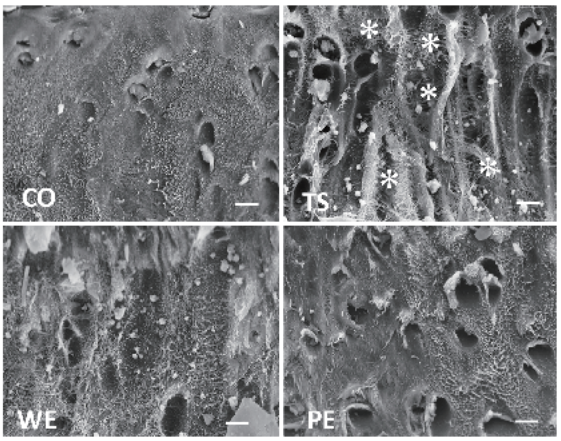

Fig.5. Structures of the intermediate layer of the articular cartilage in each group
Magnified SEM images of the specimen that were divided in sagittal direction and were treated by a sodium hypochlorite. Bar=10μm ＊： areas that the matrix fibers were observed clearly

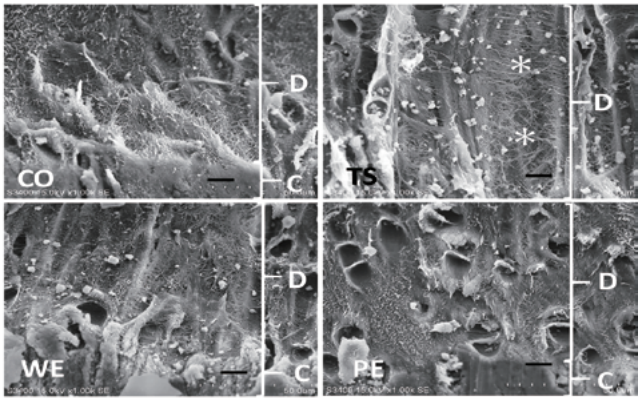

Fig.6. Structures of the intermediate layer of the articular cartilage in each group
Magnified SEM images of the specimen that were divided in sagittal direction and were treated by a sodium hypochlorite. Bar=50μm
＊： areas that the matrix fibers were observed clearly
D: deep layer, C: calcified layer

In all the areas below the deep layer close to the calcification layer, each group of thick fiber bundles extending vertically in the articular cartilage and thin fibers arranged in a network. The lower ends of the thick fiber bundles arranged in the vertical direction are buried in the matrix of the calcification layer. However, the fine fibers of CO, WE, and PE are buried in the deep substrate, and the stump of the fiber appears on the surface of the fracture surface from the etching. On the other hand, in TS, the structure of the filled fibers disappeared, and fine fibers extending laterally rather than in a network shape were clearly

observed. (Fig. 6)

4.discussion

This study aims to reduce the weight of the hind limbs of rats and examine whether treadmill walking and passive exercise can inhibit the changes in articular cartilage and joint capsule that accompany it. The thickness of the entire articular cartilage decreases with age. However, there are reports that the thickness of articular cartilage has increased due to weight loss [9] On the other hand, there are also reports that the cartilage thickness has not changed due to immobilization [10] . In this study, no differences were found between the two groups at the end of the experimental period. The intercellular matrix of cartilage contains fibrous components and proteoglycans, which are atypical substrates. The thickness of articular cartilage is closely related to the content of extracellular matrix such as collagen fibers and proteoglycans in the cartilage, which may be affected by the activity of chondrocytes. In this study, the following shows that TS has the possibility of reducing proteoglycans. High-viscosity proteoglycans provide elasticity for articular cartilage, and collagen fibers can resist the aggravation from external forces after forming bundles.

It has been shown that the chondrocytes of the growth plate become smaller as they grow and similar changes are observed with a mechanical loading by exercise for a certain period of time[11]. In this study, there was no difference in of chondrocyte density between the groups, but the chondrocyte size was the largest in TS, followed by WE, and CO and PE were smaller than those. Takahashi's report[11] shows the effect of increased the mechanical loading due to exercise on the growth plate, and is consistent with this study in examining the effect of increased that loading on chondrocytes. In the case of the epiphyseal plate, chondrocytes also line up to form cell columns. It seems that the miniaturization of chondrocytes by exercise is due to the increase in extracellular matrix between those cell columns. The chondrocytes in TS were larger than the other groups and It was supposed that this may mean that the extracellular matrix decreased compared to the other groups.

When the stainability of the extracellular matrix was observed in the toluidine blue-stained resin-embedded polished specimen, CO strongly showed metachromagy from the middle to the deep layer of the articular cartilage. However, in TS, the region showing such stainability was narrow, and the stainability of the surface and deep sides of the articular cartilage were low. From this, it was speculated that proteoglycans are present only in a limited range of the intermediate layer in TS.

In this study, a divided surface of the specimen was ground, further treated with a hypochlorous acid, and the intermediate layer was observed by magnifying with SEM. The divided surface of the intermediate layer of CO was relatively smooth, but in TS, that surface was rough, a bundle of thick matrix fibers running in the vertical direction was exposed at that face, and at the same time, many fine fibers formed a network were also observed.

It was found that, thin fibers formed the fine network are also observed in the extracellular matrix of WE and PE, but in TS, especially in PE, the exposure of the bundle of the thick fibers was little, as same as CO. In this way, many matrix fibers were clearly observed only in TS, which is considered to indicate that proteoglycan decreased in this group. As judging from resistance to sodium hypochlorite treatment, it was supposed that the proteoglycans in articular cartilage are significantly reduced by tail-suspension, but may be maintained to some extent by treadmill gait and passive movement.

It has been shown that the reason why the chondrocytes were large and their arrangement was irregular in the articular cartilage of hindlimb-immobilized rats was because the matrix decreased and as a result became the area occupied by the cells was relatively wide11). It is considered that the mechanical load on the articular cartilage was reduced due to the tail-suspension and this led to the decrease in the matrix synthesis of chondrocytes and further destroyed / removed of the matrix.

It has been reported that the calcified layer rises in both tail-suspension and hindlimb immobilization12,13). What those experimental conditions have in common is the reduction of the mechanical loading on the articular cartilage. Comparing the ratio of the thickness of the calcified layer to the total thickness of the articular cartilage in this study, that of TS is considerably higher than CO, and the data on the thickness of the calcified layer in this study supported the results of previous studies[12,13]. Moreover, the calcified layers of WE and PE also were thinner than TS, which was close to CO. Regarding the thickness of this calcified layer, as likely in the case of the extracellular matrix in the uncalcified layer described above, it has been showed that the mechanical loading by the walking exercise and the passive exercise suppressed the excessive increase in the thickness of the calcified layer.

The boundarye between the uncalcified and the calcified layers is called the tidemark, which means the calcification front[14]. It has been reported that ectopic calcification of tendons and ligaments progresses in the elderly [15,16]. Since the amount of activity decreases with aging, it is speculated that the immobilization and the accompanying load-reduction

have some effect on the promotion of substrate calcification.

It has been shown that the arrangement and the type of collagen fibers in the articular cartilage change as they grow, and type X collagen appears from the deep layer[17,18]. In this study, when the area below the deep layer of articular cartilage was magnified and observed by SEM, thick fiber bundles running vertically in the articular cartilage and thin fibers arranged in a network were observed in all groups. However, the fine fibers of CO, WE and PE were embedded in the substrate and the ends of the fibers appeared on the surface. On the other hand, in TS, the matrix that embedded the fibers disappeared, and thin fibers arranged transversally rather than reticular were clearly observed. It was thought, as judging from their location, that the thick fiber bundles in the deep layer collagen fibers may be type X collagen fibers involved in calcification[19]. This thick fiber bundles arranged vertically in the articular cartilage. From such a structure, it is considered that the lower end of the thick fiber is firmly supported by the calcified layer and the whole layers of the articular cartilage plays a role of providing resistance to the mechanical load. In the TS of this experiment, the calcified layer became thicker, but the unevenness of the boundary with the intermediate layer was not much different from that of CO, WE, and PE. The tail suspension, unlike the immobility procedure, allows flexion and extension of the knee joint. Therefore, in TS, unevenness was maintained at the interface similar to CO, and it seems that this was the same situation in WE and PE for the same reason. As mentioned above, there was no difference between the groups concerned to whole thickness of articular cartilage. However, in CO, proteoglycan that is the extracellular matrix was maintained but decreased in TS. Moreover, the proteoglycans were also maintained in WE and especially PE. The thickness of the calcified layer was thick only in TS and thin in the other groups and the matrix fibers were maintained in all groups. It was supposed that this may have been involved in maintaining the thickness of the articular cartilage in each group.

It was speculated that the articular cartilage might may be more likely to have lower elasticity than strength Since the effect of load reduction has a greater effect on proteoglycans than on substrate fibers. It was speculated that articular cartilage may cause to decrease in elasticity rather than strength, as the effect of mechanical load reduction has a greater effect on proteoglycans than on matrix fibers.

5. Conclusion

It was suggested that the structural changes of the articular cartilage caused by a loss of the mechanical stress could be suppressed by the treadmill walking and the passive

movement.

6. Committee of Animal Experiment and Ethics

This study was approved by Committee of Animal Experiment and Ethics for the research, Graduate School of Human Life Design, Toyo University.

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ラット脛骨関節軟骨の構造に及ぼす自動および他動運動の影響

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要　旨

研究はラットを用い、尾部懸垂に伴う加重低減によって引き起こされる関節軟骨の構造変化に対して、軽度または無負荷に近いトレッドミル歩行または他動運動がその変化を抑制し得るか否かについて検討した。材料として7週齢のウィスター系雄性ラット32匹を用い、それらを無作為に尾部懸垂群(HS)、トレッドミル歩行運動群(WE)、他動運動群(PE)およびコントロール群(CO)に分類した。COはケージ内で3週間正常飼育した。それ以外の3群はケージ内で尾部を3週間懸垂し、さらにWEにはトレッドミル歩行を、また、PEには他動運動を行行った。いずれの運動も1時間/日、5日/週、3週間実施した。実験期間終了後、脛骨を摘出して、組織学的に観察した。軟骨細胞の軟骨細胞の大きさはTSが最も大きく、次いでWEとなり、COおよびPEはそれらより小さかった。基質に関しては、COでは関節軟骨の中間層から深層がメタクロマジーを強く起こしたが、TSではそのような染色性を示す範囲は狭かった。次亜塩素酸処理した標本の中間層の割断面をSEMで拡大して観察すると、COの中間層は滑沢で、WEおよびPEもそれに近い状態にあったが、TSでは基質線維が露出した。これらのことから、関節軟骨の細胞外基質の構造に関しては、加重低減に伴う変化がトレッドミル歩行および他動運動で抑制される可能性があることが示唆された。

キーワード：脛骨関節軟骨、後肢懸垂、歩行および他動運動