Physiological Reloading Recovers Histologically Disuse Atrophy of the Articular Cartilage and Bone by Hindlimb Suspension in Rat Knee Joint

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Abstract

Objective. This study aimed to clarify physiological reloading on disuse atrophy of the articular cartilage and bone in the rat knee using the hindlimb suspension model. *Design.* Thirty male rats were divided into 3 experimental groups: control group, hindlimb suspension group, and reloading after hindlimb suspension group. Histological changes in the articular cartilage and bone of the tibia were evaluated by histomorphometrical and immunohistochemical analyses at 2 and 4 weeks after reloading. *Results.* The thinning and loss of matrix staining in the articular cartilage and the decrease in bone volume induced by hindlimb suspension recovered to the same level as the control group after 2 weeks of reloading. The proportion of the noncalcified and calcified layers of the articular cartilage and the thinning of subchondral bone recovered to the same level as the control group after 4 weeks of reloading. *Conclusions.* Disuse atrophy of the articular cartilage and bone induced by hindlimb suspension in the tibia of rats was improved by physiological reloading.

Keywords

Introduction

Physiological joint loading is one of the essential factors for maintenance of the functional integrity and metabolism of the articular cartilage.[1-3] The articular cartilage has a high sensitivity to these mechanical signals and adaptive response to these stimuli, such as dynamic compression, fluid shear, tissue shear, and hydrostatic pressure.[3-5] This functional process is referred to as mechanotransduction and chondrocytes quantitatively regulate the rate of matrix synthesis and degradation and change the composition of the extracellular matrix.[3] With moderate loading, the articular cartilage thickens as the surface area increases, and the matrix content increases.[5-8] Conversely, many basic studies in animals and humans reported that in the absence of load, the articular cartilage undergoes histological changes, such as thinning, decreased matrix staining, and subchondral bone expansion.[1,2,5,6,9-15] Vincent *et al.* proposed such histological changes due to lack of mechanical stress, such as loading, as cartilage atrophy.[2] In clinical practice, many patients may be affected by articular cartilage atrophy because they often require bed rest, which is temporarily associated with treatment.[11] Our previous study also reported that unloading conditions for 4 weeks by hindlimb

suspension caused significant thinning and decreased matrix staining of the articular cartilage in the tibia of the knee using the rat model.[16,17] Moreover, in the animal study, disuse atrophy of articular cartilage is a pathological condition that can lead to early-onset and severe osteoarthritis (OA).[17] The incidence of OA is increasing worldwide, making it a major medical and economic problem.[18-20] Therefore, the treatment of disuse atrophy of the cartilage is an important issue to be clarified in orthopedics, rehabilitation medicine, and physical therapy.

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In the skeletal muscle, it is widely known that inactivity causes disuse atrophy[21-23] and that reactivation improves atrophy.[24-26] In contrast, there are no previous studies on its treatment and recovery, and the treatment and recovery processes for disuse atrophy of the articular cartilage are unclear. Owing to the absence of blood vessels and dependence on nutrition from the synovial fluid, it is traditionally believed that the articular cartilage has a poor repair capacity.[4,5] For example, in both full- and partial-thickness articular cartilage defects, it is considered impossible for the hyaline cartilage to repair the defect spontaneously once it is lost.[27] However, we speculated that disuse atrophy of the articular cartilage could also be recovered by reloading. As a rationale, cartilage thickness changes depending on the load, and some studies showed that it increased with repeated exercise.[7,28] Moreover, previous studies have reported that proteoglycan metabolism is relatively fast (half-life, 8 days) and that proteoglycan synthesis increases after injury.[29,30] Moreover, our previous study provided potential data to suggest that the thinning of the articular cartilage and loss of matrix staining caused by unloading can be restored by reloading.[17]

Based on the abovementioned information, this study aimed to clarify the effect of reloading on disuse cartilage atrophy induced by unloading conditions using a rat hindlimb suspension model. In addition to the articular cartilage, the bone was included in the analysis object because the unloading condition causes histological changes in the subchondral bone, and there is a functional relationship between cartilage, subchondral bone, and bone in response to loading.

Methods

Experimental Animals and Animal Care

The study protocol was approved by the Animal Research Committee of the Graduate School of Medicine of Kanazawa University (Kanazawa, Japan; approval no. 204125) and conducted in accordance with the ARRIVE guidelines[31,32] and Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

Thirty male Wistar rats (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed under normal conditions for 5 weeks before the start of the experiments to acclimatize the animals to their new environment. One rat was housed per cage in a sanitary ventilated room under controlled temperature and humidity conditions and a 12-hour light–dark cycle with ad libitum access to food and water. The experimental animals were monitored 2 to 3 times per week to control their health status, including general food and water intake, surgical wound condition, gait, and hindlimb suspension. The experimenter cleaned the cages once or twice every 2 weeks to keep the breeding environment clean.

The rats were divided into 3 groups: control group (Con, $n = 15$, 5 rats each for 4, 6, and 8 weeks), hindlimb suspension group (HS, $n = 5$), and reloading after hindlimb suspension group (RL, $n = 10$, 5 rats each for 6 and 8 weeks). In the Con group, the rats were kept in a physiological environment. In the HS and RL groups, the rats were subjected to tail suspension for 4 weeks. Rats subjected to hindlimb suspension were allowed to walk freely in their cages using only their forelimbs. In the HS group, after the hindlimb suspension period, the rats were sacrificed and used to identify the histological changes in disuse of the cartilage due to the unloading environment and confirm the histological condition before reloading in RL group. In the RL group, after hindlimb suspension period, the rats were removed from the device on hindlimb suspension and allowed to walk freely in a physiological environment using all limbs for 2 or 4 weeks.

After starting the experiment, no further interventions, including range of motion exercise, were performed during the experimental period, and no analgesics or anti-inflammatory drugs were administered.

Hindlimb Suspension

In the HS and RL groups, the rats were subjected to tail suspension throughout the experiment. Hindlimb tail suspension was performed according to our previous study.[16,17,33,34] Briefly, under inhalation anesthesia with isoflurane, the tail of each rat was disinfected. Then, a sterile steel wire was used to drill into the proximal coccyx in which the wire remained and was shaped into a ring. Moreover, the tail ring was connected with another wire to a track hung above the cage, thereby enabling the animals to move freely on their forelimbs in the cage.

Histological Preparation

As described previously,[16,17,34,35] decalcified paraffin sections were prepared for histology. Both knees were excised frontally to evaluate the histological changes in the medial tibiofemoral joints. The right knee was used for histomorphometric analysis, and the left knee was used for immunohistochemical analysis. The specimens were serially sliced to observe the region where the articular cartilage of the femur and tibia could be in direct contact without meniscus involvement. The paraffin sections (3-μm thickness) were stained with hematoxylin–eosin and toluidine blue (0.05%, 15 min) as general staining and with immunohistochemical staining. Finally, the sections were viewed under a light microscope and imaged using a digital camera (BX51 and DP74; Olympus Corporation, Tokyo, Japan) to evaluate the histological changes in the articular cartilage.
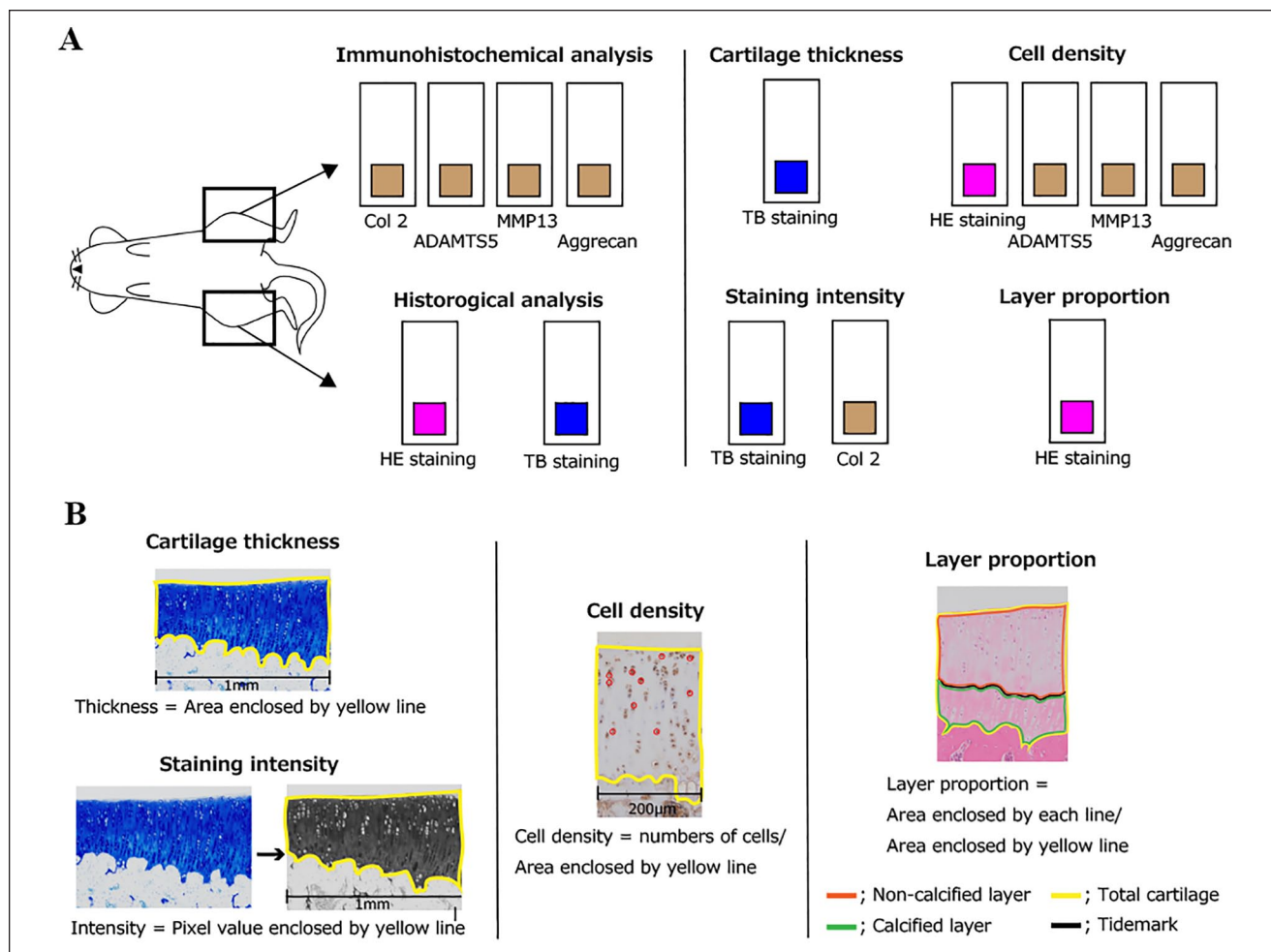
Figure 1. Schematic of histological analysis for articular cartilage: (**A**) staining method and analysis parameters and (**B**) overview of each analysis method. The right knee was used for histological analysis, and the left knee was used in the immunohistochemical analysis. For each staining, cartilage thickness, cell density, staining intensity, and layer proportion were evaluated. For details on these parameters, please refer to the text for evaluation methods. ADAMTS-5 = A disintegrin and metalloproteinase with thrombospondin motifs 5. HE, hematoxylin–eosin; TB, toluidine blue.

Histomorphometrical Analyses of the Articular Cartilage

The protocol for histomorphometrical analyses is shown in **Figure 1**. As described previously,[9,16,34-37] Adobe Photoshop CC imaging software (Adobe Systems, Inc., San Jose, CA, USA) was used to perform histomorphometrical analyses of the articular cartilage to evaluate the following 3 parameters: cartilage thickness, intensity of matrix staining with toluidine blue, and chondrocyte density.

To evaluate the total cartilage thickness, digitized images of the sections stained with toluidine blue were used. Cartilage thickness was defined as the distance between the cartilage surface and osteochondral junction. We used the measured area of the cartilage with a width of 1 mm at the center of the lesion at the tibia in the medial tibiofemoral

joint. Moreover, using the images stained with hematoxylin–eosin, the proportion of the noncalcified and calcified layers was evaluated using the tidemark as the indicator. Specifically, with a width of 200 μm, these proportions were calculated by each area of the noncalcified and calcified layers by dividing the total area of the articular cartilage.

To evaluate matrix intensity, digitized images of cartilage stained with toluidine blue were converted to grayscale (white, 255; black, 0) to assess the relative intensity of toluidine blue staining. The average staining intensity was calculated at the same area in the same manner as performed for the measurement of articular cartilage thickness.

To evaluate chondrocyte density, digitized images of the sections stained with hematoxylin–eosin were used.
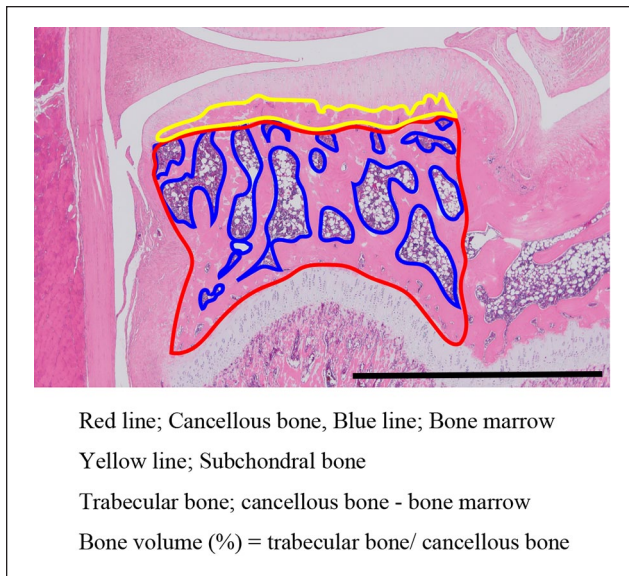
Red line; Cancellous bone, Blue line; Bone marrow

Yellow line; Subchondral bone

Trabecular bone; cancellous bone - bone marrow

Bone volume (%) = trabecular bone/ cancellous bone

Figure 2. Definition and calculation of bone volume and subchondral bone thickness. The area enclosed by the red line shows the area of the cancellous bone, and the area enclosed by the blue line shows the area of the bone marrow. The area of the cancellous bone minus the area of the bone marrow is the area of the trabecular bone. The area enclosed by the yellow line shows the area of the subchondral bone. The thickness of the subchondral bone was calculated by dividing the area of the subchondral bone by its width. Scale bar = 2 mm.

Chondrocyte density was determined as the number of chondrocytes per unit area of cartilage. This unit area was calculated using the same method as that for the abovementioned cartilage thickness, and the width to be measured was set to 200 μm. Chondrocytes with visible nuclei within the area of interest were counted manually.

Histomorphometrical Analyses of the Bone

Bone volume and thickness of the subchondral bone were measured at the tibial medial condyle according to the method of Nagira *et al.*[38] (**Fig. 2**). This is a highly reliable measurement method that shows a high correlation with the results from microcomputed tomography.[38] Bone volume (%) was defined as follows: (area of the cancellous bone − area of the bone marrow; trabecular bone)/area of cancellous bone × 100. Subchondral bone thickness was defined as the region between the osteochondral junction and bone marrow cavity.

Immunohistochemical Staining and Analyses

Paraffin sections were immunohistochemically stained using Aggrecan antibody (diluted 1:50, 13880-1-AP; Proteintech, Tokyo, Japan) and type-II collagen (diluted

1:50, ab34712; Abcam, Tokyo, Japan) as parameter of cartilage synthesis and matrix metalloproteinase 13 (MMP 13; diluted 1:50, 18165-1-AP; Proteintech Tokyo, Japan) and A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5; diluted 1:50, ab41037; Abcam, Tokyo, Japan) as a parameter of cartilage degradation. Moreover, 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) was used for the washes (5 min for 3 times) performed between each step. After deparaffined by xylenes and dehydrated by alcohols, the sections (3-μm thickness) were antigen activated by heat treatment using hot water bath (citrate buffer, pH 6.0, 70°C, 90 min) for Aggrecan, MMP 13, and ADAMTS-5 and by proteinase K (S3020, 10 min; Dako Japan, Tokyo, Japan) for type-II collagen. After inactivated endogenous peroxidase using methanol with 3% H_2O_2 for 20 min and blocked nonspecific binding of immunoglobulins using Protein Block Serum-Free (X0909; Dako Japan, Tokyo, Japan) for 15 min, the sections were incubated overnight with the primary antibody at 4°C. Then, sections were rinsed in PBS, and a subsequent reaction was made by the polymer technique using Histofine Simple Stain Rat MAX PO (MULTI) (414191, Nichirei Biosciences Inc., Tokyo, Japan) for 1 h at room temperature. Immunoreactivity was visualized using Histofine Simple Stain MAX PO (MULTI) (725191, Nichirei Biosciences Inc., Tokyo, Japan) for 5 min at room temperature. The sections were counterstained with hematoxylin for 1 min.

The protocol for immunohistochemical analyses is shown in **Figure 1**. In this analysis, the staining intensity was calculated for type-II collagen in the same way as histomorphometrical analyses described above. For Aggrecan, MMP13, and ADAMTS-5, the staining positive cells were counted, and the positive cell density was calculated in the same way as histomorphometrical analyses described above.

Statistical Analyses

All statistical analyses were performed using JMP 14 software (SAS Institute, Cary, NC, USA). All data were statistically analyzed as parametric data. The sample size was 5 for each group. Descriptive statistics were calculated as mean with standard deviation for body weight, histomorphometrical data, and immunohistochemical data. All continuous data were assessed for homoscedasticity using the Levene test. To evaluate differences between the Con and HS groups and the Con and RL groups at the same experimental period, unpaired *t*-test was used. Welch correction was applied for variables with unequal variance.

A *P* value < .05 indicated statistical significance for all analyses; exact *P* values are shown in the figures.

In this study, the total number of groups is 6, and a large number of experimental animals are needed to perform multiple comparisons. For the statistical analysis in this
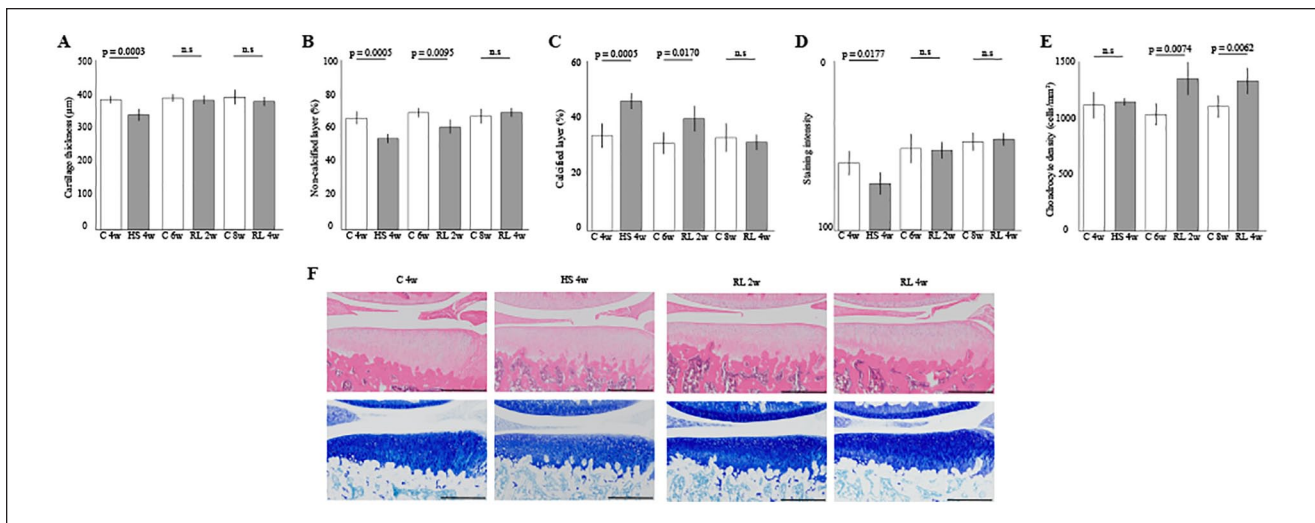
Figure 3. Histomorphometrical effect of the reloading on cartilage atrophy in the medial tibia: (**A**) cartilage thickness, (**B**) and (**C**) the percentage of the noncalcified and calcified layers in the articular cartilage, (**D**) matrix intensity of staining by toluidine blue, (**E**) chondrocyte density, and (**F**) representative histological findings. Scale bar = 500 μm. The thinning of the articular cartilage and decreases of staining intensity caused by disuse cartilage atrophy was restored by reloading for 2 weeks. Moreover, 4 weeks of reloading was required to restore the proportion of noncalcified and calcified layers. The density of chondrocytes was not affected by disuse atrophy, and significant increase in cell density was observed with reloading. HS = hindlimb suspension; RL = reloading.

study, we selected unpaired *t*-test and Welch correction between the same experimental period to reduce the number of animals used, based on the 3Rs principle of animal experiment (Reduction, Replacement, and Refinement) and calculated the number of required experimental animals using G*Power and performed the statistical analysis with the minimum number of experimental animals. Sample-size calculation was performed using the sample size and power tool in G*Power 3.1 (free; available at https://www.psy-chologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower.html),[39,40] based on the pilot experimental data of the main parameter, cartilage thickness, and matrix intensity at 4 weeks, including the first 5 rats, in the Con and HS groups. For these parameters, a minimum of 3 and 5 were required in the Con and HS groups, respectively, with a power of .80 and significance level of *P* < .05. Similarly, sample sizes were calculated for cartilage thickness and matrix intensity obtained from 5 rats in the HS group and 5 rats in the RL group at 2 weeks in the preliminary experiments. As a result, a minimum of 3 rats were required in the HS and RL groups, respectively, with a power of .80 and significance level of .05. Therefore, we set the sample size to 5 rats per group.

Results

General Condition

No experimental animals died by accident or other unexpected causes for the experimental periods. Moreover, no rats developed infections in their tail wounds. Thus, all experimental animals reached the planned experimental period safely, without adverse events. Please refer to supporting information (**S1 Table**. Body weight) for data on weight changes in the rats during the experiment.

Histomorphometrical Analyses for the Articular Cartilage

In total cartilage thickness, thickness was decreased for the hindlimb suspension for 4 weeks (**Fig. 3A**, Con 4w vs HS 4w, *P* = .0003). However, the thinning cartilage recovered to the same level as that of the Con group after 2 weeks of reloading (**Fig. 3A**, Con 6w vs RL 2w, n.s.). The proportion of the noncalcified layer was significantly decreased for 4 weeks of hindlimb suspension (**Fig. 3B**, Con 4w vs HS 4w, *P* = .0005). The proportion showed an increasing trend after 2 weeks of reloading and recovered to the same level as that of the Con group after 4 weeks of reloading (**Fig. 3B**, Con 8w vs RL 4w, n.s.). Similarly, the proportion of the calcified layer was significantly increased for 4 weeks of hindlimb suspension (**Fig. 3C**, Con 4w vs HS 4w, *P* = .0005). The proportion showed a decreasing trend after 2 weeks of reloading and recovered to the same level as that of the Con group after 4 weeks of reloading (**Fig. 3C**, Con 8w vs RL 4w, n.s.).

In the matrix intensity by toluidine blue staining, the intensity was decreased for the hindlimb suspension for 4 weeks (**Fig. 3D**, Con 4w vs HS 4w, *P* = .0177). After 2
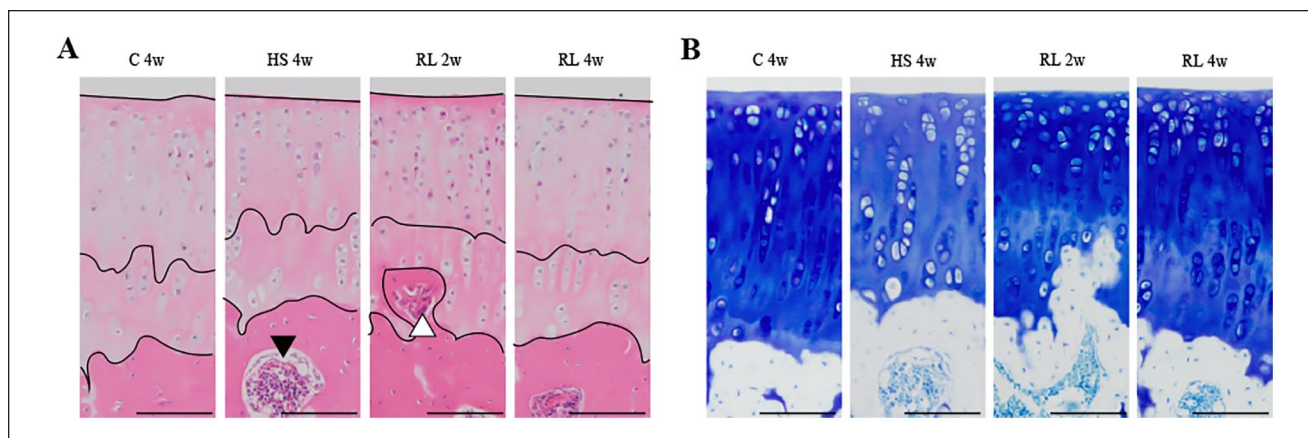
Figure 4. High magnification of representative histological finding of the tibia cartilage. Unloading and reloading did not cause any damage or fibrosis to the articular cartilage surface, which remained smooth and intact (**A** and **B**). An island of the bone marrow tissue (black triangle) is observed in the subchondral bone (**A**). Vascular invasion into the subchondral bone is suggested. Bone tissue invading the subchondral bone into the calcified layer (white triangle) is observed (**B**). Ossification of cartilage tissue is suggested. Scale bar = 100 μm. HS = hindlimb suspension; RL = reloading.
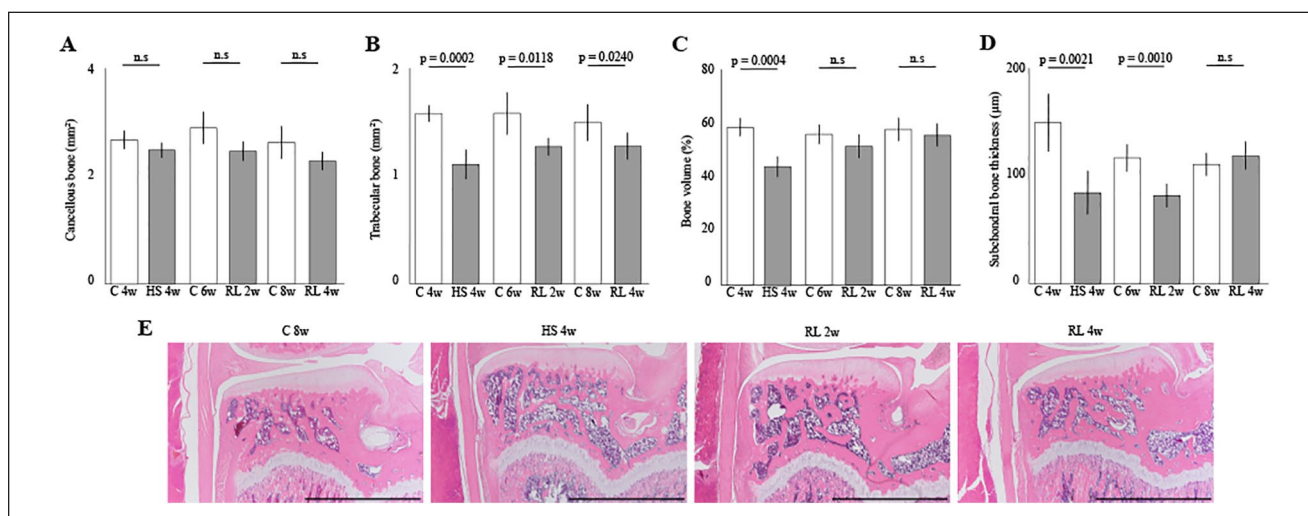

Figure 5. Histomorphometrical effect of the reloading on the bone atrophy in medial tibia: (**A** and **B**) area of the cancellous and trabecular bones, (**C**) percentage of bone volume, (**D**) thickness of subchondral bone, and (**E**) representative histological findings. Scale bar = 2 mm. The cancellous bone was not affected by unloading or reloading, and trabecular bone was significantly decreased by unloading and showed an increasing trend with reloading. As a result, the bone volume was significantly reduced by unloading and recovered to the same level as that of the Con group after 2 weeks of reloading. Similarly, the thickness of the subchondral bone was also significantly reduced by unloading, but it took 4 weeks to recover to the level of the Con group. HS = hindlimb suspension; RL = reloading.

weeks of reloading, the intensity recovered to the same level as that of the Con group (**Fig. 3D**, Con 6w vs RL 2w, n.s.).

In chondrocyte density, no significant changes in the cell density were observed after 4 weeks of hindlimb suspension (**Fig. 3E**, Con 4w vs HS 4w, n.s). At 2 and 4 weeks after reloading, the density increased significantly (**Fig. 3D**, Con 6w vs RL 2w; $P = .0074$, Con 8w vs RL 4w; $P = .0062$).

Overviews of the articular cartilage of the tibia are shown in **Figure 3F**, and high magnification images are

shown in **Figure 4**. For detailed data on histomorphometrical result in the articular cartilage, please refer to supporting information (**S2 Table**. Cartilage results).

Histomorphometrical Analyses of the Bone

In the area of the cancellous bone, no significant difference was observed in the entire experimental period (**Fig. 5A**). In the area of the trabecular bone, the area in the Con group
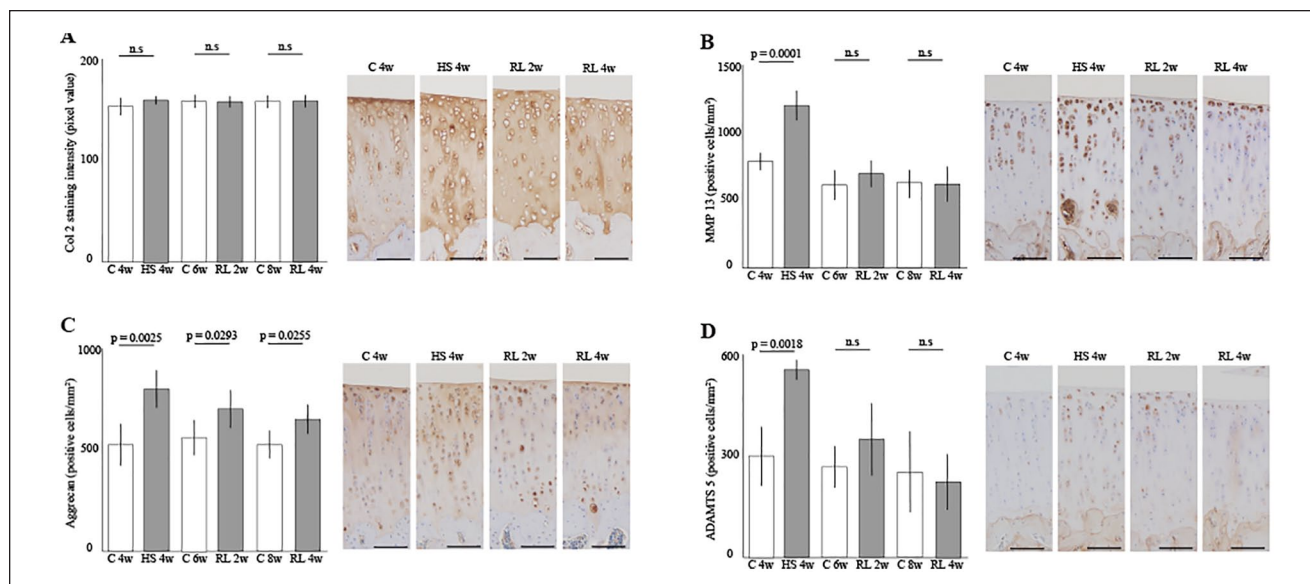
Figure 6. Immunohistochemical effect of the reloading on the cartilage atrophy in the medial tibia: (**A**) Staining intensity of type-II collagen, (**B**) MMP13-positive cell density, (**C**) Aggrecan-positive cell density, And (**D**) ADAMTS-5-positive cell density. The staining intensity of the collagen did not change significantly during the experiment. MMP13 and ADAMTS-5-positive cell densities were significantly increased by 4 weeks of unloading but returned to a steady state after 2 weeks of reloading. Aggrecan-positive cell density was significantly increased throughout the experiment. Scale bar = 100 μm. ADAMTS-5 = A disintegrin and metalloproteinase with thrombospondin motifs 5; HS = hindlimb suspension; RL = reloading.

was significantly wider than that in the HS and RL groups in the entire experimental period (**Fig. 5B**). The bone volume was decreased for the hindlimb suspension for 4 weeks (**Fig. 5C**, Con 4w vs HS 4w, $P = .0004$). However, the decreased area recovered to the same level as the Con group after 2 weeks of reloading (**Fig. 5C**, Con 6w vs RL 2w, n.s.).

In the thickness of the subchondral bone, after 4 weeks of hindlimb suspension, the thickness was significantly reduced and remained significantly reduced at 2 weeks of reloading (**Fig. 5D**, Con 4w vs HS 4w; $P = .0021$, Con 6w vs RL 2w; $P = .0010$). However, the decreased thickness recovered to the same level as that of the Con group after 4 weeks of reloading (**Fig. 5D**, Con 8w vs RL 4w, n.s.).

Overviews of the bone and subchondral bone of the tibia are shown in **Figure 5E**. For detailed data on histomorphometrical result in the bone and subchondral bone, please refer to supporting information (**S3 Table**. Bone results).

Immunohistochemical Analyses of the Articular Cartilage

The resulting graph and histology are shown in **Figure 6**. The staining intensity of type-II collagen was not significantly changed by unloading and reloading (**Fig. 6A**). MP13-positive cell density showed a significant increase with unloading for 4 weeks but was comparable to that of the control group after 2 and 4 weeks of reloading (**Fig. 6B**, Con 4w vs HS 4w; $P = .0001$). Aggrecan-positive cell

density was significantly higher than the control group at all unloading and reloading times (**Fig. 6C**, Con 4w vs HS 4w; $P = .0025$, Con 6w vs RL 2w; $P = .0293$, Con 8w vs RL 4w; $P = .0255$). ADAMTS-5-positive cell density showed a significant increase with unloading for 4 weeks but was comparable to that of the control group after 2 and 4 weeks of reloading (**Fig. 6B**, Con 4w vs HS 4w; $P = .0018$). For detailed data on immunohistochemical result in the articular cartilage, please refer to supporting information (**S4 Table**. IHS results).

Discussion

This study aimed to examine the effect of reloading on the recovery of articular cartilage and bone from disuse atrophy caused by the unloading environment for 4 weeks in the rat knee joint. As a result, 2 weeks was required for recovery of cartilage thinning and decrease of matrix staining, and 4 weeks was required for recovery of the proportion of cartilage layers. In the bone, recovery of the bone mass required 2 weeks, and recovery of subchondral bone thickness required 4 weeks. With regard to the bone, unloading causes bone loss, and reloading improves temporary osteoporosis.[14,41] However, in the articular cartilage, there have been no reports of such atrophy and recovery by reloading. Although it was previously recognized that the repair capacity of the articular cartilage, especially in traumatic cartilage injury, is poor, the results of this study clearly show

that it has sufficient recovery capacity by reloading for disuse atrophy. This new finding is novel and informative and could have an impact on medicine in articular cartilage. Moreover, it has been reported that the articular cartilage becomes thinner and softer due to the unloading environment, making it more susceptible to damage, and that disuse atrophy of the articular cartilage is a factor in the development and severity of OA.[17] Furthermore, Haapala *et al.* explained the importance of prevention of articular cartilage atrophy after joint immobilization, since it did not recover sufficiently by remobilization.[42] Therefore, the need for and importance of prevention and treatment of disuse atrophy of the articular cartilage will increase in clinical practice in the future.

In this study, 3 interesting histological results were obtained. First, reloading increased the density of chondrocytes. In this study and previous studies,[9,34] disuse atrophy of the articular cartilage results in thinning with no change in density. Thus, the actual number of chondrocytes decreases. We speculate that reloading may have increased the density of chondrocytes due to the need for matrix production to recover atrophic cartilage. This is because Lotz *et al.* reported that chondrocyte density defines not only the cartilage volume but also its composition and repair capacity,[43] and Eckstein *et al.* reported that exercise load level correlates with cartilage volume and that articular cartilage has a functional adaptive capacity to change its structure depending on the specific environment.[5]

Second, the result on the ratio of the layers of articular cartilage is shown. In the previous studies[9,14] and the present study, when the articular cartilage atrophied, the ratio of the noncalcified layer decreased, and the ratio of the calcified layer increased. The noncalcified layer is responsible for friction reduction in the superficial layer and shock absorption of load in the deep layer.[4,43-45] We speculate that the unloading environment may have reduced the need for these functions, resulting in a selective decrease of noncalcified layer. Moreover, 4 weeks of reloading restored this ratio to the same level as that in the control group, which may be one reason for this speculation.

Third, the immunohistochemical results indicate a balance between matrix synthesis and destruction. The number of matrix disrupting MMP13- and ADAMTS-5-positive cells increased in the unloading environment and returned to a steady state upon reloading at 2 weeks. Conversely, the collagen did not change significantly with or without loading, while Aggrecan increased significantly throughout the experiment with unloading and reloading. Thus, although MMP13 and collagen and Aggrecan and ADAMTS-5 were paired for synthesis and destruction,[6,44,46] they showed different changes, suggesting that the activation of Aggrecan and collagen by reloading may be through different pathways. The different degradation rates, synthesis rates, and half-lives of collagen and Aggrecan may also have an effect. Furthermore, it may be related to the fact that both chondrocyte density and number of Aggrecan-positive cells remain significantly increased after reloading, despite the recovery of cartilage thickness and percentage of layers.

The histological results of this study revealed that disuse atrophy of the articular cartilage can be recovered by reloading. However, the histological changes of this disuse atrophy are minor compared with those of traumatic cartilage damage, OA, and joint contracture,[47-49] and the results of this study do not suggest that articular cartilage may recover from these conditions with severe histological changes. Moreover, the animals used in this study were rats, which are rodents. Generally, it has been reported that small animals and humans differ in metabolism, maturation age, thickness, chondrocyte density, and layer proportions.[50-53] Therefore, further research is required to apply the recovery of reloading against disuse atrophy of the articular cartilage to humans.

There are 2 major limitations in this study. First, the sample size may be relatively small. We used G*Power to calculate the required sample size to study the statistical size (power of .80 and significance level of $P < .05$). Then, we used the unpaired *t*-test and performed statistical analysis. However, multiple comparisons may be more appropriate to account for within- and between-group errors. If multiple comparisons were used, a larger sample size would be required because of the large number of groups to be compared, 6 in our protocol. Second, we have not been able to evaluate the mechanical stresses occurring in the knee joints of rats during the experiment. Although hindlimb suspension can achieve an unloading condition of the knee joint, it does not limit joint movements, such as flexion and extension of the knee joint. It has been reported that such joint motion without pressure from loading promotes repair of the articular cartilage in traumatic cartilage damage and OA.[54,55] Therefore, in this study, it is possible that joint motion during the non-loading period had some effect on the recovery of the articular cartilage during reloading.

In conclusion, disuse atrophy of the articular cartilage and bone caused by unloading can be recovered quantitatively and qualitatively after 4 weeks of reloading. This is a useful finding with many implications for basic and clinical medicine and may modify disease and treatment concepts in articular cartilage. Further studies are needed to analyze whether active exercise therapy can further accelerate recovery and whether intermittent loading during unloading periods can prevent disuse atrophy of the articular cartilage.

Authors' Note

Author Contributions

All of the authors made substantial contributions to (1) the conception and design of the study, data acquisition, and analysis and interpretation of the data; (2) drafting the article or critically revising it for important intellectual content; and (3) providing final approval of the submitted version of the manuscript.

The authors' specific contributions were as follows:

(1) Conception and design of the study: IT, TM, HK, and MH
(2) Analysis and interpretation of the data: IT, TM, HK, and MH
(3) Drafting of the article: IT, HK, and MH
(4) Critical revision of the article for important intellectual content: IT, HK, and MH
(5) Final approval of the article: IT, TM, HK, and MH
(6) Obtaining funding: IT, TM, HK, and MH
(7) Collection and assembly of the data: IT, TM, and MH

Ikufumi Takahashi (t_ikuhumi@med.kanazawa-u.ac.jp) is responsible for the integrity of the work as a whole from inception to the finished article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was approved by our institutional review board.

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