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Regular Article

Effects of Weight Bearing on Marrow Adipose Tissue and Trabecular Bone after Anterior Cruciate Ligament Reconstruction in the Rat Proximal Tibial Epiphysis

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The effects of mechanical unloading after anterior cruciate ligament (ACL) reconstruction on bone and marrow adipose tissue (MAT) are unclear. We investigated weight bearing effects on bone and MAT after ACL reconstruction. Rats underwent unilateral knee ACL transection and reconstruction, followed by hindlimb unloading (non-weight bearing), no intervention (low-weight bearing, the hindlimb standing time ratio (STR; operated/contralateral) during treadmill locomotion ranging from 0.55 to 0.91), or sustained morphine administration (moderate-weight bearing, STR ranging from 0.80 to 0.95). Untreated rats were used as controls. At 7 or 14 days after surgery, changes in trabecular bone and MAT in the proximal tibial were assessed histologically. Histological assessments at 7 or 14 days after surgery showed that ACL reconstruction without post-operative intervention did not significantly change trabecular bone and MAT areas. Hindlimb unloading after ACL reconstruction induced MAT accumulation with adipocyte hyperplasia and hypertrophy within 14 days, but did not significantly affect trabecular bone area. Increased weight bearing through morphine administration did not affect trabecular bone and MAT parameters. Our results suggest that early weight bearing after ACL reconstruction is important in reducing MAT accumulation, and that reduction in weight bearing alone is not sufficient to induce bone loss early after ACL reconstruction.

Key words: ACL reconstruction, weight bearing, trabecular bone, marrow adipose tissue, apoptosis

I. Introduction

Bones have multiple functions, such as providing structural support for the body and serving as the environment for hematopoiesis within the marrow [6]. Bone marrow consists of two types of tissue: hematopoietic tissue and marrow adipose tissue (MAT). Traditionally, MAT has been considered as a filler of marrow cavities when hematopoietic tissue and/or bone are decreased [9]. However, recent studies have revealed that MAT plays various roles in bone and cartilage metabolism, as well as in systemic metabolism and hematopoiesis [1, 9, 20, 27, 32, 38]. For example, MAT can contribute to age-related bone loss through its repressive effect on osteoblasts and favorable effect on osteoclasts [1]. Furthermore, the presence of high amounts of fat in the femoral heads of hip osteoarthritis patients indicates that MAT may play a role in osteoarthritis by inducing inflammation [31, 38].

Mechanical loading is believed to play a crucial role in the regulation of bone and MAT mass. Unloading caused by microgravity, bedridden conditions, or hindlimb unloading in rodent models generally results in bone loss and MAT accumulation [4, 32], while increased mechanical

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loading through exercise can lead to a decrease in MAT volume associated with increased bone formation [7]. However, the effects of mechanical loading on bone and MAT may differ between the intact limb and the injured and/or operated limb. In rodent models without joint injury or surgery, reduced mechanical loading due to hindlimb unloading induced bone loss and MAT accumulation in the tibia [2, 5, 29]. In contrast, a previous study reported that hindlimb unloading after anterior cruciate ligament (ACL) injury attenuated bone loss in the distal femur in mice [10], although another study reported opposite results (i.e., hindlimb unloading after ACL injury accelerated bone loss in the distal femur in mice) [3]. Regarding exercise, treadmill exercise in intact rats increased bone formation and decreased MAT [7], while treadmill exercise in ACL reconstructed rats did not affect trabecular bone and MAT mass in the proximal tibia [18].

ACL injury is the most common knee injury and can result in bone loss around the knee in human patients [25]. In a rat ACL transection model, bone loss in the proximal tibial epiphysis was accompanied by MAT accumulation [16]. The most common treatment for ACL injury is ligament reconstruction surgery. A previous study reported that immediate ligament reconstruction after ACL transection inhibited trabecular bone loss but could not block MAT accumulation in the proximal tibial epiphysis in rats [16]. Another study reported decreased bone mineral density in the proximal tibia after ACL reconstruction in human patients [23]. These inconsistent results may be attributed to differences in rehabilitation protocols, animal species used, and timing of surgery.

Rehabilitation protocols for patients with ACL reconstruction are generally divided into traditional and accelerated rehabilitation [8]. Traditional rehabilitation involves non-weight bearing and joint immobilization in the early post-surgery period to protect the graft [8]. In contrast, accelerated rehabilitation allows for immediate weight bearing and joint motion to avoid the adverse effects of disuse due to non-weight bearing and immobilization [8]. A previous study reported that joint immobilization after ACL reconstruction induced trabecular bone loss and MAT accumulation in the rat proximal tibia, in which rats could move in the cage so that their immobilized limbs were at least partially weight-loaded [18]. Nonetheless, no studies have examined the effects of differences in the amount of weight bearing after ACL reconstruction on bone and MAT.

This study aimed to examine the effects of differences in the amount of weight bearing after ACL reconstruction on trabecular bone and MAT in the rat proximal tibia. We hypothesized that reduced weight bearing due to hindlimb unloading would decrease trabecular bone mass and promote MAT accumulation, while increased weight bearing due to pain relief would mitigate or attenuate the reduction in trabecular bone mass and MAT accumulation.

II. Materials and Methods

Experimental animals

Fifty-two 8-week-old male Wistar rats (Japan SLC, Shizuoka, Japan) were used, all of which were also used in our previous study [19]. Rats were randomly divided into four groups: untreated control (n = 8), ACL reconstruction (ACLR; n = 15, low-weight bearing), ACL reconstruction plus hindlimb unloading (ACLR + HU; n = 15, non-weight bearing), and ACL reconstruction plus morphine administration (ACLR + M; n = 14, moderate-weight bearing) groups (Fig. 1). In the ACLR group, the mean of the one-hindlimb standing time ratio (operated/contralateral) during treadmill locomotion, an index of weight bearing (a lower one-hindlimb standing time ratio indicates reduced weight bearing), was 0.58, 0.55, 0.76, and 0.91 at 1, 3, 7, and 14 days after ACL reconstruction, respectively [19]. These results indicate that weight bearing was instantly decreased just after ACL reconstruction and gradually recovered during the 14 days post-surgery period. We confirmed that the reduction in weight bearing after ACL reconstruction was partially counteracted by morphine administration, and the mean of the one-hindlimb standing time ratio in the ACLR + M group was 0.80, 0.84, 0.91, and 0.95 at 1, 3, 7, and 14 days after ACL reconstruction, respectively [19]. Rats were sacrificed at day 7 or 14 after starting the experiment (n = 4 at each time point in the control group, and n = 7 or 8 at each time point in the other groups). In the control group, data from the right and left knees of eight rats were analyzed separately, so eight knees of four rats were used for analyses at each time point. Rats were housed in a temperature-controlled room (20–25°C) with a 12-hour light/dark cycle. Standard rodent food and water were provided ad libitum. This study was approved by the animal experimentation committee of Hiroshima International University (permission number: AE20-014) and was performed in accordance with standard ethical guidelines for the care and use of laboratory animals.

ACL reconstruction surgery

Rats in the ACLR, ACLR + HU, and ACLR + M groups underwent ACL reconstruction surgery on their right knees. The surgery was performed using previously described methods [14, 15, 19]. Briefly, rats were anesthetized with intraperitoneal injection of ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively), and a quadruple-bundle autograft was prepared using the tail tendons. The knee was opened through the medial parapatellar approach, and the ACL was transected using a surgical knife. Bone tunnels were then drilled from the anteromedial side of the proximal tibia to the lateral side of the distal femur using a Kirschner wire (0.8 mm diameter), and the autograft was passed through the tunnels. The proximal end was fixed to the femur with a stainless-steel interference screw (0.8 mm diameter and 2.0 mm length, TE-00001; Matsumoto, Chiba, Japan). Subsequently, the autograft was

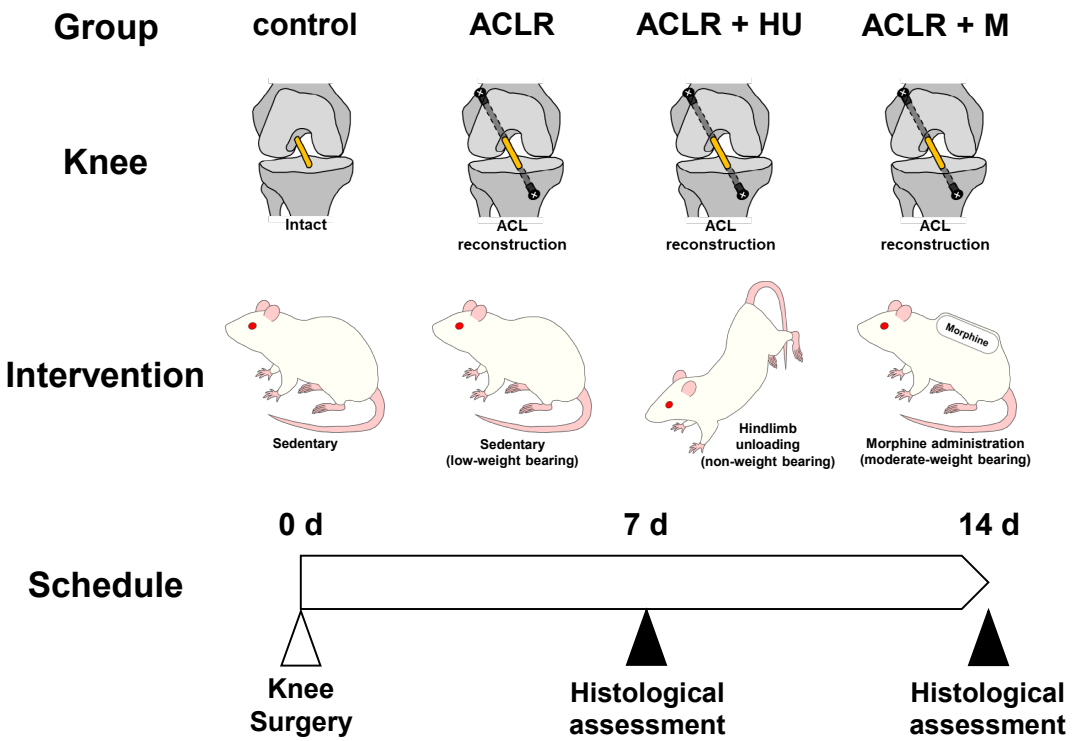

Fig. 1. Experimental protocol.

manually tensioned, and the distal end was fixed to the tibia with a stainless-steel interference screw. Finally, the joint capsule and skin were sutured with silk sutures.

Hindlimb unloading

Hindlimb unloading was performed on rats in the ACLR + HU group using previously described methods [19]. Immediately after ACL reconstruction, a Kirschner wire was inserted into the proximal region of the tail of an anesthetized rat, and both ends were bent to form rings. One end of the stainless-steel wire was attached to the rings of the Kirschner wire, and the other end was attached to the top of a cylindrical cage (height 36 cm, diameter 32 cm) using a clip. The length of the stainless-steel wire was adjusted to prevent contact between the hindlimbs and the floor. The forelimbs were in contact with the floor, and the rats were able to move freely in the cage using their forelimbs.

Morphine administration

To increase weight bearing through pain relief, morphine was administered to rats in the ACLR + M group, following the methods described in a previous study [19]. An ALZET osmotic mini-pump (model 2ML2; Durect Corporation, Cupertino, CA, USA) was filled with morphine hydrochloride (Daiichi-Sankyo Co. Ltd, Tokyo, Japan) and implanted subcutaneously immediately after ACL reconstruction. Morphine hydrochloride was continuously administered at a rate of 4.8 mg/day throughout the experimental period.

Histological analysis

At the end of the experimental period, rats were anesthetized and sacrificed by exsanguination. Knees were immersion-fixed in 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4) for two days. Samples were decalcified with 17.7% ethylenediaminetetraacetic acid (pH 7.2, Osteosoft; Merck Millipore, Darmstadt, Germany) and embedded in paraffin. Sagittal sections (4 μm thick) were cut at the medial midcondylar level using a microtome and stained with Safranin-O Fast Green. Parameters of trabecular bone and MAT in the proximal tibial epiphysis were assessed using previously described methods [16, 18]. Six fields of view located between the articular cartilage and the growth plate were photographed at 20× magnification using a light microscope (BX51; Olympus, Tokyo, Japan) equipped with a digital microscope camera (WRAYCAM-NOA2000; WRAYMER, Tokyo, Japan). The trabecular bone area was measured manually and expressed as a percentage of the total tissue area. Additionally, the numbers of total and empty lacunae in the trabecular bone were counted, and the empty lacuna ratio was calculated by dividing the number of empty lacunae by the number of total lacunae [37].

Adipocytes were identified morphologically as described in a previous study [21]. The number of adipocytes was counted manually. If the entire cell border was not included in the field, the number of adipocytes was counted as 0.5. The number of adipocytes was expressed as cells per mm^2 of tissue area. Cross-sectional areas were measured in adipocytes and summed. Mean adipocyte size

was calculated by dividing the sum of the cross-sectional areas by the number of adipocytes. MAT area was calculated by dividing the sum of cross-sectional areas by the total tissue area. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure adipocyte and trabecular bone areas and count bone lacunae.

Immunohistochemistry for caspase-3

Immunohistochemistry for caspase-3 was performed to detect apoptotic osteocytes. Deparaffinized sections of the knee were incubated with methanol containing 3% H_2O_2 for 30 min, followed by incubation with 1% normal horse serum in 0.01 M phosphate-buffered saline (pH 7.4) for 30 min. After sections were incubated with an anti-caspase-3 antibody (No. GTX110543, 1:4000 dilution; GeneTex, California, LA, USA) overnight at 4°C, a secondary antibody (horse biotinylated anti-mouse/rabbit IgG, 1:250 dilution; BA-1400, Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at room temperature. The negative control was incubated with vehicle (1% bovine serum albumin), instead of an anti-caspase-3 antibody. The sections were then incubated with a streptavidin-biotin complex (1:50 dilution; Elite ABC, Vector Laboratories, Burlingame, CA, USA) for 30 min, and the immunoreaction was visualized using Dako EnVision + kit/HRP (DAB) (Dako Japan, Tokyo, Japan). Finally, counterstaining with hematoxylin was performed to stain nuclei. Six fields of view located between the articular cartilage and the growth plate (similar regions to those used to assess marrow adiposity and trabecular bone area) were photographed at 40× magnification. Total and caspase-3 positive osteocytes were counted, and the percentage of caspase-3 positive osteocytes was calculated.

TRAP staining

To visualize osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed using the TRAP/ALP stain kit (294-67001; FUJIFILM Wako, Tokyo, Japan). Six fields of view located between the articular cartilage and the growth plate (similar regions to those used to assess marrow adiposity and trabecular bone area) were photographed at 20× magnification. Osteoclast surface length was manually measured using ImageJ software, calculated as the length of the TRAP-positive bone surface divided by the total bone surface [11].

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses were performed using Dr. SPSS II for Windows (SPSS Japan, Tokyo, Japan). Two-way analyses of variance (ANOVAs) were employed to identify significant main effects and interactions. When significant main effects, but not interactions, were detected, Bonferroni tests were used for post hoc comparisons between levels. When significant interactions were detected, Bonferroni tests were used to identify simple main effects. In addition, Pearson's correlation coefficient (when normality could be assumed based on the Shapiro-Wilk test) or Spearman's correlation coefficient (when normality could not be assumed based on the Shapiro-Wilk test) was calculated between empty lacuna ratio and caspase-3 positive cell ratio, empty lacuna ratio and osteoclast surface length, trabecular bone area and empty lacuna ratio, and trabecular bone area and MAT area. A p-value of < 0.05 was considered to indicate statistical significance.

III. Results

Trabecular bone area

Figure 2 shows representative images of sections stained with Safranin-O Fast Green. In the trabecular bone area, neither the interaction ($P = 0.242$) nor the main effects of time ($P = 0.513$) and intervention ($P = 0.232$) were found to be statistically significant (Fig. 3A).

Empty lacuna ratio

Two-way ANOVA revealed a significant main effect for intervention in the empty lacuna ratio (Fig. 3B, $P = 0.001$). The empty lacuna ratio was significantly higher in all ACL reconstructed groups (Fig. 2F–H and N–P) than in the control group (Fig. 2E and M, Fig. 3B, $P \leq 0.012$). There were no differences in the empty lacuna ratio between the ACL reconstructed groups ($P = 1.000$ in all comparisons). The interaction ($P = 0.474$) and the main effect of time ($P = 0.214$) were not significant.

Caspase-3 positive cell ratio

Figure 4 shows representative images of caspase-3-immunostained sections. In the negative control, osteocytes were not stained (data not shown). Two-way ANOVA revealed a significant main effect for intervention in the caspase-3 positive cell ratio (Fig. 3C, $P = 0.002$). The caspase-3 positive cell ratio was significantly higher in all ACL reconstructed groups (Fig. 4B–D and F–H) than in the control group (Fig. 3C, Fig. 4A and E, $P \leq 0.041$). There were no differences in the caspase-3 positive cell ratio between the ACL reconstructed groups ($P = 1.000$ in all comparisons). The interaction ($P = 0.442$) and the main effect of time ($P = 0.529$) were not significant.

Osteoclast surface length

Figure 5 shows representative images of TRAP-stained sections. Two-way ANOVA revealed a significant main effect for intervention in the osteoclast surface length (Fig. 3D, $P = 0.001$). The osteoclast surface length was significantly longer in all ACL reconstructed groups (Fig. 5B–D and F–H) than in the control group (Fig. 3D, Fig. 5A and E, $P \leq 0.009$). There were no differences in the osteoclast surface length between the ACL reconstructed groups ($P = 1.000$ in all comparisons). The interaction ($P = 0.132$) and the main effect of time ($P = 0.263$) were not significant.

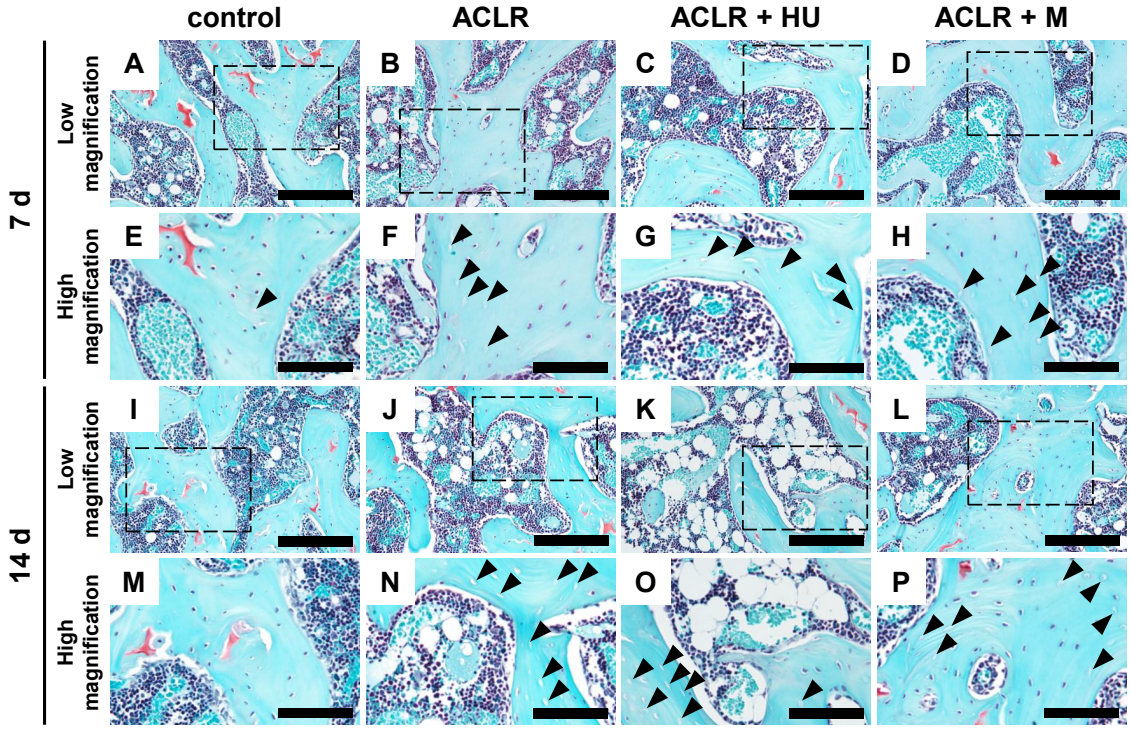

Fig. 2. Histological features in the epiphysis of the proximal tibia. Representative images of Safranin-O Fast Green-stained sections are presented for the control (**A, E, I, M**), ACLR (**B, F, J, N**), ACLR + HU (**C, G, K, O**), and ACLR + M (**D, H, L, P**) groups. Images in panels (**A–H**) and (**I–P**) represent 7 and 14 days post-surgery, respectively. Images in panels (**E–H**) and (**M–P**) show high magnification views of the boxes in images (**A–D**) and (**I–L**), respectively. Arrowheads indicate empty lacunae. Circular cavities with absence of cytoplasmic staining are adipocytes. Bars = 200 μm and 100 μm in low magnification images (**A–D** and **I–L**) and high magnification images (**E–H** and **M–P**), respectively.

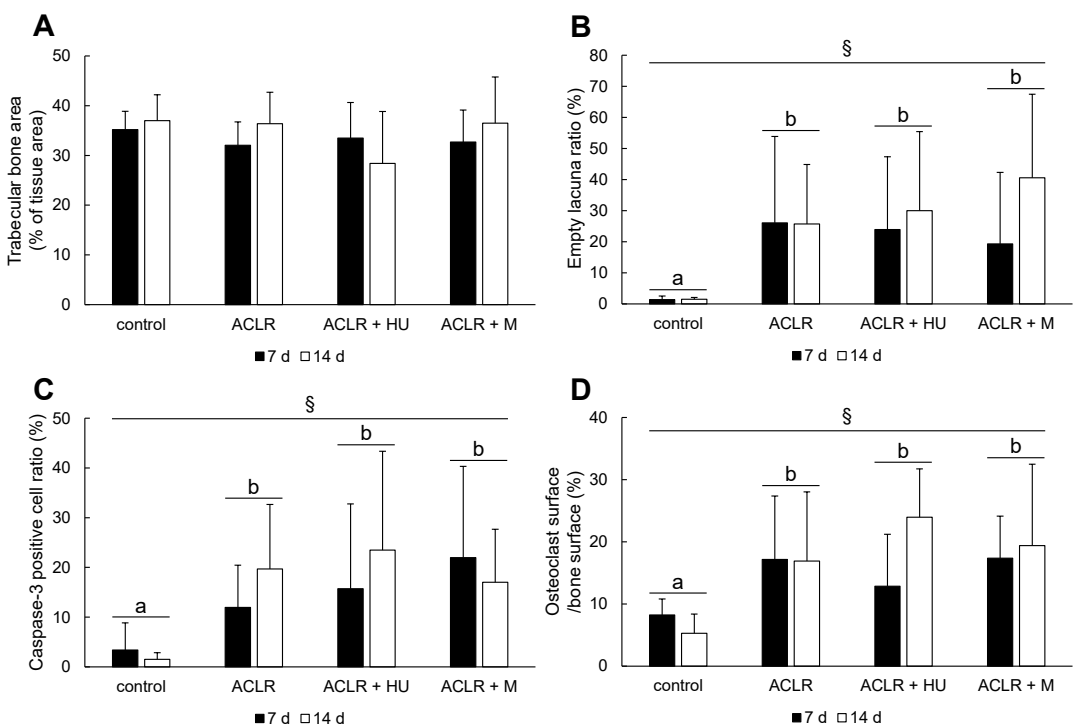

Fig. 3. Trabecular bone parameters. (**A**) Trabecular bone area, (**B**) empty lacuna ratio, (**C**) caspase-3 positive cell ratio, and (**D**) osteoclast surface length. Values are presented as mean ± standard deviation. §: significant main effect of intervention. Different letters indicate statistically significant differences between groups, whereby groups not sharing the same letter are significantly different from one another.

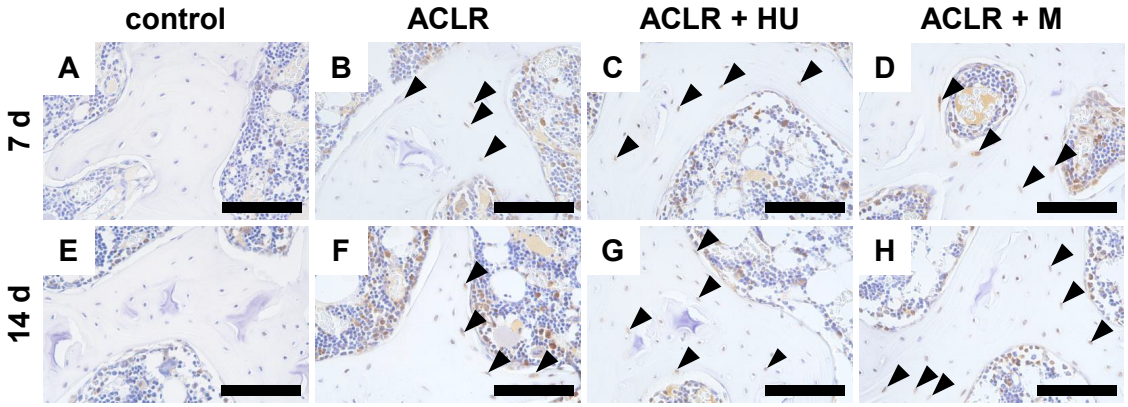

Fig. 4. Expression of caspase-3. Representative images of caspase-3-immunostained sections are presented for the control (**A, E**), ACLR (**B, F**), ACLR + HU (**C, G**), and ACLR + M (**D, H**) groups. Images in panels (**A–D**) and (**E–H**) represent 7 and 14 days post-surgery, respectively. Arrowheads indicate caspase-3-positive cells. Bars = 100 μm.

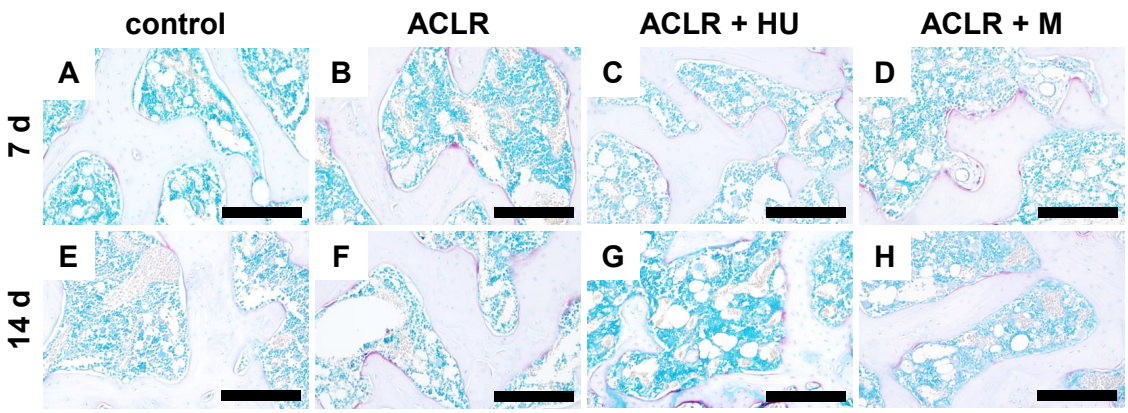

Fig. 5. TRAP staining. Representative images of TRAP-stained sections are presented for the control (**A, E**), ACLR (**B, F**), ACLR + HU (**C, G**), and ACLR + M (**D, H**) groups. Images in panels (**A–D**) and (**E–H**) represent 7 and 14 days post-surgery, respectively. Osteoclasts are stained in purple. Bars = 200 μm.

Adipocyte number

Two-way ANOVA revealed a significant interaction between time and intervention ($P = 0.038$), and main effects of both time ($P = 0.003$) and intervention ($P < 0.001$) on adipocyte number (Fig. 6A). At seven days post-surgery, the number of adipocytes in the ACLR + HU group (Fig. 2C) was significantly higher than that in the ACLR + M group (Fig. 2D, Fig. 6A, $P = 0.014$). At 14 days post-surgery, the number of adipocytes in the ACLR + HU group further increased compared to seven days post-surgery (Fig. 2K, Fig. 6A, $P < 0.001$), and was significantly higher than those in the control, ACLR, and ACLR + M groups (Fig. 2I, J, and L, Fig. 6A, $P < 0.001$ in all comparisons).

Adipocyte size

Two-way ANOVA revealed significant main effects for time ($P < 0.001$) and intervention ($P < 0.001$) on adipocyte size (Fig. 6B). Adipocyte size was significantly larger at 14 days compared to 7 days post-surgery. Among the groups, adipocyte size in the ACLR + HU group (Fig. 2C and K) was significantly larger than in the control, ACLR, and ACLR + M groups (Fig. 2A, B, D, I, J, and L, Fig. 6B, $P \leq 0.003$). Interaction was not significant ($P = 0.082$).

MAT area

Two-way ANOVA revealed a significant interaction between time and intervention ($P = 0.002$) and significant main effects of both time ($P < 0.001$) and intervention ($P < 0.001$) on MAT area (Fig. 6C). At seven days post-surgery, the MAT area in the ACLR + HU group (Fig. 2C) was significantly larger than in the ACLR + M group (Fig. 2D, Fig. 6C, $P = 0.004$). At 14 days post-surgery, the MAT area in the ACLR + HU group further increased compared to that at seven days post-surgery (Fig. 2K, Fig. 6C, $P < 0.001$) and was significantly larger than in the control, ACLR, and ACLR + M groups (Fig. 2I, J, and L, Fig. 6C, $P < 0.001$ in all comparisons). In the ACLR group, the MAT area at 14 days post-surgery was significantly larger than at

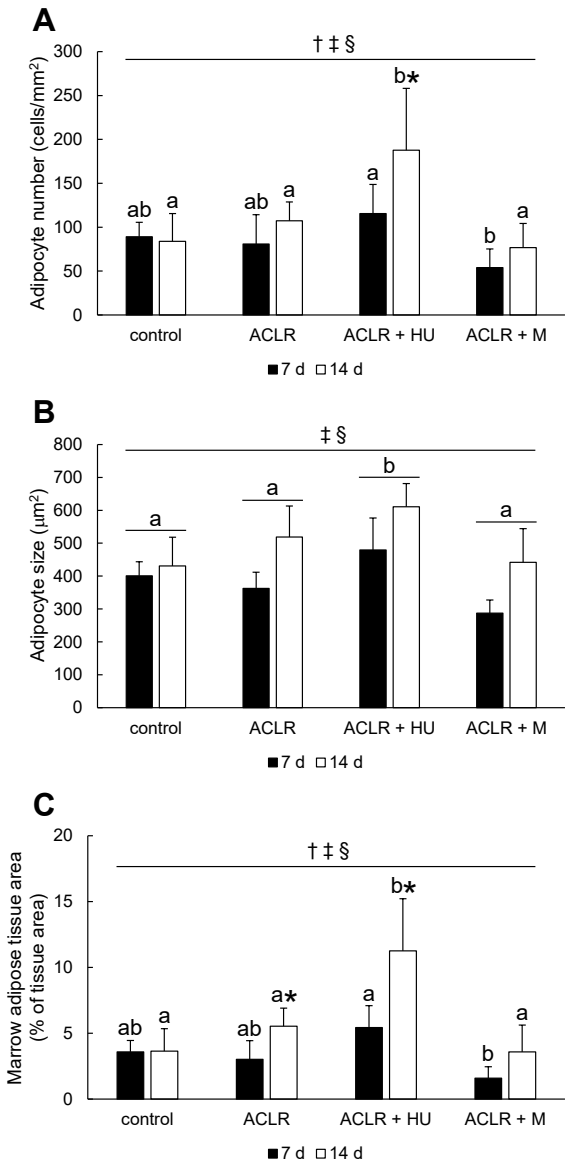

Fig. 6. Bone marrow adiposity. (**A**) Adipocyte number, (**B**) adipocyte size, and (**C**) marrow adipose tissue area. Values are presented as mean ± standard deviation. †: significant interactions between time and intervention. ‡: significant main effect of time. §: significant main effect of intervention. Different letters indicate statistically significant differences between groups, whereby groups not sharing the same letter are significantly different from one another. *: significant difference compared to seven days post-surgery.

seven days post-surgery (Fig. 2B and J, Fig. 6C, P = 0.018), but was not significantly different from that in the control group (Fig. 2I, Fig. 6C, P = 0.372).

Correlation analysis

At seven days post-surgery, a strong positive correlation was found between empty lacuna ratio and caspase-3 positive cell ratio (Fig. 7A, $r_s = 0.713$, $P < 0.001$). Similarly, at 14 days post-surgery, a strong positive correlation was found between empty lacuna ratio and caspase-3 positive cell ratio (Fig. 7B, $r_s = 0.729$, $P < 0.001$)

A moderate positive correlation was observed between empty lacuna ratio and osteoclast surface length at 14 days post-surgery (Fig. 7D, $r_s = 0.651$, $P < 0.001$), while the correlation at seven days post-surgery was not significant (Fig. 7C, $r_s = 0.136$, $P = 0.482$).

There were no significant correlations between trabecular bone area and empty lacuna ratio at both seven (Fig. 7E, $r_s = 0.327$, $P = 0.083$) and 14 days post-surgery (Fig. 7F, $r_s = 0.327$, $P = 0.072$).

At 14 days post-surgery, a weak but significant negative correlation was observed between trabecular bone area and MAT area (Fig. 7H, $r_s = -0.392$, $P = 0.029$), but not at seven days post-surgery (Fig. 7G, $r = -0.345$, $P = 0.067$).

IV. Discussion

We aimed to examine the effects of weight bearing after ACL reconstruction on the trabecular bone and MAT in the rat proximal tibia. Differences in weight bearing did not significantly affect any parameters of trabecular bone. Regarding the MAT, parameters in the ACLR and ACLR + M groups were comparable to the control group, whereas MAT accumulation was detected in the ACLR + HU group.

In this study, trabecular bone and MAT areas in the ACLR group were comparable to those of the control group, although a previous study reported MAT accumulation in the rat proximal tibial epiphysis at 4 and 12 weeks after ACL reconstruction [16]. Another study reported that trabecular bone and MAT areas in the proximal tibial epiphysis did not change significantly after two and four weeks of ACL reconstruction in rats [18]. Our experimental periods (within 14 days) may have been too short to detect significant changes in trabecular bone and MAT mass. After ACL reconstruction, however, a qualitative change in trabecular bone was detected; the empty lacuna ratio was significantly increased. The increase in the empty lacuna ratio can be induced by apoptosis of osteocytes [13]. We confirmed that the caspase-3 positive cell ratio was significantly higher in the ACLR group than in the control group. Furthermore, a strong positive correlation was found between the empty lacuna ratio and caspase-3 positive cell ratio. These results suggest that ACL reconstruction induced a reduction in osteocytes (increase in empty lacuna ratio) through apoptosis. It is generally believed that apoptosis of osteocytes can induce bone loss via the induction of osteoclasts [34]. In our study, the osteoclast surface length significantly increased after ACL reconstruction, and a significant positive correlation between empty lacuna ratio and osteoclast surface length was observed at 14 days post-surgery. However, in this experimental condition, trabecular bone area was not reduced by ACL reconstruction. Osteocyte viability contributes to bone strength independently of bone mass [12, 30]. Therefore, ACL reconstruction-induced apoptosis of osteocytes might induce a reduction in bone strength. Although we did not

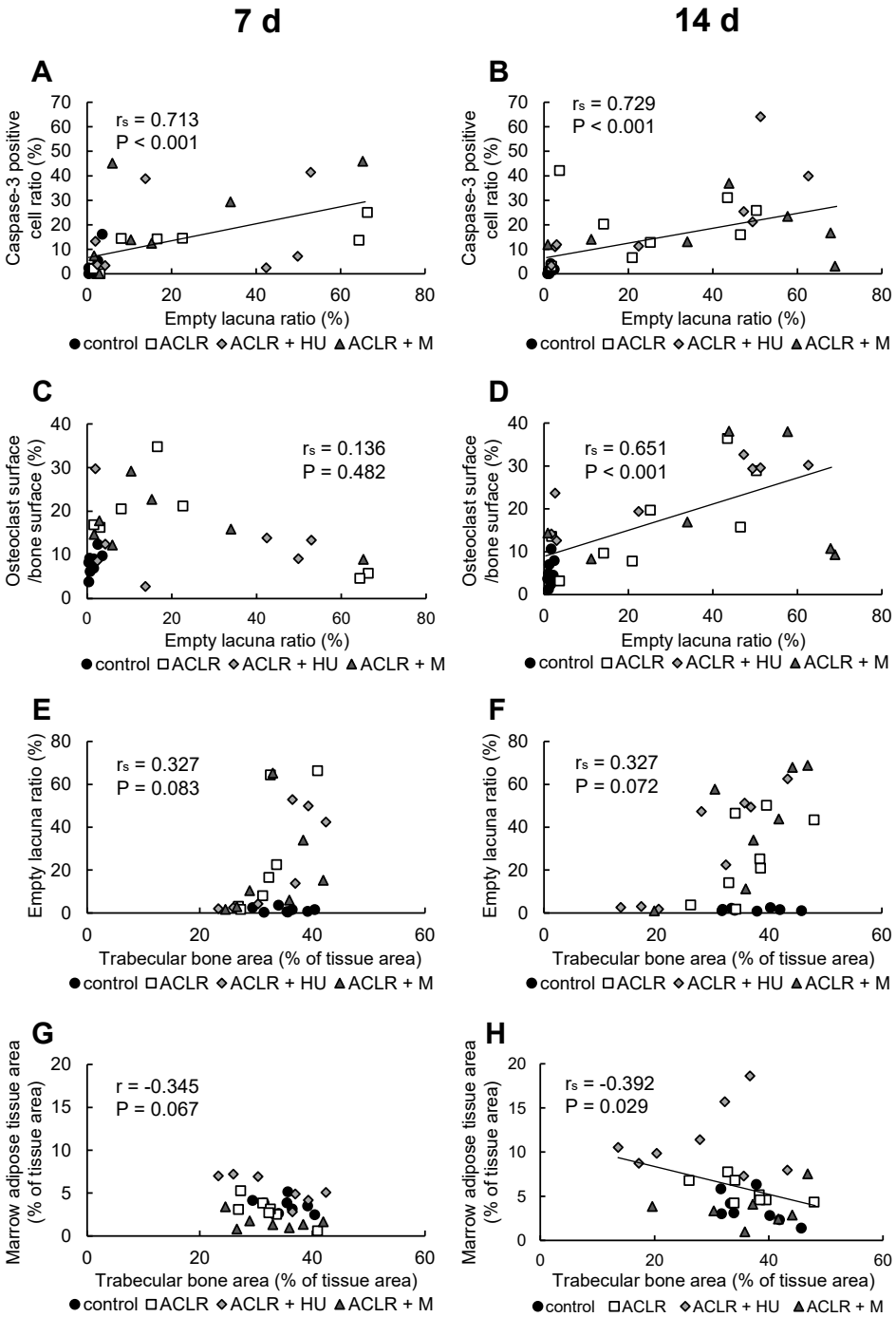

Fig. 7. Correlations between empty lacuna ratio and caspase-3 positive cell ratio (**A, B**), empty lacuna ratio and osteoclast surface length (**C, D**), trabecular bone area and empty lacuna ratio (**E, F**), and trabecular bone area and marrow adipose tissue area (**G, H**). (**A**), (**C**), (**E**), and (**G**) represent 7 days post-surgery, and (**B**), (**D**), (**F**), and (**H**) represent 14 days post-surgery.

evaluate bone strength, several cases of tibial plateau fractures after ACL reconstruction have been reported [22, 28].

Hindlimb unloading after ACL reconstruction significantly increased MAT area at 14 days post-surgery. Theoretically, an increase in MAT area can be induced by an increase in adipocyte hyperplasia, adipocyte hypertrophy, or a combination of these [33]. Because hindlimb unloading increased both adipocyte number and adipocyte size, the increased MAT area in the ACLR + HU group would be due to a combination of adipocyte hyperplasia and adipocyte hypertrophy. In general, MAT accumulation is thought to have negative effects on bone mass through osteoclastogenesis induced by the receptor activator of NF-κB ligand derived from MAT [26]. MAT accumulation

observed in aging, post-menopausal osteoporosis, and anorexia nervosa is associated with bone loss [24]. However, in our study, increased MAT due to hindlimb unloading was not accompanied by an increase in osteoclast surface length and a reduction in trabecular bone area. Because bone metabolism is relatively slow, our experimental periods (within 14 days) might be too short to detect the effects of hindlimb unloading on trabecular bone parameters. A significant negative correlation was found between trabecular bone area and MAT area at 14 days post-surgery. Thus, we cannot exclude the possibility that increased MAT by hindlimb unloading may lead to bone degeneration in the longer term. A recent review suggested that MAT plays a role in osteoarthritis [38]. We previously reported that cartilage degeneration after ACL reconstruction was accelerated by hindlimb unloading [19], and increased MAT might contribute to this adverse effect of hindlimb unloading on cartilage degeneration. In addition, MAT plays an endocrine role in producing leptin, adiponectin, and cytokines that target distant skeletal muscle [9]. The accumulation of MAT may also contribute to the exacerbation of muscle atrophy during hindlimb unloading after ACL reconstruction [17].

Increased weight bearing by morphine administration did not affect all the examined parameters of trabecular bone and MAT. The main reason for this may be that ACL reconstruction alone did not change the trabecular bone area and MAT parameters within 14 days. Separately, increased weight bearing by morphine administration could not attenuate ACL reconstruction-induced osteocyte apoptosis. It is believed that the relationship between osteocyte apoptosis and mechanical loading is U-shaped (i.e., both hypo- and hyper-mechanical loading can induce osteocyte apoptosis) [12]. However, in our study, differences in weight bearing did not affect the empty lacunae ratio and caspase-3 positive cell ratio, suggesting that factors other than mechanical loading affect osteocyte apoptosis after ACL reconstruction. Several factors, including inflammation, can also induce osteocyte apoptosis [34]. Further studies are needed to elucidate the mechanisms of osteocyte apoptosis induced by ACL reconstruction.

This study has limitations. The follow-up periods in this study were short (up to 14 days), potentially explaining why differences in the amount of weight bearing did not significantly affect any parameter of trabecular bone. Longer-term follow-up might be better to detect the effects of differences in weight bearing on trabecular bone parameters. Clinically, if non-weight bearing is employed after ACL reconstruction, the period is often within 14 days [35, 36]. Thus, we designed the experimental periods to align with this clinical timeframe. We did not examine changes in trabecular bone and MAT due to reloading after the hindlimb unloading period, even though patients typically return to relatively normal levels of physical activity after a period of post-operative non-weight bearing. A previous study reported that increased MAT in the tibia due to hindlimb unloading was reversed by reloading in rats [5]. Whether the adverse effects of non-weight bearing after ACL reconstruction on MAT can be reversed by reloading, as observed in the intact limb, remains an open question.

In conclusion, hindlimb unloading after ACL reconstruction increased MAT area through adipocyte hyperplasia and hypertrophy. Therefore, early weight bearing after ACL reconstruction will be important in reducing MAT accumulation. However, an increase in weight bearing did not affect trabecular bone parameters such as trabecular bone area, osteocyte apoptosis, and osteoclastogenesis.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Funding

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VII. Acknowledgments

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