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Regular Article

The Effects of Corticosteroid Administration and Treadmill Exercise on Marrow Adipose Tissue and Trabecular Bone after Anterior Cruciate Ligament Reconstruction in Rats

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We aimed to investigate the effects of short-term corticosteroid administration after anterior cruciate ligament (ACL) reconstruction on marrow adipose tissue (MAT) and trabecular bone mass, as well as to examine whether treadmill exercise can mitigate MAT increase and trabecular bone deterioration caused by corticosteroid. ACL-reconstructed rats were divided into groups: no intervention, daily treadmill exercise (60 min/day), administration of the steroidal drug dexamethasone (250 μg/kg on days 0–5, 7, and 9 post-operatively), or dexamethasone administration combined with treadmill exercise. Untreated rats were served as controls. At day 10 or 30 post-operatively, histological assessments were performed in the proximal tibial epiphysis. MAT accumulation and trabecular bone loss were observed after ACL reconstruction. Dexamethasone promoted MAT accumulation at day 10 post-operatively but did not affect the trabecular bone loss. The MAT accumulation caused by dexamethasone reversed within 21 days after discontinuation. Treadmill exercise did not influence the changes in the MAT and trabecular bone areas. Short-term corticosteroid administration after ACL reconstruction promoted MAT accumulation while not affecting trabecular bone area. The MAT accumulation resulting from corticosteroid administration was reversible after discontinuation. Treadmill exercise could not mitigate the accumulation of MAT caused by corticosteroid administration and did not affect trabecular bone area.

Key words: ACL reconstruction, marrow adipose tissue, bone, steroids, exercise

I. Introduction

Anterior cruciate ligament (ACL) injury is the most common ligament injury, resulting in knee instability [7]. Surgical reconstruction is the primary treatment for ACL injury, restoring knee stability [7]. However, various side effects, including pain and joint contracture, which are triggered by inflammation, are observed after ACL reconstruction surgery [11, 12, 18, 29]. Steroidal anti-inflammatory drugs (corticosteroids) have been used short-term to alleviate pain and joint contracture after ACL reconstruction, and their effectiveness has been reported [18, 28]. Long-term corticosteroid administration is known to have various adverse metabolic effects [9]. However, the side effects of short-term corticosteroid administration after ACL reconstruction remain poorly understood.

ACL reconstruction can cause marrow adipose tissue (MAT) accumulation and bone loss in the bones around knee joint [13, 21]. For example, MAT accumulation in the proximal tibia has been observed in rats after ACL reconstruction [13]. Another study reported a decrease in bone mineral density in the proximal tibia three months after ACL reconstruction in human patients [21]. MAT is

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believed to have negative effects on bone and cartilage metabolism through the secretion of adipokines [26, 35]. Accordingly, bone loss due to aging or unloading and hip osteoarthritis are associated with an increase in MAT [25, 26]. Therefore, the increased MAT observed after ACL reconstruction may contribute to bone loss and osteoarthritis development. Considering that corticosteroids can cause lipid metabolism abnormalities and osteoporosis [2, 27], short-term corticosteroid administration after ACL reconstruction may potentially promote MAT accumulation and bone loss. However, this possibility has not been investigated thus far.

Weight-bearing exercises, such as walking and running, are known to have positive effects on MAT and bone metabolism. For instance, treadmill exercise in intact rats increased bone formation rate and reduced MAT in the proximal tibia [5]. Running exercise has been shown to suppress increased MAT caused by a high-fat diet or diet-induced obesity [32, 33]. Furthermore, weight-bearing exercise is recommended to counteract corticosteroid-induced osteoporosis [8, 19]. Based on these findings, we hypothesize that weight-bearing exercise can mitigate the adverse effects of corticosteroid administration after ACL reconstruction on MAT and bone metabolism.

The aims of this study were: 1) to investigate the effects of short-term corticosteroid administration after ACL reconstruction on MAT and trabecular bone, and 2) to determine whether treadmill exercise can attenuate the increase in MAT and deterioration of trabecular bone caused by corticosteroid administration. We hypothesized that 1) short-term corticosteroid administration after ACL reconstruction would promote MAT accumulation and trabecular bone loss, and 2) treadmill exercise would mitigate MAT accumulation and trabecular bone loss induced by corticosteroid administration.

II. Materials and Methods

Experimental animals

Sixty-six male Wistar rats (eight weeks old; Japan SCL, Shizuoka, Japan) were randomly assigned to five groups as follows: untreated control (n = 8), ACL reconstruction (ACLR; n = 15), ACL reconstruction plus treadmill exercise (ACLR + T; n = 14), ACL reconstruction plus administration of the steroidal drug dexamethasone (ACLR + D; n = 14), and ACL reconstruction plus dexamethasone administration and treadmill exercise (ACLR + DT; n = 15) (Fig. 1). Data were collected at either day 10 or day 30 after the start of 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) (Fig. 1). In the control group, the right and left knees were analyzed as individual samples, resulting in eight knees from four rats being analyzed at each time point. The rats were housed in standard cages 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: AE20-027) 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 + T, ACLR + D, and ACLR + DT groups underwent ACL reconstruction surgery on their right knee, following a previously described method [11]. Briefly, rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and a quadruple-bundle autograft was prepared using the tail tendons. The knee joint was accessed through a medial parapatellar approach, and the ACL was completely transected. Subsequently, bone tunnels were drilled from the lateral side of the distal femur to the anteromedial side of the proximal tibia using a 0.8-mm-diameter Kirschner wire. After passing the autograft through the bone tunnels, the proximal end was fixed to the femur tunnel using a stainless-steel interference screw (0.8-mm-diameter and 2.0-mm-long; TE-00001; Matsumoto, Chiba, Japan). The autograft was manually tensioned, and the distal end was fixed to the tibia tunnel using another stainless-steel interference screw. Finally, the joint capsule and skin were sutured. Rats in the control group did not receive anesthesia or undergo knee surgery.

Dexamethasone administration

The rats in the ACLR + D and ACLR + DT groups received a subcutaneous injection of 250 μg/kg of dexamethasone (Sigma–Aldrich, St. Louis, MI, USA) on days zero (immediately after surgery) to five, seven, and nine post-operatively. The timing and dosage of dexamethasone administration were selected based on previous studies to mitigate inflammation and joint contracture [12, 22]. A previous study demonstrated that this administration method reduced joint swelling and contracture in ACL-reconstructed rats [16].

Treadmill exercise

Rats in the ACLR + T and ACLR + DT groups underwent treadmill exercise starting from day three post-operatively, following a previously described method [14–17]. Briefly, rats were subjected to forced walking on a treadmill machine (Rat runner; Agawa, Shimane, Japan) at a low speed of 12 m/minute. The exercise protocol consisted of six 10-minute exercise sessions with one-minute intervals, resulting in a total of 60 minutes of walking per day. Treadmill exercise was performed six days per week.

Histological analysis

At the end of the experimental period, rats were euthanized by exsanguination under anesthesia. The knee joints were collected and fixed by immersion in 0.1 M phosphate-buffered 4% paraformaldehyde at 4°C for two days. After

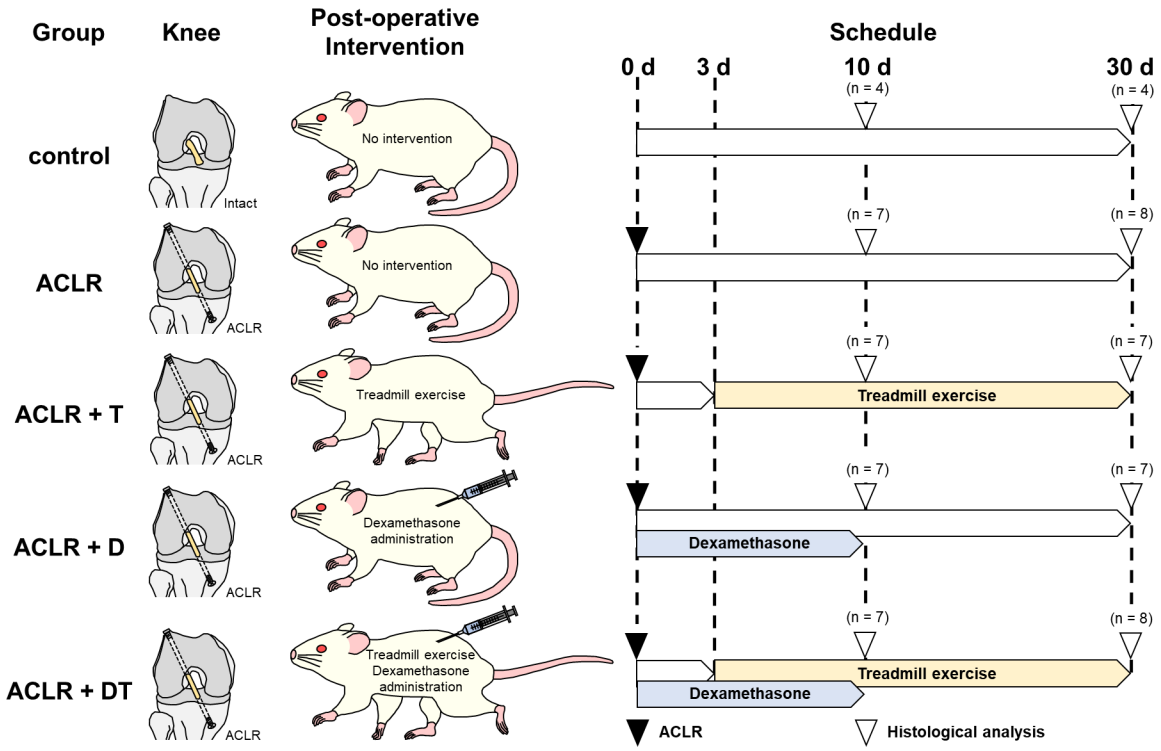

Fig. 1. Experimental protocol. ACLR, anterior cruciate ligament reconstruction; T, treadmill exercise; D, dexamethasone administration.

decalcification with ethylenediaminetetraacetic acid (Osteosoft; Merck Millipore, Darmstadt, Germany), sagittal paraffin sections (4-μm-thick) were prepared at the medial midcondylar level and stained with Safranin-O Fast Green.

MAT and trabecular bone parameters in the proximal tibial epiphysis were assessed using previously described methods [13, 17]. Briefly, six fields of view, located between the articular cartilage and the growth plate, were captured at 20× magnification. The trabecular bone area was manually measured in each image and expressed as a percentage of the total tissue area.

Adipocytes were morphologically identified according to a previous study [20], and the number of adipocytes was manually counted. If the entire cell border was not visible in the image, the number of adipocytes was considered as 0.5. The number of adipocytes was expressed as cells per mm^2 of tissue area. The cross-sectional area of each adipocyte was manually measured and summed. The mean adipocyte size was calculated by dividing the sum of cross-sectional areas by the number of adipocytes. MAT area was determined by dividing the sum of cross-sectional areas by the total tissue area. Measurements of trabecular bone and adipocyte areas were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

TRAP staining

Tartrate-resistant acid phosphatase (TRAP) staining was performed to visualize osteoclasts 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 trabecular bone area and marrow adiposity), were captured at 20× magnification. The TRAP-positive bone surface length and total bone surface length were manually measured using ImageJ software. Osteoclast surface length was calculated by dividing the TRAP-positive bone surface length by the total bone surface length [10].

Statistical analysis

Data were presented 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 conducted to identify main effects and interactions. In cases where significant main effects were observed without interactions, Bonferroni tests were used for post hoc comparisons between levels. In cases where significant interactions were detected, Bonferroni tests were employed to examine simple main effects. Given that normality assumptions were not met based on the Shapiro-Wilk test, Spearman's correlation coefficients were calculated to assess the relationship between trabecular bone area and MAT area, osteoclast surface length and MAT area, and osteoclast surface length and trabecular bone area. A p-value of < 0.05 was considered statistically significant.

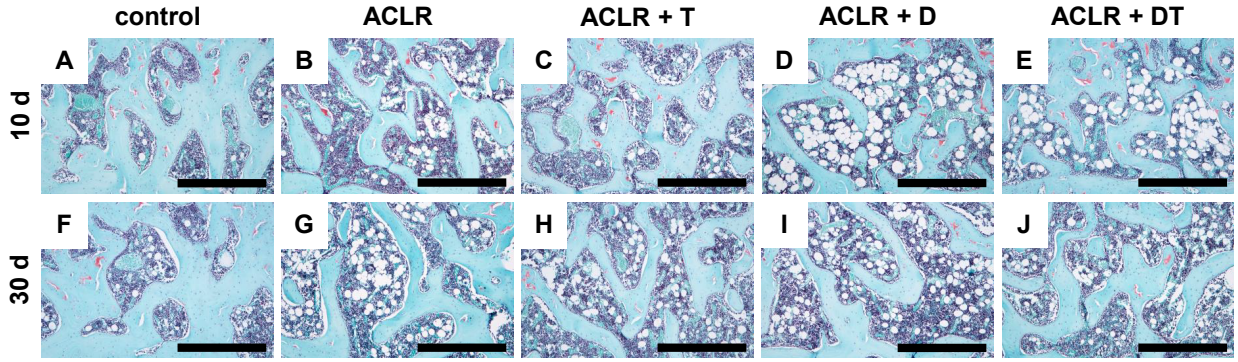

Fig. 2. Histological features in the proximal tibial epiphysis. Representative images of Safranin-O Fast Green-stained sections from the control (**A, F**), ACLR (**B, G**), ACLR + T (**C, H**), ACLR + D (**D, I**), and ACLR + DT (**E, J**) groups. **A–E** and **F–J** represent at day 10 and 30 post-operatively, respectively. Bars = 500 μm. ACLR, anterior cruciate ligament reconstruction; T, treadmill exercise; D, dexamethasone administration.

III. Results

Trabecular bone area

Compared to the control group (Fig. 2A, F), all ACL-reconstructed groups (Fig. 2B–E, G–J) exhibited reduced trabecular bone density at both time points. A two-way ANOVA revealed a significant main effect of intervention on trabecular bone area ($P = 0.001$). Trabecular bone area was significantly smaller in all ACL-reconstructed groups compared to the control group (Fig. 3A, $P \leq 0.036$). No significant differences were observed in trabecular bone area between the ACL-reconstructed groups ($P = 1.000$ for all comparisons). The interaction ($P = 0.576$) and the main effect for time ($P = 0.399$) were not significant.

Osteoclast surface length

Figure 4 shows representative images of TRAP-stained sections. A two-way ANOVA revealed a significant main effect of intervention on osteoclast surface length ($P = 0.023$). In all ACL-reconstructed groups (Fig. 4B–E and G–J), osteoclast surface length tended to be longer than that in the control group (Fig. 3B, Fig. 4A, F), but the difference was significant only between the control and ACLR + D groups ($P = 0.039$). No significant differences were observed in osteoclast surface length between the ACL-reconstructed groups ($P = 1.000$ for all comparisons). The interaction ($P = 0.744$) and the main effect for time ($P = 0.950$) were not significant.

Bone marrow adiposity

A two-way ANOVA revealed a significant main effect of intervention on adipocyte number ($P < 0.001$). Compared to the control group (Fig. 2A, F), all ACL-reconstructed groups (Fig. 2B–E, G–J) showed significantly higher adipocyte numbers (Fig. 3C, $P \leq 0.022$). Among the ACL-reconstructed groups, the ACLR + D group had significantly higher adipocyte numbers compared to the ACLR and ACLR + T groups ($P \leq 0.002$). There were no significant differences in adipocyte numbers between the ACLR vs. ACLR + T groups ($P = 1.000$) and ACLR + D vs. ACLR + DT groups ($P = 0.077$). The interaction ($P = 0.167$) and the main effect for time ($P = 0.398$) were not significant.

A two-way ANOVA revealed a significant interaction between time and group ($P < 0.001$) and significant main effects for both time ($P < 0.001$) and intervention ($P < 0.001$) on adipocyte size. At day 10 post-operatively, the ACLR + D (Fig. 2D) and ACLR + DT (Fig. 2E) groups showed significantly larger adipocyte size compared to the control (Fig. 2A), ACLR (Fig. 2B), and ACLR + T (Fig. 2C) groups (Fig. 3D, $P < 0.001$). At day 30 post-operatively, the ACLR group (Fig. 2G) exhibited significantly larger adipocyte size than the control group (Fig. 2F, Fig. 3D, $P = 0.039$). In the ACLR + D (Fig. 2I) and ACLR + DT (Fig. 2J) groups, adipocyte size at day 30 post-operatively was significantly smaller than at day 10 post-operatively ($P < 0.001$) and not significantly different from all other groups ($P \geq 0.109$). There were no significant differences in adipocyte size between the ACLR vs. ACLR + T groups ($P \geq 0.072$) and ACLR + D vs. ACLR + DT groups ($P \geq 0.790$) at both time points.

A two-way ANOVA revealed a significant interaction between time and group ($P < 0.001$) and a main effect for intervention ($P < 0.001$) on MAT area. At day 10 post-operatively, the ACLR + D (Fig. 2D) and ACLR + DT (Fig. 2E) groups showed significantly larger MAT area compared to the control (Fig. 2A), ACLR (Fig. 2B), and ACLR + T (Fig. 2C) groups (Fig. 3E, $P < 0.001$). MAT area in the ACLR group at day 30 post-operatively (Fig. 2G) was significantly increased compared to day 10 post-operatively ($P = 0.042$) and significantly larger than that in the control group (Fig. 2F, Fig. 3E, $P = 0.003$). MAT area in the ACLR + D group at day 30 post-operatively (Fig. 2I) was significantly decreased compared to day 10 post-operatively ($P = 0.003$) but still significantly larger than that in the control group ($P < 0.001$). MAT area in the ACLR + DT group at day 30 post-operatively (Fig. 2J) was significantly decreased compared to day 10 post-operatively ($P < 0.001$) and not significantly different from all other groups ($P \geq 0.074$). There were no significant dif-

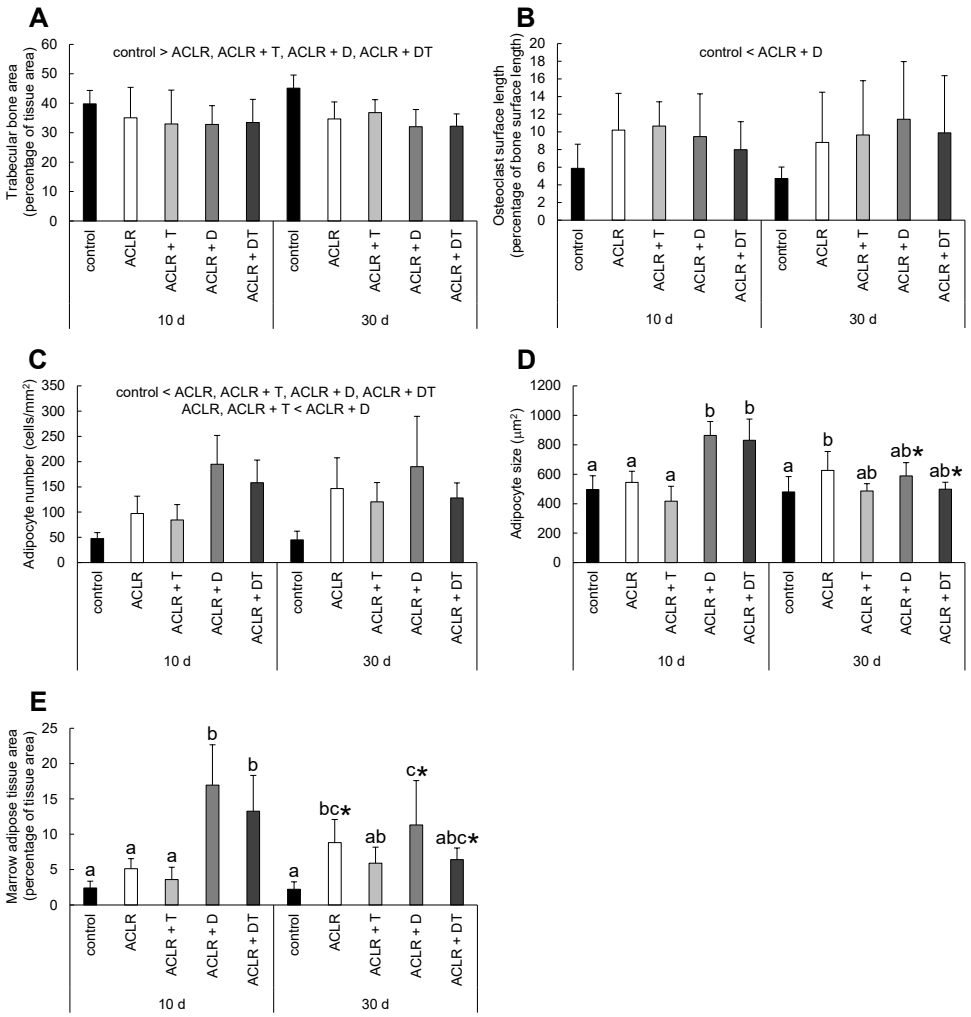

Fig. 3. Trabecular bone area, osteoclast surface length, and bone marrow adiposity. (**A**) Trabecular bone area, (**B**), osteoclast surface length, (**C**) adipocyte number, (**D**) adipocyte size, and (**E**) marrow adipose tissue area. Values are mean ± standard deviation. Different letters indicate statistically significant differences between groups at same timepoint, whereby groups not sharing the same letter are significantly different from one another. *: significant difference compared with day 10 post-operatively. ACLR, anterior cruciate ligament reconstruction; T, treadmill exercise; D, dexamethasone administration.

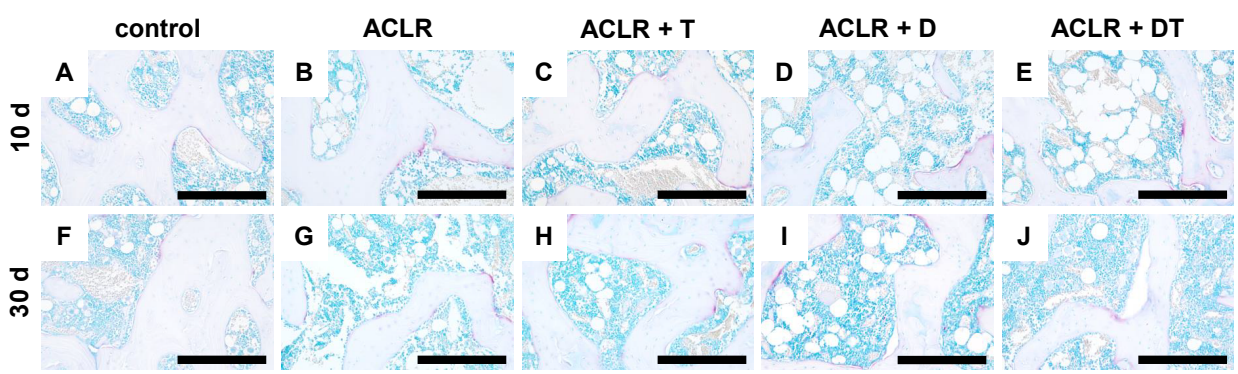

Fig. 4. Osteoclasts in the proximal tibial epiphysis. Representative images of tartrate-resistant acid phosphatase-stained sections from the control (**A, F**), ACLR (**B, G**), ACLR + T (**C, H**), ACLR + D (**D, I**), and ACLR + DT (**E, J**) groups. **A–E** and **F–J** represent at day 10 and 30 post-operatively, respectively. Osteoclasts are stained in purple. Bars = 250 μm. ACLR, anterior cruciate ligament reconstruction; T, treadmill exercise; D, dexamethasone administration.

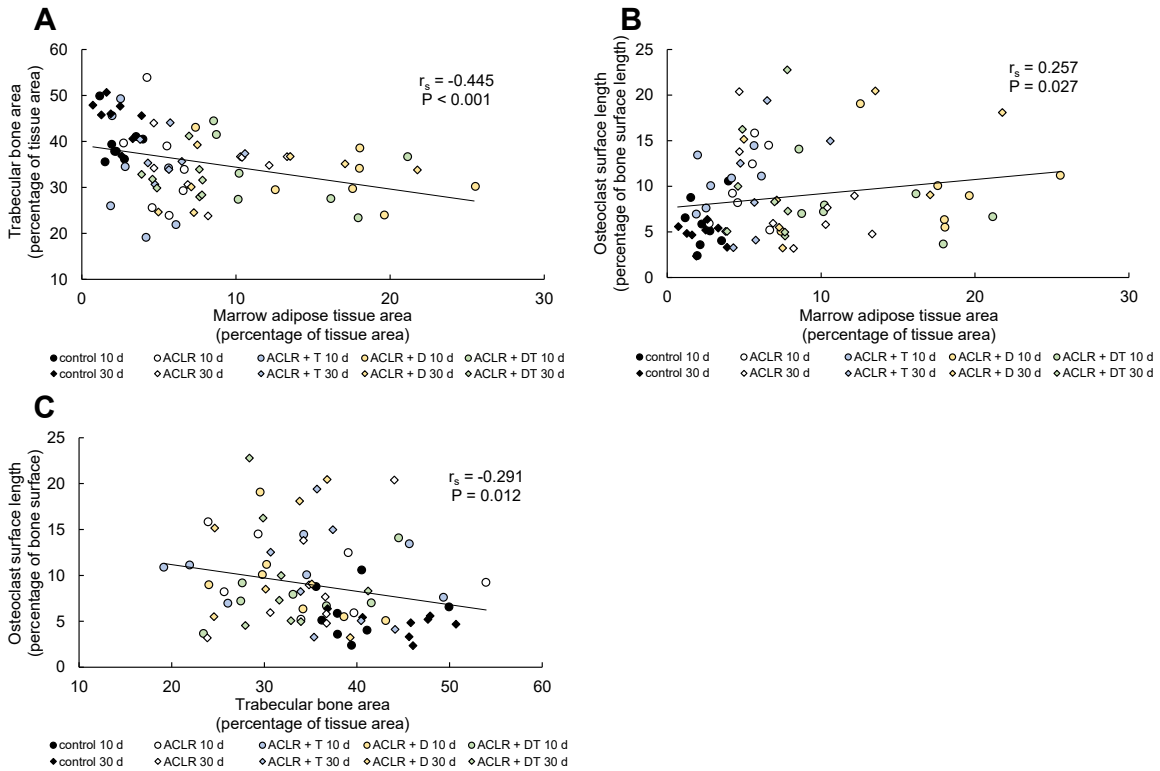

Fig. 5. Correlations between trabecular bone area and marrow adipose tissue area (**A**), osteoclast surface length and marrow adipose tissue area (**B**), and osteoclast surface length and trabecular bone area (**C**). A moderate negative correlation was observed between trabecular bone area and marrow adipose tissue area (**A**). A weak positive correlation was observed between osteoclast surface length and marrow adipose tissue area (**B**). A weak negative correlation was observed between osteoclast surface length and trabecular bone area (**C**). ACLR, anterior cruciate ligament reconstruction; T, treadmill exercise; D, dexamethasone administration.

ferences in MAT area between the ACLR vs. ACLR + T groups (P = 1.000) and ACLR + D vs. ACLR + DT groups (P ≥ 0.074) at both time points. The main effect for time was not significant (P = 0.099).

Correlation analysis

A moderate negative correlation was observed between trabecular bone area and MAT area (Fig. 5A, r_s = −0.445, P < 0.001). A weak positive correlation was observed between osteoclast surface length and MAT area (Fig. 5B, r_s = 0.257, P = 0.027). A weak negative correlation was observed between osteoclast surface length and trabecular bone area (Fig. 5C, r_s = −0.291, P = 0.012).

IV. Discussion

The primary aim of this study was to investigate the effects of short-term administration of corticosteroid after ACL reconstruction on MAT and trabecular bone. Administration of dexamethasone, a corticosteroid, after ACL reconstruction promoted MAT accumulation when administered up to day nine post-operatively, while it did not affect trabecular bone area. The MAT accumulation induced by dexamethasone administration reversed within 21 days after discontinuation. The secondary aim of this study was to examine whether treadmill exercise could mitigate the changes in MAT and trabecular bone caused by corticosteroid administration. Concurrent use of treadmill exercise with dexamethasone did not counteract the effects of dexamethasone on MAT accumulation and did not affect trabecular bone area.

Previous studies have reported MAT accumulation [13] and a decrease in bone mineral density [21] in the proximal tibia after ACL reconstruction. Consistent with these findings, we also observed an increase in MAT area and a decrease in trabecular bone area in the proximal tibia in the ACLR group. It is considered that MAT accumulation has negative effects on bone mass through osteoclastogenesis [23]. In our study, a weak but significant positive correlation was detected between osteoclast surface length and MAT area, and osteoclast surface length in the ACLR group was tended to be longer than that in the control group, although the difference was not significant. Furthermore, significant negative correlations between trabecular bone area vs. MAT area and trabecular bone area vs. osteoclast surface length were identified, suggesting that the increased MAT may contribute, at least partially, to the decrease in trabecular bone area observed after ACL reconstruction through osteoclastogenesis.

Short-term administration of dexamethasone after

ACL reconstruction promoted MAT accumulation. Compared to the control group, the ACLR + D group exhibited a significant increase in MAT area at day 10 post-operatively, whereas the ACLR group did not show such an increase. Furthermore, the MAT area at day 10 post-operatively was significantly larger in the ACLR + D group than in the ACLR group. These findings indicate that dexamethasone administration promotes MAT accumulation when administered up to day nine post-operatively. Adipocytes are differentiated from marrow stromal cells, and dexamethasone stimulates adipocyte differentiation in rat marrow stromal cell cultures [1]. Another study showed that corticosteroid (methylprednisolone) administration increased not only adipocyte number but also adipocyte size in the rabbit femur [30]. Consistent with these studies, our results showed a significantly higher number of adipocytes and larger adipocyte size in the ACLR + D group compared to the control and ACLR groups at day 10 post-operatively, indicating that MAT accumulation in the ACLR + D group was mediated by adipocyte hyperplasia and hypertrophy.

Upon discontinuation of dexamethasone administration, the MAT area decreased. Specifically, the MAT area in the ACLR + D group significantly decreased at day 30 compared to day 10 post-operatively, and there was no significant difference in the MAT area between the ACLR and ACLR + D groups at day 30 post-operatively. This reduction in MAT area after discontinuation of dexamethasone administration appeared to be primarily attributed to adipocyte atrophy, as evidenced by the significant decrease in adipocyte size in the ACLR + D group at day 30 compared to day 10 post-operatively. Our findings suggest that adipocyte hypertrophy and MAT accumulation induced by short-term corticosteroid administration after ACL reconstruction are reversible upon discontinuation of administration.

The accumulation of MAT is often linked to bone loss [26]. Consistent with this, a moderate negative correlation was observed between MAT area and trabecular bone area, as mentioned earlier. However, the increase in MAT area observed in the ACLR + D group at day 10 post-operatively was not accompanied by an increase in osteoclast surface length and a decrease in trabecular bone area when compared to the ACLR group. This suggests that the transient increase in MAT resulting from short-term dexamethasone administration may not be sufficient to cause significant bone loss, as sustained MAT accumulation would be required for that.

No significant differences in MAT and trabecular bone parameters were found between the ACLR vs. ACLR + T groups and the ACLR + D vs. ACLR + DT groups, indicating that treadmill exercise had no significant impact on the changes in MAT and trabecular bone induced by ACL reconstruction and dexamethasone administration. A previous study also reported that two or four weeks of treadmill exercise after ACL reconstruction did not affect MAT and trabecular bone parameters [17]. Treadmill exercise did not suppress the increase in MAT area caused by dexamethasone administration. Previous studies have shown that running exercise can inhibit MAT increase induced by high-fat diet or diet-induced obesity [32, 33]. Conversely, patients with anorexia nervosa, despite engaging in aerobic exercise and high energy expenditure, exhibit MAT accumulation [6]. These findings suggest that the regulation of MAT mass is complex, and the effects of exercise on MAT may vary depending on the context. MAT accumulation during caloric restriction is associated with elevated blood corticosteroid levels [3]. In conjunction with our results, this suggests that exercise is unable to counteract corticosteroid-induced MAT accumulation.

Our study has limitations. We employed histological methods to assess MAT and trabecular bone mass. While histological analysis allows for detailed information on adipocyte number and size [13, 17, 36, 37], it has a limited analysis area compared to osmium micro-computed tomography and magnetic resonance imaging, which are alternative methods for evaluating MAT and trabecular bone mass in rodent models [24, 33]. The administration of dexamethasone was short-term (up to day nine post-operatively). This may explain why dexamethasone had no significant effect on trabecular bone parameters. Clinically, if corticosteroids are used to alleviate pain and joint contracture after ACL reconstruction, the duration of administration is usually less than one week [18, 28]. Thus, we set the duration of dexamethasone administration up to day nine post-operatively. A previous study reported that MAT accumulation in the distal femur induced by two weeks of dexamethasone administration was not reversed even eight weeks after discontinuation of administration [34]. Thus, longer-term dexamethasone administration may induce more sustained MAT changes. The dose of dexamethasone in this study (250 μg/kg dose) may be higher than the dose typically administered to patients after ACL reconstruction (8–10 mg dose) [4, 18], and thus, the short-term effects of dexamethasone might be overestimated. However, in postmenopausal women with corticosteroid-induced osteoporosis, there was no correlation between the accumulated corticosteroid dose and MAT area [31]. Treadmill exercise was initiated on day three post-operatively when limping was observed [14]. The insufficient weight-bearing on the knee due to limping might contribute to the lack of effects of treadmill exercise on MAT and trabecular bone parameters. Treadmill exercise started after the resolution of limping may have different effects.

In conclusion, our findings demonstrate that short-term corticosteroid administration after ACL reconstruction promotes MAT accumulation while not affecting trabecular bone area and osteoclastogenesis. The MAT accumulation resulting from short-term corticosteroid administration is reversible upon discontinuation of treatment. Treadmill exercise did not mitigate the MAT accumulation induced by corticosteroid administration after ACL reconstruction.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Funding

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