

# Effects of Joint Immobilization and Treadmill Exercise on Articular Cartilage After ACL Reconstruction in Rats

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**Background:** The development of osteoarthritis after anterior cruciate ligament (ACL) reconstruction (ACLR) is an important issue. However, the appropriate rehabilitation protocol to prevent cartilage degeneration due to postoperative osteoarthritis is unclear.

**Purpose:** To examine the effects of joint immobilization and treadmill exercise on articular cartilage after ACLR.

**Study Design:** Controlled laboratory study.

**Methods:** A total of 55 rats received unilateral knee ACL transection and reconstruction surgery using tail tendon autografts. After surgery, rats were reared without intervention, with joint immobilization, or with daily treadmill exercise (12 m/minute, 60 minutes/day, 6 days/week). Treadmill exercise was initiated at 3 or 14 days postoperatively. After 2 weeks of immobilization, the fixation device was removed from some of the immobilized rats, and the knee was allowed to move freely for 2 weeks. Untreated, age-matched rats ( $n = 8$ ) were used as controls. At 2 or 4 weeks after starting the experiment, cartilage degeneration in the medial tibial plateau was histologically assessed using a modified Mankin score, cartilage thickness, chondrocyte density, and immunohistochemistry for cyclooxygenase-2 (COX-2) in the anterior, middle, and posterior regions.

**Results:** After ACLR, cartilage degeneration in the anterior region characterized by increased Mankin score, accompanied with increased COX-2 expression, was detected. Joint immobilization after ACLR facilitated cartilage degeneration, which is detected by histological changes such as reductions in cartilage thickness, chondrocyte density, and high Mankin scores. Enhanced COX-2 expression in all degenerated cartilage regions was also detected. It was found that 2 weeks of remobilization could not restore cartilage degeneration induced by 2 weeks of immobilization after ACLR. Treadmill exercise after ACLR did not affect most articular cartilage parameters, regardless of the timing of exercise.

**Conclusion:** Our results indicated that (1) immobilization after ACLR accelerates cartilage degeneration, even when applied only for 2 weeks, and (2) mild exercise during early phases after ACLR does not facilitate cartilage degeneration.

**Clinical Relevance:** To reduce cartilage degeneration, periods of joint immobilization after ACLR should be minimized. Mild exercise during the early phases after ACLR will not negatively affect articular cartilage.

**Keywords:** ACL reconstruction; cartilage degeneration; exercise; joint immobilization; osteoarthritis

Anterior cruciate ligament (ACL) injury is one of the most common knee injuries and induces joint instability.<sup>5</sup> Patients with ACL injuries are significantly more likely to develop knee osteoarthritis.<sup>39</sup> Post-traumatic osteoarthritis accounts for nearly 12% of symptomatic osteoarthritis<sup>49</sup> and thus is an important problem in the field of orthopaedics.

ACL reconstruction (ACLR) surgery is the most common treatment for ACL injury and can restore knee stability.<sup>4</sup> ACLR may reduce the risk of developing knee osteoarthritis, but it cannot fully eliminate the risk.<sup>20,37</sup> Consequently, after more than 20 years of ACLR, moderate-to-severe radiographic osteoarthritis is present in about 30% of

cases.<sup>12,43</sup> Therefore, the development of treatment strategies to prevent osteoarthritis after ACL injury and reconstruction is an important issue.

Rehabilitation protocols after ACLR are roughly divided into traditional and accelerated rehabilitation.<sup>14</sup> Traditional rehabilitation uses joint immobilization and prohibits weightbearing activities in early postoperative periods to protect the graft, then gradually permits joint motion and weightbearing.<sup>14</sup> In accelerated rehabilitation, weightbearing activities, as tolerated, and joint motion are permitted immediately after surgery to avoid the negative effects of disuse.<sup>14</sup> Beynon et al<sup>2</sup> examined biomarkers of cartilage metabolism in synovial fluid and reported that biomarkers did not differ between traditional and accelerated rehabilitation protocols. However, they did not examine articular cartilage itself; thus, the appropriate rehabilitation protocol

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to prevent cartilage degeneration due to postoperative osteoarthritis is unclear.

Some animal studies report effects of joint immobilization or exercise on articular cartilage after ACL transection and reconstruction/repair. Palmoski and Brandt<sup>36</sup> reported that joint immobilization after ACL transection prevented articular cartilage degeneration. Conversely, 6 weeks of joint immobilization after ACLR or repair can aggravate cartilage degeneration.<sup>38,42</sup> However, it is unknown whether a shorter duration of joint immobilization after ACLR also aggravates cartilage degeneration.

Regarding the effects of exercise, several studies indicate that treadmill exercise after ACL transection can facilitate cartilage degeneration.<sup>9,10,33,46,51</sup> Conversely, treadmill exercise performed with reduced joint instability by extra-articular bracing (surgical stabilization using nylon thread) does not facilitate cartilage degeneration.<sup>33</sup> Moreover, Sun and Peng<sup>46</sup> reported that treadmill exercise combined with hinged external fixator attenuated ACL transection-induced cartilage degeneration. These findings suggest that treadmill exercise for ACL-deficient knees with joint instability negatively affects articular cartilage, while exercise for restabilized knees does not facilitate cartilage degeneration and may generate positive impacts. However, the effects of exercise for ACL-reconstructed knees on articular cartilage have not been investigated.

In this study, we aimed to examine the effects of joint immobilization and treadmill exercise on articular cartilage degeneration after ACLR in rats. We hypothesized that cartilage degeneration after ACLR is facilitated by joint immobilization, even if it is performed in the short term, while cartilage degeneration is attenuated by treadmill exercise.

## METHODS

### Experimental Animals

The study protocol was approved by the animal experimentation committee of our institution. We used 8-week-old male Wistar rats (Japan SLC) in this study. A total of 77 rats were randomly divided into 7 groups: control (n = 8), immobilization (IM; n = 14), ACLR plus sedentary (ACLR + SED; n = 14), ACLR + SED followed by treadmill exercise (ACLR + SED/EX; n = 6), ACLR + EX (n = 13), ACLR plus IM (ACLR + IM; n = 15), and ACLR + IM followed by SED (ACLR + IM/SED; n = 7) (Figure 1).

Until 2 weeks postoperatively, rats in the ACLR + SED and ACLR + SED/EX groups were identical (Figure 1). Similarly, rats in the ACLR + IM and ACLR + IM/SED groups were identical until 2 weeks postoperatively

(Figure 1). In the control group, data from the right and left knees were considered individual samples. Therefore, we analyzed data from 8 knees in 4 rats at each time point. The unoperated left knees in the ACLR + EX group were used as the EX group to assess the effects of treadmill exercise alone (Figure 1). Data were collected at 2 or 4 weeks after starting the experiment. Rats were housed in standard cages in a temperature-controlled room (20°C -25°C) with a 12-hour light/dark cycle. Standard rodent food and water were provided ad libitum.

### ACLR Reconstruction

For rats that underwent ACLR, we performed ACL transection and reconstruction surgery on the right knee using the methods described in previous studies.<sup>24,26</sup> Briefly, tail tendons were collected from anesthetized rats (ketamine; 80 mg/kg, xylazine; 10 mg/kg, intraperitoneally), and quadruple-bundle autografts were prepared. After opening the knee joint via a medial parapatellar approach, the ACL was exposed and transected using a surgical knife. Bone tunnels from the anteromedial side of the proximal tibia to the lateral side of the distal femur were drilled using a 0.8 mm-diameter Kirschner wire. Autografts were then passed through the bone tunnels and the proximal and distal ends were fixed to the femur and tibia, respectively, by stainless steel interference screws (0.8 mm-diameter and 2.0 mm-length, TE-00001; Matsumoto). Finally, the joint capsule and skin were sutured. The knees of rats in the control and IM groups remained intact.

### Joint Immobilization

The right knees of rats in the IM, ACLR + IM, and ACLR + IM/SED groups were immobilized according to methods described previously.<sup>21,29</sup> Briefly, rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), after which 0.8 mm-diameter Kirschner wires were screwed into the proximal femur and distal tibia and fixed with wire and resin (Provincine Fast; Shofu) at a flexion angle of 140°. Immobilization for rats in the ACLR + IM and ACLR + IM/SED groups was performed immediately after ACLR surgery. During joint immobilization, rats could move freely using 4 limbs. After 2 weeks of immobilization, the fixation device was removed from rats in the ACLR + IM/SED group, and the knee was allowed to move freely for 2 weeks.

### Treadmill Exercise

Rats in the ACLR + EX and ACLR + SED/EX groups performed daily treadmill exercises on a low-speed (12 m/minute)

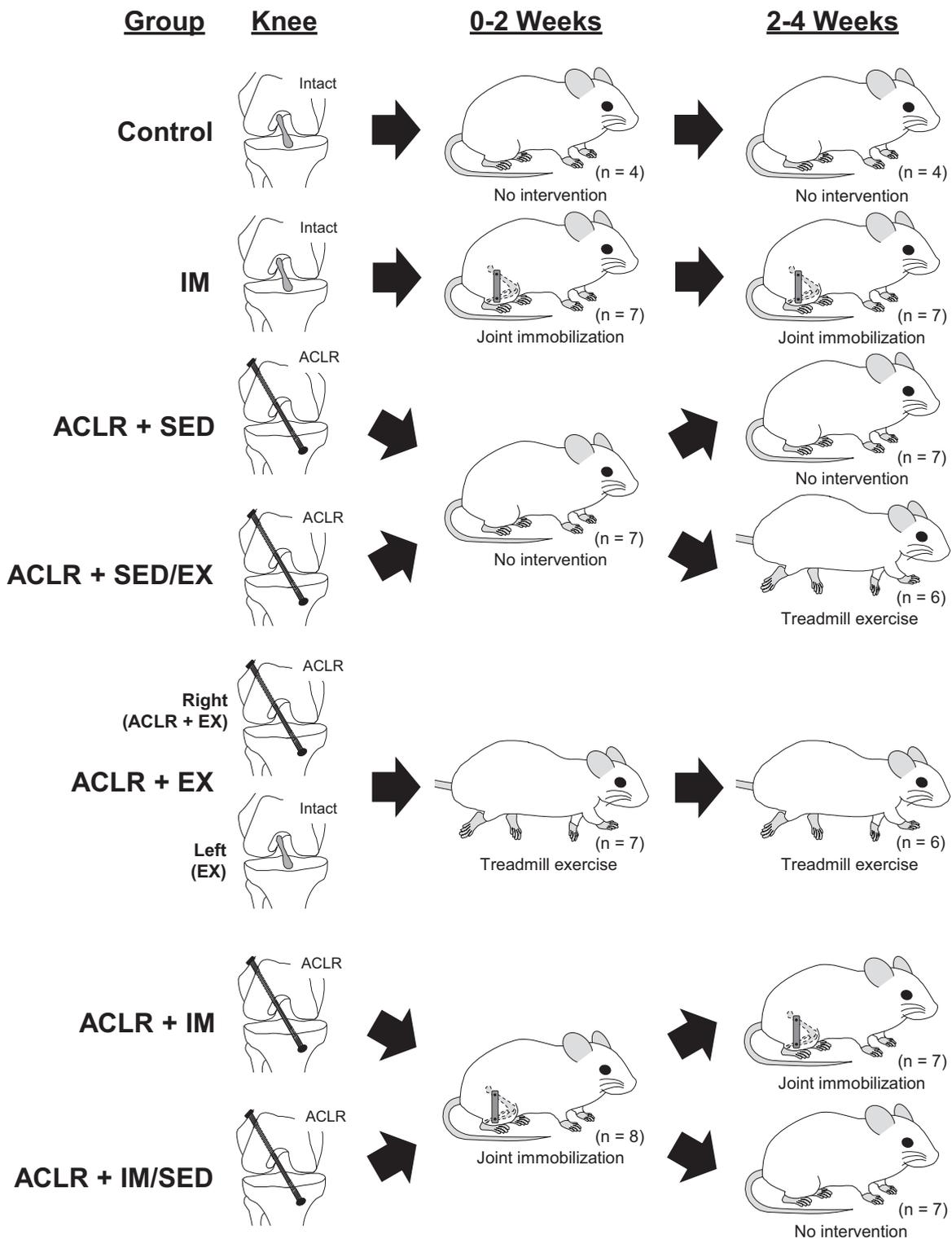
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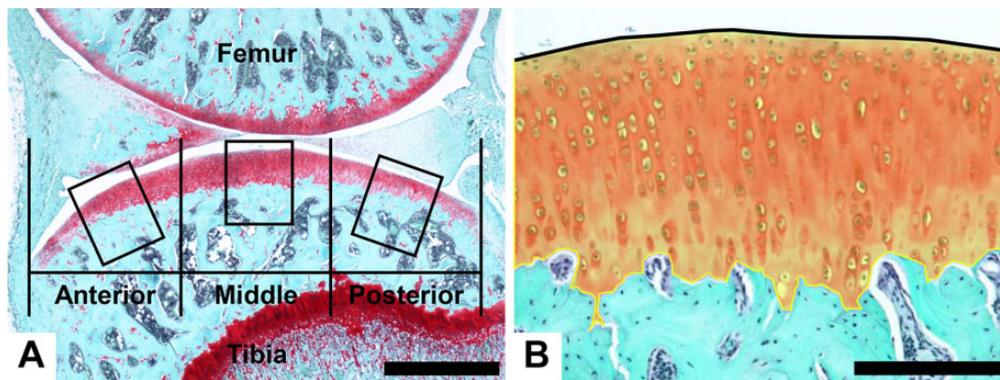
Ethical approval for this study was granted by Hiroshima International University (ref No. AE20-022).



**Figure 1.** Experiment protocol (N = 77 male Wistar rats). ACLR, anterior cruciate ligament reconstruction; EX, exercise; IM, immobilization; SED, sedentary.

rodent treadmill machine (Rat runner; Agawa), according to methods described previously.<sup>22,23</sup> The intervention consisted of 6, 10-minute exercise sessions separated by

1-minute intervals; thus, the rats walked for a total of 60 minutes per day. The intervention was initiated at 3 days postoperatively in the ACLR + EX group and at



**Figure 2.** Methods of regional analysis and measurement of cartilage thickness in the medial tibial plateau. (A) The patellar tendon insertion onto the tibia and the posterior capsule tibial insertion were used as anterior and posterior landmarks, respectively. Articular surface was divided equally into the anterior, middle, and posterior regions. The central region in each region was then photographed at 10 $\times$  magnification (boxes) and used to measure cartilage thickness and chondrocyte density. In addition, histopathological scoring in each region was performed using a modified Mankin scoring system. (B) Cartilage thickness was calculated by dividing the area of cartilage matrix (yellow stained area) by the length of the cartilage surface (black solid line). Scale bars: (A) 1 mm; (B) 200  $\mu$ m.

14 days in the ACLR + SED/EX rats and performed 6 days per week. Control rats and rats in the ACLR + SED group were allowed to move freely in their cage without any intervention.

### Histological Analysis

At the end of the experimental period (2 or 4 weeks postoperatively), rats were sacrificed by exsanguination under anesthesia. The knees were sampled and immersion-fixed in 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4) for 2 days at 4°C. Samples were then decalcified using 17.7% EDTA (pH 7.2; Osteosoft; Merck Millipore) for 1 month at room temperature and embedded in paraffin. Sagittal sections (4  $\mu$ m thickness) were made at the medial midcondylar level and stained with Safranin O–Fast Green.

We focused our analyses on the articular cartilage in the medial tibial plateau due to osteoarthritis after ACLR's being most frequently observed in the medial tibiofemoral joint.<sup>8,12,28</sup> The medial tibial plateau was divided into the anterior, middle, and posterior regions according to methods described by Campbell et al<sup>7</sup> and the center of each region was photographed at 10 times magnification (Figure 2A). Cartilage thickness and chondrocyte density were then assessed as described previously.<sup>35</sup> Cartilage thickness was calculated by dividing the area of cartilage matrix by the length of the cartilage surface (Figure 2B). To calculate chondrocyte density, chondrocyte number was counted manually and represented as cells per square millimeter of cartilage matrix area. ImageJ software (National Institutes of Health) was used to measure cartilage matrix area, cartilage surface length, and chondrocyte number.

Histopathological scoring in the anterior, middle, and posterior regions of the articular cartilage was performed using a modified Mankin scoring system.<sup>48</sup> Three criteria, including (1) structure, (2) cellular abnormalities, and (3)

matrix staining, were scored and a total score was calculated (Figure 3). This system used a scale of 0 to 13, with 0 being normal and 13 being the most degenerated. Histopathological scoring was conducted by 2 examiners (A.K. and J.O.), and the means were calculated. Histological analysis was performed using 1 slide per sample.

### Immunohistochemistry for Anti-Cyclooxygenase 2

After deparaffinization and rehydration, sections were incubated with methanol containing 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes to block endogenous peroxidase activity. After nonspecific binding was blocked by incubating with 1% normal horse serum in 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 30 minutes, sections were incubated with an anti-cyclooxygenase-2 (COX-2) antibody (No. 18955, 0.5  $\mu$ g/ml; Immuno-Biological Laboratories) overnight at 4°C. The negative control was incubated with vehicle (1% bovine serum albumin) instead of an anti-cyclooxygenase 2 (anti-COX 2) antibody. After rinsing with PBS, sections were incubated with a secondary antibody (horse biotinylated anti-mouse/rabbit IgG, 1:250 dilution; BA-1400; Vector Laboratories) for 30 minutes, followed by incubation with a streptavidin-biotin complex (1:50 dilution; Elite ABC; Vector Laboratories) for 30 minutes. The immunoreaction was visualized by Dako EnVision + kit/HRP (DAB) (Dako Japan). Finally, counterstaining with hematoxylin was performed.

Semiquantitative assessment of COX-2 staining was performed according to methods described previously.<sup>18</sup> Briefly, the anterior, middle, and posterior regions were photographed at 20 $\times$  magnification. As shown in Table 1, the extent of COX-2 positive cells was scored 0 to 4, and the staining intensity was scored 0 to 3. The scores of extent and intensity were multiplied, and the total score was calculated. Higher scores indicate a higher expression of COX-2.

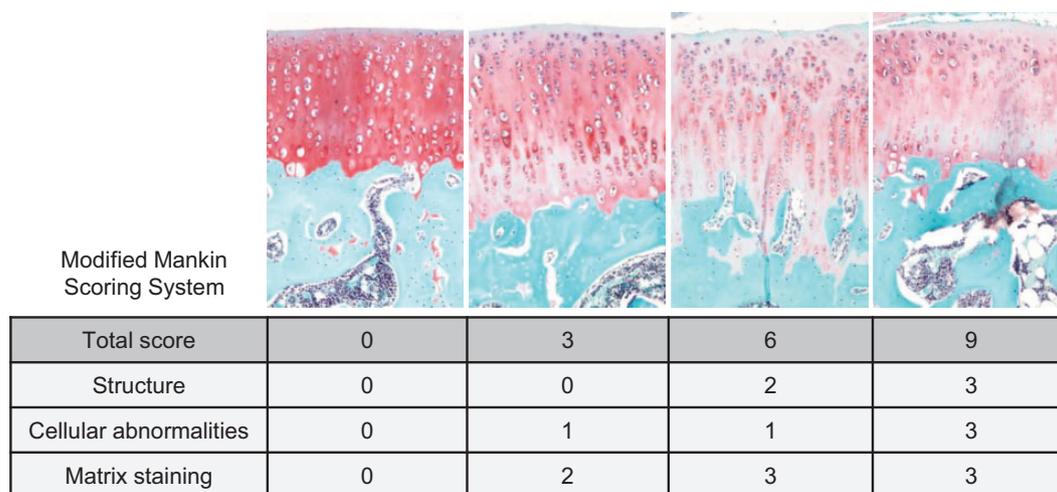


Figure 3. Examples of modified Mankin scoring system.

TABLE 1  
Semiquantitative Scoring System for COX-2 Staining

Extent	Score	Intensity	Score
No cells stained	0	Negative	0
1%-10% of cells stained	1	Weak	1
11%-50% of cells stained	2	Moderate	2
51%-80% of cells stained	3	Strong	3
81%-100% of cells stained	4		

### Statistical Analysis

Figures show data of cartilage thickness and chondrocyte density expressed as means and standard deviations using bar graphs for the continuous variables. Mankin score and COX-2 expression score data are expressed as medians and interquartile range using boxplots and tables, respectively, for the ordinal variables. Statistical analyses were performed using Dr. SPSS II for Windows (SPSS Japan) and SPSS Version 22.0 for Macintosh (IBM). Two-way analyses of variance were used to detect significant main effects or interactions. Upon detecting significant main effects, Bonferroni tests were performed to compare among levels. If significant interactions were detected, Bonferroni tests were performed to detect simple main effects. A *P* value of < .05 was considered statistically significant. The interrater reliability coefficient for Mankin score was calculated with the intraclass correlation coefficient (ICC[2, 1]) using SPSS Version 22.0 for Macintosh.

## RESULTS

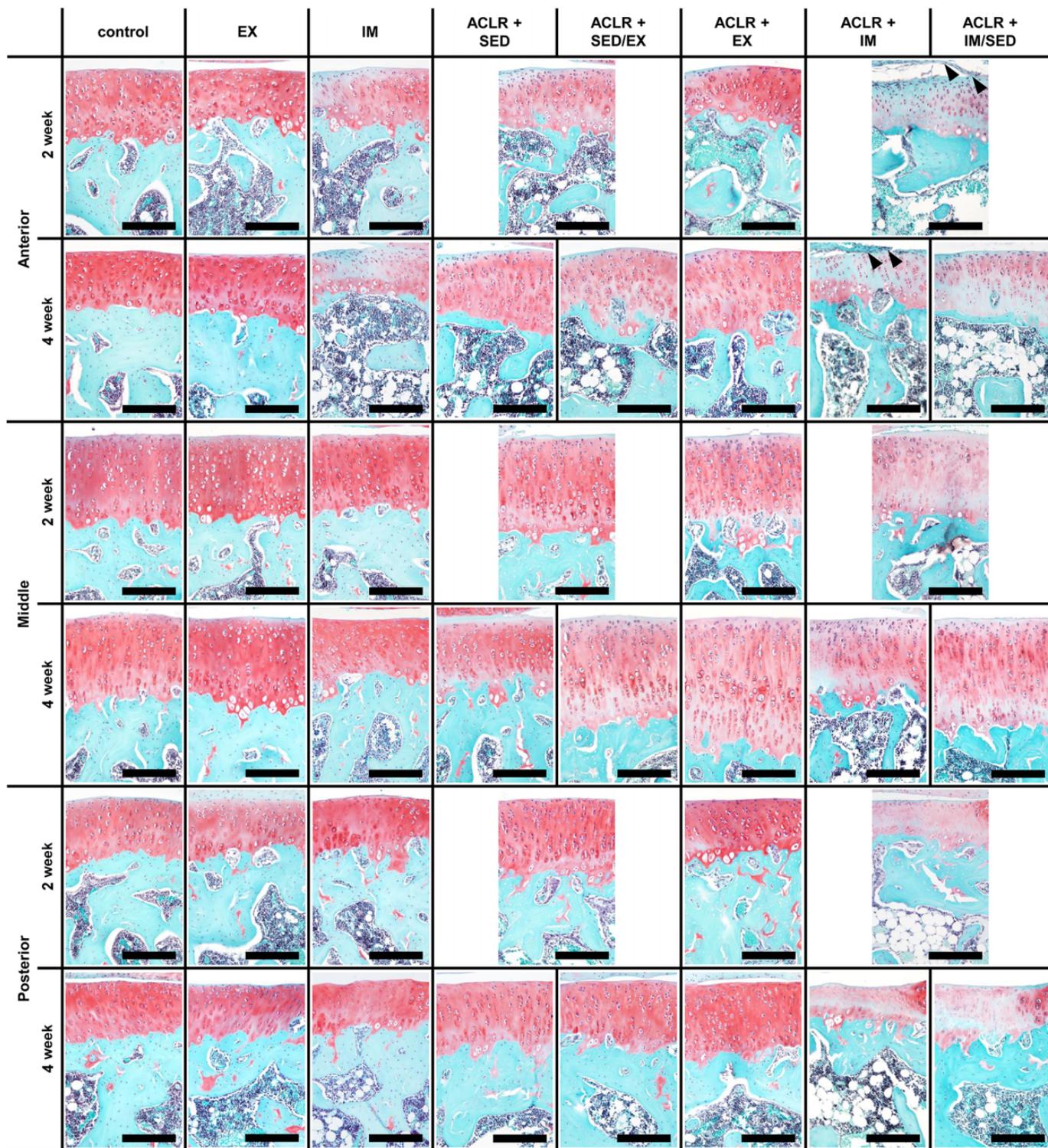
A total of 1 rat in the IM group and 1 rat in the ACLR + IM group that were scheduled to be sacrificed at 2 weeks postoperatively died due to anesthesia during surgery. Thus, 6 and 7 rats were included in the analyses in the IM and ACLR + IM groups, respectively, at 2 weeks postoperatively.

### Histological Features of the Articular Cartilage in the Medial Tibial Plateau

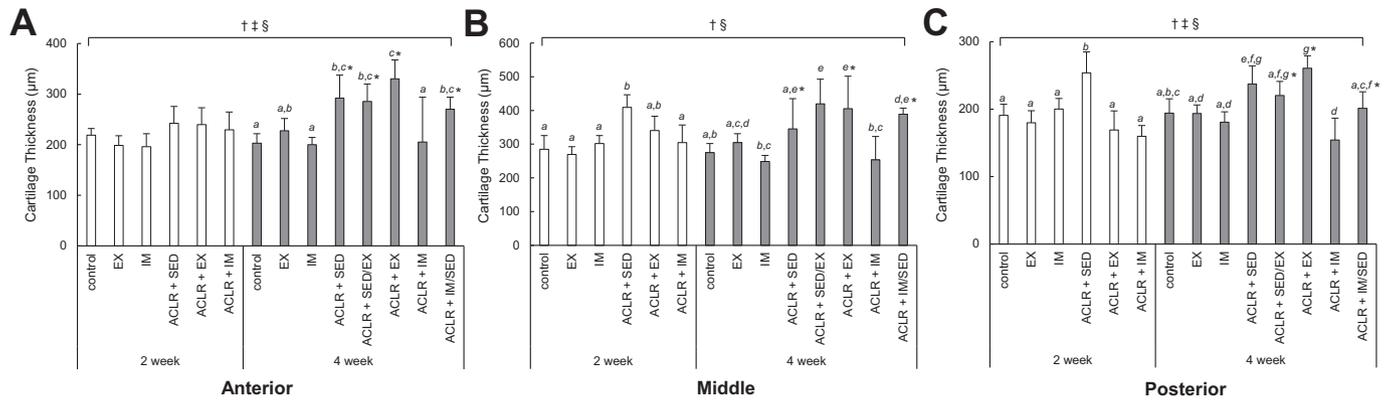
Figure 4 shows representative images of the sections stained using Safranin O–Fast Green. In the control and EX groups, no apparent abnormalities were observed for any regions. In the IM group, the staining intensity of Safranin O in the anterior region decreased, and hypocellularity was observed in the posterior region. In the ACLR + SED group, reduced Safranin O staining intensity was detected, especially in the anterior and middle regions. Articular cartilage thickness in all regions tended to be thicker in the ACLR + SED group than in the control group. In the ACLR + SED/EX group, histological features were similar to the ACLR + SED group. In the ACLR + EX group, the number of chondrocytes increased in the middle and posterior regions at 2 weeks postoperatively, and Safranin O staining intensity decreased in the middle region. In the ACLR + EX group, articular cartilage in all regions was thicker at 4 weeks postoperatively. In the ACLR + IM group, formation of pannus-like tissue on the articular cartilage, as well as an apparent reduction in Safranin O staining intensity, was observed in the anterior region. In the middle and posterior regions of the ACLR + IM group, hypocellularity and decreased Safranin O staining intensity were observed. In addition, cartilage thickness in the posterior region tended to be thinner compared with all other groups. In the ACLR + IM/SED group, overall histological features of the articular cartilage were similar to the ACLR + IM group, but articular cartilage thickness in the anterior and middle regions tended to be thicker than those in the control and ACLR + IM groups.

### Cartilage Thickness

In the EX and IM groups, cartilage thickness was not different from the control group in all regions (Figure 5). Cartilage thickness in the ACLR + SED, ACLR + SED/EX, and ACLR + EX groups was significantly thicker than that



**Figure 4.** Histological features of articular cartilage in the medial tibial plateau, as shown in sections stained with Safranin O–Fast Green. Cartilage in the control group had strong Safranin O staining, indicating the presence of rich proteoglycan, and the cartilage surface was smooth in all regions. In the EX group, histological features matched those of the control group. In the IM group, we observed reduced Safranin O intensity in the anterior region and hypocellularity in the posterior region. In the ACLR + SED group, some signs of degeneration (reduced Safranin O intensity and a small surface irregularity) were detected in all regions. Articular cartilage thickness in all regions tended to be thicker in the ACLR + SED group than in the control group. In the ACLR + SED/EX and ACLR + EX groups, histological features in all regions were similar to the ACLR + SED group. In the ACLR + IM group, formation of pannus-like tissue on the articular cartilage (arrowheads), as well as an apparent reduction in Safranin O intensity, was observed in the anterior region. In the middle region in the



**Figure 5.** Cartilage thickness in the (A) anterior, (B) middle, and (C) posterior regions. Values are expressed as means  $\pm$  SD. †Significant time  $\times$  intervention interaction. ‡Significant main effect of time. §Significant main effect of intervention. Different lowercase letters indicate statistically significant differences between groups within the same time point, such that groups not sharing the same letter are significantly different from one another. \*Significant difference versus 2 weeks postoperatively. Cartilage in the ACLR + SED, ACLR + SED/EX, and ACLR + EX groups was significantly thicker versus the control group in all regions. Cartilage thickness in the ACLR + IM group was significantly thinner versus the ACLR + SED group in all regions. Cartilage thickness in the ACLR + IM/SED was significantly thicker versus the ACLR + IM group in all regions. ACLR, anterior cruciate ligament reconstruction; EX, exercise; IM, immobilization; SED, sedentary.

in the control group in all regions at 2 and/or 4 weeks postoperatively. Between the ACLR + SED and ACLR + SED/EX or ACLR + EX groups, there were no differences in cartilage thickness, except in the posterior region at 2 weeks (ACLR + SED group > ACLR + EX group). In the ACLR + IM group, cartilage thickness in all regions was significantly thinner than that in the ACLR + SED group at 2 and/or 4 weeks postoperatively, and cartilage thickness in the posterior region was significantly thinner than that in the control group at 4 weeks postoperatively. Cartilage thickness in the ACLR + IM/SED was significantly thicker than that in the ACLR + IM group in all regions at 4 weeks postoperatively.

### Chondrocyte Density

Chondrocyte density in the EX, ACLR + SED, ACLR + SED/EX, and ACLR + EX groups was not different from the control group in all regions (Figure 6). There were no differences in chondrocyte density between the ACLR + SED and ACLR + SED/EX or ACLR + EX groups. In the middle region, chondrocyte density in the ACLR + IM group was significantly lower than other groups, except for the control and ACLR + IM/SED groups. In the posterior region, chondrocyte density in the IM, ACLR + IM, and ACLR + IM/SED groups was significantly lower than that in the control and ACLR + SED groups. There were no

differences in chondrocyte density between the ACLR + IM and ACLR + IM/SED groups.

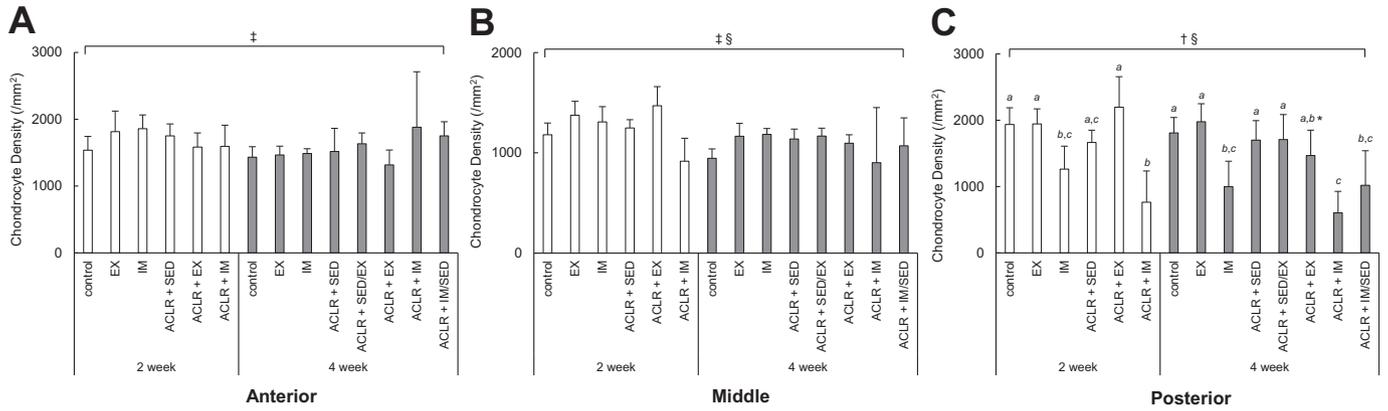
### Mankin Score

The Mankin score in the EX group was not different from the control group in all regions (Figure 7). In the IM group, Mankin scores in the anterior and posterior regions were significantly higher than those in the control group. In the ACLR + SED group, Mankin scores in the anterior region was significantly higher than that in the control group. In the ACLR + SED/EX and ACLR + EX groups, Mankin scores in the anterior and middle regions were significantly higher than those in the control group. There were no differences in Mankin scores between the ACLR + SED and ACLR + SED/EX or ACLR + EX groups. The Mankin score in the ACLR + IM group was significantly higher than those in the control, IM, and ACLR + SED groups in all regions. In the ACLR + IM/SED group, the Mankin score was not different from the ACLR + IM group in all regions. The interrater ICCs for the Mankin scores were 0.917, 0.875, and 0.965 in the anterior, middle, and posterior regions, respectively, indicating excellent agreement.

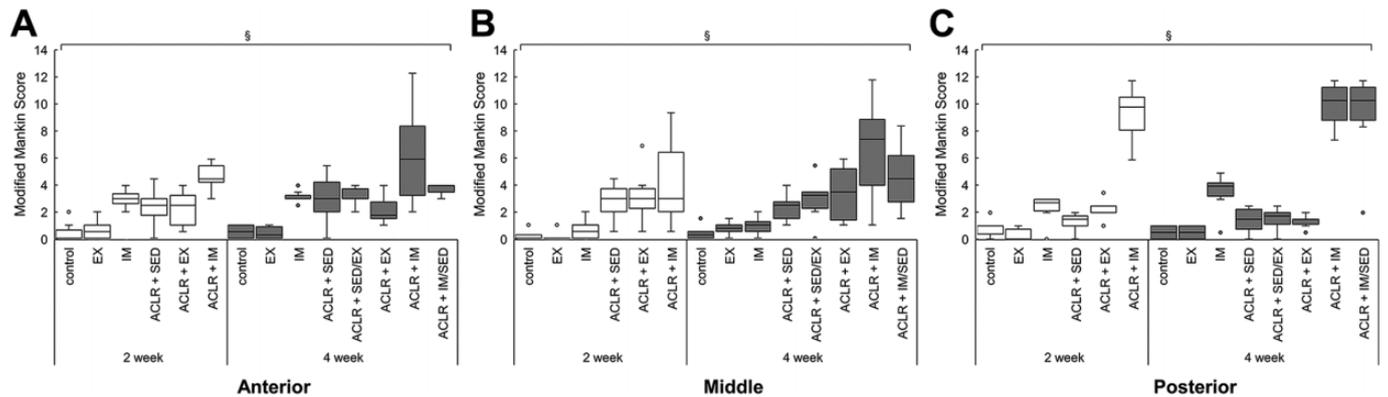
### COX-2 Expression

Figure 8 shows representative images of the COX-2-immunostained sections. In the posterior region, 1 sample

**Figure 4.** (Continued). ACLR + IM group, a small surface irregularity, hypocellularity, and decreased Safranin O intensity were observed. In the posterior region in the ACLR + IM group, the number of chondrocytes and Safranin O intensity were markedly reduced at both time points, and cartilage thickness in the posterior region appeared thinner compared with all other groups. In the ACLR + IM/SED group, histological features were similar to the ACLR + IM group, although articular cartilage thickness in the anterior and middle regions appeared thicker compared with the ACLR + IM group. Scale bars, 200  $\mu$ m. ACLR, anterior cruciate ligament reconstruction; EX, exercise; IM, immobilization; SED, sedentary.



**Figure 6.** Chondrocyte density in the (A) anterior, (B) middle, and (C) posterior regions. Values are expressed as means  $\pm$  SD. †Significant time  $\times$  intervention interaction. ‡Significant main effect of time. §Significant main effect of intervention. Different lowercase letters indicate statistically significant differences between groups within the same time point, such that groups not sharing the same letter are significantly different from one another. \*Significant difference versus 2 weeks postoperatively. In the anterior and middle regions, chondrocyte density at 4 weeks postoperatively was significantly lower than at 2 weeks. Chondrocyte density in the middle region was significantly lower in the ACLR + IM group than other groups, except for the control and ACLR + IM/SED groups. Chondrocyte density in the posterior region was significantly lower in the IM, ACLR + IM, and ACLR + IM/SED groups than in the control and ACLR + SED groups. ACLR, anterior cruciate ligament reconstruction; EX, exercise; IM, immobilization; SED, sedentary.



**Figure 7.** Modified Mankin scores for the (A) anterior, (B) middle, and (C) posterior regions. The boxplots show the median as a horizontal line inside the box and the interquartile range as the length of the box. The whiskers represent the minimum and maximum values within 1.5 times the interquartile range from either end of the box. Circles indicate outliers. §Significant main effect of intervention. In the IM, ACLR + SED, ACLR + SED/EX, and ACLR + EX groups, Mankin scores in 1 or 2 regions were significantly higher versus the control group. In the ACLR + IM group, Mankin scores in all regions were significantly higher versus the control, IM, and ACLR + SED groups. In the ACLR + IM/SED group, the Mankin score was not different versus the ACLR + IM group. ACLR, anterior cruciate ligament reconstruction; EX, exercise; IM, immobilization; SED, sedentary.

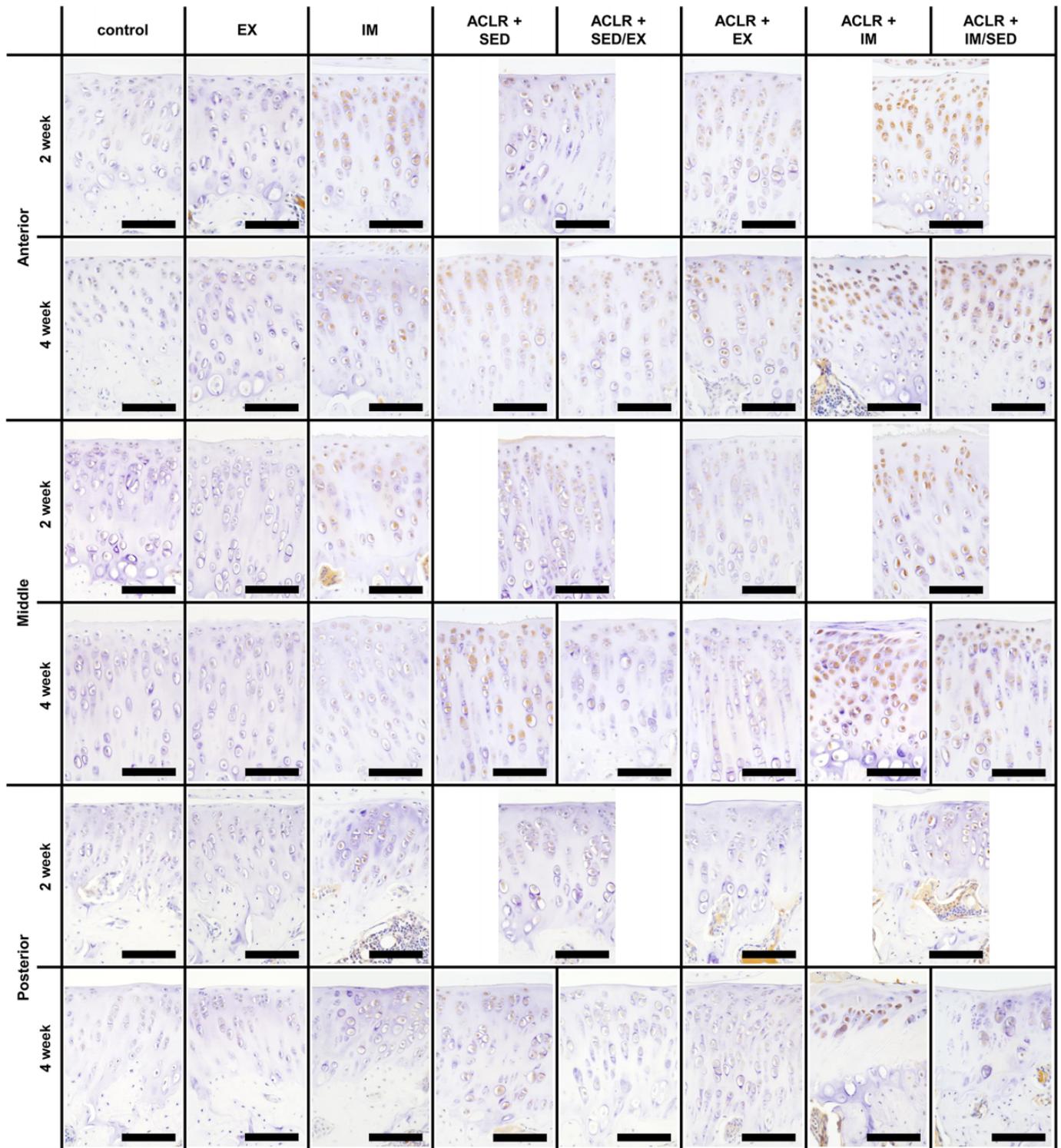
in the ACLR + IM group at 4 weeks postoperatively was excluded from the analysis, because there were almost no cells. COX-2 expression in the EX group was not different from the control group in all regions (Tables 2-4). In the IM, ACLR + SED, ACLR + IM, and ACLR + IM/SED groups, COX-2 expression was significantly higher than that in the control group in all regions. In the ACLR + SED/EX and ACLR + EX groups, COX-2 expression in the anterior and middle regions was significantly higher than that in the control group. There were no significant differences in COX-2 expression between the ACLR + SED and ACLR + SED/EX or ACLR + EX groups. Between the ACLR + SED and ACLR + IM groups, COX-2 expression was higher in the ACLR +

IM group in all regions. There were no significant differences in COX-2 expression between the ACLR + IM and ACLR + IM/SED groups. In the negative control, cells were not stained (data not shown).

DISCUSSION

Natural Course After ACLR and Sedentary Activity

After ACLR, increased Mankin score accompanied with increased COX-2 expression was detected in the anterior region, confirming induced cartilage degeneration. In



**Figure 8.** COX-2 expression in the medial tibial plateau. Representative images of the COX-2 immunostained sections. Scale bars, 100  $\mu$ m. ACLR, anterior cruciate ligament reconstruction; COX-2, cyclooxygenase-2; EX, exercise; IM, immobilization; SED, sedentary.

addition, articular cartilage thickening was detected in all regions at 2 and/or 4 weeks postoperatively. Therefore, we showed ACLR can experimentally induce cartilage degeneration using a rat model. This finding was also clinically

supported by a recent report in which thickness or volume of the cartilage in the femur and tibia increased after ACLR.<sup>15,50</sup> Accordingly, articular cartilage thickening is considered a sign of early osteoarthritis.<sup>11</sup>

TABLE 2  
COX-2 Expression in the Anterior Region<sup>a</sup>

	Control	EX	IM	ACLR + SED	ACLR + SED/ EX	ACLR + EX	ACLR + IM	ACLR + IM/ SED	P <sup>b</sup>
Extent									
2 weeks	2.5 (2-3)	2 (1.5-2)	3 (2.25-3)	3 (3-3.5)		3 (2-3)	4 (4-4)		Time: .980
4 weeks	2 (2-3)	2 (2-2)	3 (3-4)	3 (3-4)	3 (3-3)	3 (3-3)	3 (3-3)	3 (3-4)	Intervention: <b>&lt;.001</b>
Intensity									
2 weeks	1 (1-2) <sup>a,b</sup>	1 (1 -1) <sup>a</sup>	2.5 (2-3) <sup>c,d</sup>	2 (2-2.5) <sup>c,d</sup>		2 (2-2) <sup>b,c</sup>	3 (3-3) <sup>d</sup>		Interaction: .079
4 weeks	1.5 (1-2) <sup>a,b</sup>	1 (1 -1) <sup>a</sup>	3 (2.5-3) <sup>c,d</sup>	3 (2-3) <sup>c,d</sup>	2 (2-2.75) <sup>b,c</sup>	3 (3-3) <sup>c,d*</sup>	3 (3-3) <sup>c,d</sup>	3 (3-3) <sup>d</sup>	Time: <b>.006</b> Intervention: <b>&lt;.001</b>
Total									
2 weeks	2.5 (2-6) <sup>a</sup>	2 (1.5-2) <sup>a</sup>	7.5 (4.5-9) <sup>b</sup>	6 (6-8.5) <sup>b</sup>		6 (4-6) <sup>a,b</sup>	12 (12-12) <sup>c</sup>		Interaction: <b>.015</b>
4 weeks	3 (2-6) <sup>a,b</sup>	2 (2-2) <sup>a</sup>	9 (7.5-12) <sup>c</sup>	9 (7-10.5) <sup>c</sup>	6 (6-8.25) <sup>b,c</sup>	9 (9-9) <sup>c*</sup>	9 (9-9) <sup>c*</sup>	9 (9-12) <sup>c</sup>	Time: .241 Intervention: <b>&lt;.001</b> Interaction: <b>.004</b>

<sup>a</sup>Values are provided as median (interquartile range). Different letters indicate statistically significant differences between groups at the same time point, such that groups not sharing the same letter are significantly different from one another. \*Significant difference compared with 2 weeks postoperatively. Compared with the control group, the COX-2 expression was significantly higher in the IM, ACLR + SED, ACLR + SED/EX, ACLR + EX, ACLR + IM, and ACLR + IM/SED groups. At 2 weeks postoperatively, the COX-2 total score in the ACLR + IM group was significantly higher than all other groups. ACLR, anterior cruciate ligament reconstruction; COX-2, cyclooxygenase-2; EX, exercise; IM, immobilization; SED, sedentary.

<sup>b</sup>Boldface P values indicate statistical significance for effect as shown ( $P < .05$ ).

TABLE 3  
COX-2 Expression in the Middle Region<sup>a</sup>

	Control	EX	IM	ACLR + SED	ACLR + SED/ EX	ACLR + EX	ACLR + IM	ACLR + IM/ SED	P <sup>b</sup>
Extent									
2 weeks	2 (1-2)	2 (1-2)	3 (3-3)	3 (3-3.5)		3 (2-3)	4 (4-4)		Time: .318
4 weeks	2 (2-2.25)	2 (2-2)	3 (3-3.5)	3 (3-4)	3 (3-3)	3 (3-3)	4 (3.5-4)	3 (3-4)	Intervention: <b>&lt;.001</b>
Intensity									
2 weeks	1 (1 -1)	1 (1 -1)	2.5 (2-3)	2 (2-2.5)		2 (1-2)	3 (3-3)		Interaction: .203
4 weeks	1 (1 -1)	1 (1 -1)	2 (2-2)	3 (2-3)	1.5 (1-2)	2 (2-2)	3 (3-3)	3 (2.5-3)	Time: .914 Intervention: <b>&lt;.001</b>
Total									
2 weeks	2 (1-2)	2 (1-2)	7.5 (6-9)	6 (6-9)		6 (2-6)	12 (12-12)		Interaction: .090
4 weeks	2 (2-2.25)	2 (2-2)	6 (6-7)	9 (8-9)	4.5 (3-6)	6 (6-6)	12 (10.5-12)	9 (7.5-12)	Time: .657 Intervention: <b>&lt;.001</b> Interaction: .186

<sup>a</sup>Values are provided as median (interquartile range). Compared with the control group, the COX-2 expression was significantly higher in the IM, ACLR + SED, ACLR + SED/EX, ACLR + EX, ACLR + IM, and ACLR + IM/SED groups. The COX-2 intensity score in the ACLR + IM group was significantly higher versus the IM and ACLR + SED groups. The COX-2 total score in the ACLR + IM group was significantly higher versus all groups except for the ACLR + IM/SED group. ACLR, anterior cruciate ligament reconstruction; COX-2, cyclooxygenase-2; EX, exercise; IM, immobilization; SED, sedentary.

<sup>b</sup>Boldface P values indicate statistical significance for effect as shown ( $P < .05$ ).

## Effects of Immobilization

We aimed to examine the effects of joint immobilization on cartilage degeneration after ACLR. Previous studies reported that immobilization for intact joints induced cartilage degeneration, increased COX-2 expression in chondrocytes, and decreased chondrocyte density.<sup>32,44,47</sup> We also detected higher Mankin scores accompanied by higher expression of COX-2 in the anterior and posterior regions and decreased chondrocyte density in the posterior region in the IM group. In the ACLR + IM group, Mankin scores in

all regions, especially in the posterior region, apparently increased compared with the ACLR + SED and IM groups. Moreover, in the posterior region, thinning of the articular cartilage and decreased chondrocyte density were also observed. These results indicate that ACLR and joint immobilization synergistically facilitate cartilage degeneration. Previous studies have revealed that 6 weeks of joint immobilization after ACLR or ACL repair can aggravate cartilage degeneration.<sup>38,42</sup> In our study, joint immobilization after ACLR greatly facilitated cartilage degeneration, even if performed for only 2 weeks. Between the ACLR +

TABLE 4  
COX-2 Expression in the Posterior Region<sup>a</sup>

	Control	EX	IM	ACLR + SED	ACLR + SED/ EX	ACLR + EX	ACLR + IM	ACLR + IM/ SED	P <sup>b</sup>
<b>Extent</b>									
2 weeks	1.5 (1-2)	1 (0-2)	3 (2.25-3)	3 (2-3)		2 (2-2.5)	3 (3-3.5)		Time: <b>.009</b>
4 weeks	2 (2-2)	2 (2-2)	3 (3-3)	3 (3-4)	3 (2.25-3)	3 (2.25-3)	3.5 (3-4)	3 (3-3.5)	Intervention: <b>&lt;.001</b>
Interaction: .327									
<b>Intensity</b>									
2 weeks	1 (1 -1)	1 (0 -1)	2 (1.25-2)	1 (1 -1.5)		1 (1 -1)	2 (2-2.5)		Time: .774
4 weeks	1 (1 -1)	1 (1 -1)	1 (1 -1.5)	2 (1-2)	1 (1 -1)	1 (1 -1.75)	2.5 (2-3)	2 (1.5-2)	Intervention: <b>&lt;.001</b>
Interaction: .085									
<b>Total</b>									
2 weeks	1.5 (1-2)	1 (0-2)	6 (3-6)	3 (2-4.5)		2 (2-2.5)	6 (6-9)		Time: .858
4 weeks	2 (2-2)	2 (2-2)	3 (3-4.5)	6 (3-8)	3 (2.25-3)	3.5 (2.25-5.5)	8 (4-10.5)	6 (4.5-7)	Intervention: <b>&lt;.001</b>
Interaction: .262									

<sup>a</sup>Values are provided as median (interquartile range). The COX-2 extent score at 4 weeks postoperatively was significantly higher than at 2 weeks. Compared with the control group, the COX-2 expression was significantly higher in the IM, ACLR + SED, ACLR + IM, and ACLR + IM/SED groups. The COX-2 intensity score in the ACLR + IM group was significantly higher versus all groups except for the ACLR + IM/SED group. ACLR, anterior cruciate ligament reconstruction; COX-2, cyclooxygenase-2; EX, exercise; SED, sedentary; IM, immobilization.

<sup>b</sup>Boldface *P* values indicate statistical significance for effect as shown ( $P < .05$ ).

IM and ACLR + IM/SED groups, there were no significant differences in chondrocyte density, Mankin score, or COX-2 expression, although cartilage thickness was significantly thicker in the ACLR + IM/SED group. These results indicate that 2 weeks of remobilization in the ACLR + IM/SED group could not restore cartilage degeneration facilitated by 2 weeks of immobilization after ACLR. Therefore, joint immobilization after ACLR should be minimized to reduce cartilage degeneration. Previous studies suggested that short-term (within 4 weeks) immobilization after ACLR improves tendon-to-bone healing in rats and mice.<sup>1,6,13,30</sup> It is unknown if decreased graft laxity after immobilization will result in long-term improvement in cartilage status.

COX-2 plays an important role in the development of cartilage degeneration.<sup>52</sup> COX-2 can increase prostaglandin E2 levels, and increased prostaglandin E2 induces cartilage degeneration through increased expression of proteolytic enzymes such as metalloproteinases and decreased proteoglycan synthesis.<sup>52</sup> We demonstrated that joint immobilization after ACLR enhanced COX-2 expression (intensity score or total score) in all regions. Thus, enhanced COX-2 production may be one of the mechanisms of the facilitation of cartilage degeneration by joint immobilization.

Cartilage degeneration in the ACLR + IM group was region-specific and most severe in the posterior region. We speculate this may be explained by region-specific blood clot formation and its interaction with joint immobilization. Hemarthrosis is commonly observed in patients after ACLR surgery.<sup>27,41</sup> In the rat ACLR model, blood clots were observed in the posterior joint space at 3 days postoperatively.<sup>24</sup> In a previous study, blood clots were observed in the posterior joint space until 3 days after blood injection in both immobilized and nonimmobilized knees.<sup>34</sup> Thus, we speculate that joint immobilization did not affect the location of blood clots formation, and blood clots would be formed in the posterior joint space of the immobilized knee

early after ACLR. Blood exposure to cultured human articular cartilage induces chondrocyte death via apoptosis.<sup>17</sup> Sogi et al<sup>44</sup> reported that blood injection into immobilized rat knees reduces chondrocyte density. Accordingly, combination of ACLR and joint immobilization dramatically decreased chondrocyte density in the posterior region, although ACLR alone did not decrease chondrocyte density. Chondrocytes synthesize all matrix components in the articular cartilage, such as proteoglycans, and regulate matrix metabolism.<sup>3</sup> Hemarthrosis also negatively affects proteoglycan synthesis by chondrocytes. Previous studies revealed that blood exposure of rat knee articular cartilage or cultured human articular cartilage decreased proteoglycan synthesis.<sup>17,40</sup> Therefore, in the posterior region in the ACLR + IM group, a dramatic decrease in chondrocyte density and blood clot formation would result in proteoglycan loss and cartilage thinning.

#### Effects of Treadmill Exercise

Another aim of this study was to examine the effects of treadmill exercise on articular cartilage degeneration after ACLR. Treadmill exercise did not induce cartilage degeneration in the intact knee, as cartilage thickness, chondrocyte density, Mankin score, and COX-2 expression were not significantly different between the control and EX groups. A previous study indicated that cartilage degeneration was caused by high-speed (26.8 m/minute) treadmill exercise, but not moderate (19.3 m/minute) or low-speed (15.2 m/minute) exercise in the rat knee.<sup>31</sup> In our study, treadmill exercise speed was low (12 m/minute), and this level of exercise did not have any negative effects on cartilage health.

Treadmill exercise had almost no impact on cartilage degeneration after ACLR, regardless of the timing of exercise, as there were no differences in cartilage thickness (except in the posterior region at 2 weeks), chondrocyte

density, Mankin score, or COX-2 expression between the ACLR + SED and ACLR + EX or ACLR + SED/EX groups. A previous study using the same rat ACLR model reported that ACL transection-induced joint instability was reduced significantly by reconstruction surgery, but residual instability remained even after reconstruction.<sup>25,26</sup> Therefore, deterioration of joint stability by ACL transection will not be restored completely by reconstruction surgery, which may explain why treadmill exercise did not positively affect joint cartilage.

### Limitations

This study has several limitations. First, our methods of joint immobilization were different from the methods used in clinical practice. Human knees after ACLR are typically immobilized by knee brace at a flexion angle of 0° to 60°, if immobilization is applied.<sup>14,16</sup> On the other hand, rat knees were immobilized by external fixator at a flexion angle of 140°. Differences in immobilization methods may affect cartilage degeneration. Second, exercise effects may vary with intensity. In this study, we chose low-speed treadmill exercise because performing high-intensity exercise is difficult during the early phase after surgery. Third, the unoperated contralateral knees of rats in the ACLR + EX group were used as the EX group. Although the contralateral knees would receive compensatory overload, cartilage degeneration was not detected in the EX group. Fourth, the follow-up periods were relatively short (up to 4 weeks). In our study, cartilage degeneration after ACLR developed within 2 weeks and did not progress between 2 and 4 weeks. These findings suggest that early cartilage degeneration after ACLR largely develops within 2 weeks in our model. However, additional long-term studies will be needed, because osteoarthritis after ACLR is a long-term process in human patients.<sup>19</sup>

A fifth limitation was that we did not assess joint laxity. Although increased joint laxity after ACLR has been correlated with osteoarthritis progression,<sup>45</sup> the effects of joint immobilization, sedentary activity, and treadmill exercise on joint laxity are unknown. Sixth, whether the negative changes due to joint immobilization after ACLR are reversible are unclear. Most patients return to a relatively normal level of physical activity after a period of time, but we did not examine whether degenerative changes induced by immobilization are reversed by exercise. Seventh, we could not separate out the effects of immobilization and limited weightbearing. During joint immobilization, rats could move freely using 4 limbs. However, weightbearing on the immobilized hindlimb would be limited. It is unclear whether the changes observed after joint immobilization were purely the result of immobilization or whether they included the effects of limited weightbearing.

### CONCLUSION

Joint immobilization greatly facilitated cartilage degeneration after ACLR, even when used for only 2 weeks. Two weeks of remobilization could not restore cartilage

degeneration induced by 2 weeks of immobilization after ACLR. To reduce cartilage degeneration, periods of joint immobilization after ACLR should be minimized. Treadmill exercise during early phases (~4 weeks) after ACLR did not facilitate cartilage degeneration, but also did not prevent cartilage effects caused by ACLR, regardless of the timing of exercise. Therefore, mild exercise during the early phases post ACLR will not negatively affect articular cartilage.

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