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Effects of aerobic exercise at different intensities on articular cartilage in mice

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ABSTRACT

Background: Maintaining intrinsic articular cartilage homeostasis is essential for the health of cartilage. However, the impact of aerobic exercise of varying intensities on the articular cartilage homeostasis has never been studied. This study aims to elucidate the influence of different aerobic exercise intensities on the anabolic and catabolic processes within articular cartilage.

Methods: Forty-eight male C57BL/6J mice, aged 7 weeks, were divided into 4 aerobic exercise groups and 1 control group. The aerobic exercise groups were subjected to both acute and chronic exercise protocols with varying intensities of 8, 12, 16, 20, and 24 m/min. Total RNA from the knee joint cartilage was extracted in both phases to quantify mRNA of anabolic (Sox9, Col2a1, and Acan) and catabolic (MMP-13 and ADAMTS5) markers. In the chronic exercise, articular cartilage thickness and chondrocyte density were histologically assessed. Additionally, immunohistochemical staining quantified relevant molecules involved in cartilage metabolism.

Results: In the acute exercise, the 8 m/min group exhibited reduced ADAMTS5 expression compared to the control, 16 m/min, and 24 m/min groups. Chronic exercise showed enhanced articular cartilage thickness in both the 8 and 12 m/min groups relative to the control group. Moreover, the 8 m/min group demonstrated elevated aggrecan levels in comparison to both the control and 24 m/min groups. Additionally, the 24 m/min group exhibited significantly higher ADAMTS5 levels than the control group.

Conclusion: Our findings suggest that consistent low-intensity aerobic exercise suppresses catabolic molecule expression in articular cartilage, thereby fostering anabolic activity. Conversely, continuous high-intensity aerobic exercise can potentially disrupt cartilage homeostasis by enhancing catabolic processes. This dichotomy underscores the need for balanced exercise regimens to maintain cartilage health. (243/250)

Keywords: Aerobic exercise, Exercise intensity, Cartilage, OA, Anabolism and catabolism

Introduction

Homeostasis of articular cartilage is important for the structural integrity of the joint. The balance between cartilage anabolism and catabolism is crucial for maintaining cartilage health [1].

Exercise has been identified as a critical factor in influencing the health and integrity of articular cartilage. Animal research indicates that high-intensity aerobic exercise may lead to a decrease in extracellular matrix (ECM) content and articular cartilage thickness, whereas low- and moderate-intensity aerobic exercise may lead to an increase in ECM content and articular cartilage thickness [2–5]. The balance between cartilage anabolism and catabolism is therefore affected by exercise. Elevated levels of degradative enzymes, including matrix metalloproteinase-13 (MMP-13) and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), are associated with cartilage deterioration. Conversely, exercise can help regulate these enzyme levels, potentially reducing their harmful effects on the ECM and maintaining cartilage thickness and density [6,7]. Sex-determining region Y-Box 9 (Sox9) is a crucial transcription factor for chondrocytes as it inhibits their proliferation and hypertrophy, which is essential for cartilage formation. Some studies have shown that Sox9 and type II collagen (Col2a1) genes are co-expressed during the process of cartilage formation [8,9]. According to a pioneering report on exercise as an aid to chondrocyte regeneration, human-related exercise upregulates the cartilage-related gene Sox9 and the transcription factor Col2a1 [10].

A previous study has pointed out that different intensities of aerobic exercise lead to different results in cartilage degradation [11]. Animal studies have reported that prolonged continuous training at a speed of 18 m/min may promote cartilage degeneration and the progression of osteoarthritis (OA) [11–13]. On the other hand, joint injuries are unlikely to occur even with prolonged running if the running speed is lower than 13-15 m/min [14,15]. Human studies have shown that moderate recreational running and physical activity do not increase the risk of cartilage degradation. However, competitive running, while not causing cartilage damage, does increase the risk of cartilage degradation [16,17]. So far, the impact on articular cartilage has varied depending on the intensity of exercise.

The intensity of aerobic exercise may play an important role in cartilage degeneration. However, it is currently unknown how different intensities of aerobic exercise affect the

anabolic and catabolic metabolism of articular cartilage. The aim of this study was to investigate the impact of varying intensities of aerobic exercise on the balance between anabolic and catabolic processes in healthy articular cartilage.

Materials and Methods

1. Experimental design

This study was conducted following the Regulations for the Conduct of Animal Experiments at Kobe University and was approved by the Animal Experimentation Committee (approval number: P190814).

This experiment used 48 male mice (7 weeks old) purchased from SLC (SLC Inc., Shizuoka, Japan). The mice were housed in microchip-lined polyethylene terephthalate cages and provided with free access to water and food. The temperature for rearing was maintained at 22 ± 1 °C, while a 12-hour light-dark cycle (8:00 am-8:00 pm) was used for light control. After a one-week acclimatization period, the mice were randomly divided into 2 groups: acute and chronic exercise. Each group consisted of 24 animals, of which 4 mice were chosen as controls and did not participate in any exercise regimen. The remaining 20 mice were assigned to different exercise regimens, which involved running at various intensities.

2. Aerobic exercise protocol

A universal treadmill (MK-680C, Muromachi Machinery Co., Ltd.) equipped with a mild electric stimulus was used. After a week of general training for acclimation (Fig. 1), 2 distinct exercise training regimens were implemented. The acute exercise regimen consisted of a single session lasting 60 minutes with a 0-degree incline, conducted once during the week. The chronic exercise regimen involved daily sessions of 60 minutes at a 0-degree incline, repeated over 6 weeks. Both exercise protocols utilize motion speeds of 8, 12, 16, 20, and 24 m/min, with an exercise duration of 60 minutes per session. Preliminary results confirm that mice can run without falling back at a minimum exercise intensity of 8 m/min and can complete a 60-minute run at a maximum exercise intensity of 24 m/min [18]. The two simulated running intensities corresponded to walking and high-intensity endurance exercises, respectively. Earlier studies have demonstrated that significant morphological alterations in the articular cartilage are

achievable with 60 minutes of daily exercise over six weeks [19–21]. Starting on the first day of the exercise group, the control group mice were placed on a treadmill daily without exercise.

3. Sample preparation

The mice were euthanized under deep anesthesia with 40 mg/kg of sodium pentobarbital following a subcutaneous injection of 0.02 mg/kg of buprenorphine. At 12 hours after acute exercise and 24 hours after chronic exercise, the animals were killed by exsanguination under anesthesia. The knee joints were then harvested and positioned at 80-90° flexion. The knee joints were positioned uniformly at 80-90° of flexion and cryo-embedded in a 1:1 mixture of n-hexane and isopentane at -75°C [22]. These cryopreserved samples were stored at -80°C until sectioning. Coronal knee joint sections, 5 μm thick, were prepared with a cryomicrotome (Leica Microsystems Japan, CM1860). These sections were subsequently used for histological or immunohistochemical evaluations.

4. Histological analysis

Histological analysis was performed solely on the chronic exercise group to examine changes in tissue morphology resulting from prolonged exercise.

4.1 Assessment area definition

The target of assessment in this experiment was the tibial plateau of the knee. Our previous study has defined the articular cartilage region to be assessed as the medial femoral-tibial joint based on its position in the embedded joint [23]. The tibial central zone (TM) is defined as the area adjacent to the cartilage of the femoral head located at the medial edge of the anterior and posterior corners of the meniscus. The anterior tibial area (TA) is defined as the anterior horn of the meniscus. The posterior tibial region (TP) is defined as the area below the posterior horn of the meniscus. Sections for histological analysis varied from 50-250 μm outward from the level of the meniscus, divided into anterior and posterior corners in sagittal sections.

4.2 Analysis of organizational patterns

Articular cartilage thickness was measured using digital images of toluidine blue-stained tissue sections. Toluidine blue staining reveals a tidemark in the articular cartilage and a distinct demarcation between the articular cartilage and subchondral bone. The area of the non-calcified layer of articular cartilage below 200 μm of the articular surface and the area of the entire cartilage layer was measured in all of these regions. The thickness of each area was calculated by dividing its area by 200. The thickness of the calcified layer was determined by subtracting the thickness of the non-calcified layer from the thickness of the full layer. The average articular cartilage thickness for each sample was calculated by averaging three slices taken at 50 ,100, and 150 μm from the level at which the meniscus was divided into anterior and posterior corners. Chondrocyte density was measured by analyzing thromboxane-eosin (HE) stained tissue sections. The thickness of the articular cartilage and the area of cartilage were measured using the same method. The chondrocytes within the measured area were evaluated manually. The density of chondrocytes was determined by calculating the number of chondrocytes per unit area of cartilage.

4.3 Immunohistochemical analysis

The sections were prepared using the method established in our laboratory [24]. The frozen sections were incubated with anti-Sox 9 (diluted 1:500, ab3639, Abcam, Cambridge, UK), anti-type II collagen (diluted 1:200, ab21291, Abcam), anti-aggrecan (diluted 1:400, AB1031, Chemicon, Tokyo, Japan), anti-MMP-13 (diluted 1:400, ab39012, Abcam), and anti-ADAMTS5 (diluted 1:500, ab41037, Abcam) antibodies. Goat biotin-conjugated anti-rabbit IgG antibody (1:200 dilution, Vector Laboratories, BA-1000) was used as the secondary antibody, and the immunoreaction was visualized with 3,3'-tetrachlorobenzidine (Vector Laboratories, SK-4105). Hematoxylin was used as a contrast stain to differentiate the noncalcified layer from the calcified layer.

A subsequent reaction was made using the streptavidin-biotin-peroxidase complex technique with an Elite ABC kit (diluted 1:50, PK-610, Vector Laboratories, Burlingame, USA). ImmPACT DAB (SK-4105, Vector Laboratories) was then used for visualizing the immunoreaction. Finally, the sections were counterstained with Dako Real Hematoxylin, washed in water, and mounted. The immunolabelled sections were captured with a light

microscope (BX53; Olympus, Tokyo, Japan) and camera (DP73; Olympus, Tokyo, Japan) at 4 or 20× magnification in the central 400 μm $\times$ 200 μm area of the medial tibial plateau. For type II collagen and aggrecan, the tissue images were grayscaled using Adobe Photoshop CS2, and the average gray value (0-255) within the region was calculated using ImageJ. Staining intensity is defined as 255 – average gray value. For MMP-13, ADAMTS5, Sox9, aggrecan, TNF- α , and IL-6, the number of immunopositive cells was counted manually, and the positive cell density was calculated by dividing the number of positive cells by the area. All slides were stained for each primary antibody in one session (same day, same reagents, and same protocol).

5. Analysis of molecular biology

To evaluate the acute effects of acute interventions as well as the homeostatic changes of chronic exercise, the molecular biological changes induced by acute and chronic exercise regimes were analyzed.

5.1 Total RNA extraction

We evaluated the mRNA expression levels of Sox9, Col2a1, Acan (gene expression levels of aggrecan), MMP-13 (matrix metalloproteinase-13), and ADAMTS5 in articular cartilage. Sagittal sections of 20 μm were prepared in the same manner as the specimens described above (25 medial and 25 lateral sections per specimen, for a total of 50 sections per knee). Total RNA was extracted from frozen sections of knee cartilage, and articular cartilage was separated from the sections with forceps under a stereomicroscope. Total RNA was extracted from the collected articular cartilage using the RNeasy Universal Mini Kit (Qiagen) according to the product specifications. The extracted total RNA was stored at -80°C until analysis.

5.2 Analysis by real-time PCR

Real-time PCR analysis was conducted using the StepOne™ Real-time PCR system (Thermo Fisher Scientific). The TaqMan Gene Expression Assay Kit (Applied Biosystems) was used for the primers, and Col2a1, Acan, MMP-13, ADAMTS5, and Sox9. Using the $\Delta\Delta\text{CT}$ method, the mRNA expression levels of each group were corrected for 18s expression levels and compared

to each target with reference to the control group. The relative expression of each target gene was calculated using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the internal reference.

6. Statistical methods

The results are presented as mean $\pm$ standard deviation. Statistical analysis was conducted using EZR. The normality of all data was assessed using the Shapiro-Wilk test. If normality was confirmed, one-way ANOVA and Tukey HSD test were used to compare the results of all groups. If normality was rejected, the Kruskal-Wallis and Steel-Dwas tests were used. $P < 0.05$ was considered statistically significant.

Results

The body weight and muscle wet weight of the chronic experimental group

After 6 weeks of aerobic exercise, the body weights of mice in the 16 m/min and 24 m/min groups decreased in comparison to those in the control and 8 m/min groups. The wet weight of the gastrocnemius muscle did not differ significantly among the groups.

Histologic evaluation of articular cartilage

Toluidine blue staining, which reflects proteoglycan content, did not decrease in any region or group. The noncalcified layer of articular cartilage in the TM region showed an increase in thickness in the 8 m/min ($P = 0.0316$) and 12 m/min groups ($P = 0.0460$) when compared to the control group (Fig. 2A and C). However, in the TA and TP regions there was no significant difference in the thickness of articular cartilage in the noncalcified and calcified layers among the two groups (Table 1).

Hematoxylin and HE staining showed no significant difference in chondrocyte density in any region (TA, TP, and TM) or layer (calcified and noncalcified layers) among the groups (Fig. 2A and Table 1). Although there were no overlapping findings of chondrocyte clusters or tidemarks, subchondral bone proliferation was observed and the thickness increased only in the 24 m/min group ($P = 0.0118$) (Fig. 2B and C).

Immunohistologic Evaluation of Articular Cartilage

Type II collagen was evenly distributed in both the noncalcified and calcified layers of cartilage, with no significant difference in staining intensity among the groups (Fig. 3A and B). MMP-13 was not detected in the noncalcified layer, and there was no significant difference between the groups in the calcified layer (Fig. 3A and B). Aggrecan expression increased in the non-calcified layer in the 8 m/min group ($P = 0.0389$) compared to the control group. Additionally, in the calcified layer, aggrecan expression increased in the 8 m/min ($P = 0.0221$) and 12 m/min groups ($P = 0.0239$) compared to the control group. Aggrecan expression was also increased in the 8 m/min ($P = 0.0212$) and 12 m/min ($P = 0.0229$) groups compared with the 24 m/min group (Fig. 4A and B). In the non-calcified layer, ADAMTS5 expression increased in the 16 m/min ($P = 0.0321$) and 24 m/min groups ($P = 0.0112$) and tended to increase in the 20 m/min group ($P = 0.0586$) compared to the control group. Positive cells were also observed in the deep layer of articular cartilage in the 16, 20, and 24 m/min groups. No significant difference was observed between the groups in the calcified layer (Fig. 4A and B). In both the noncalcified and calcified layers, there was no significant difference in Sox9 expression between the groups (Fig. 4A and B).

Expression of articular cartilage genetic factors

In the acute exercise group, there were no significant differences in the expression levels of Sox9, Col2a1, Acan, and MMP-13 between groups. On the other hand, the expression level of ADAMTS5 was significantly lower in the 8 m/min ($P = 0.0120$) group compared to the control group. ADAMTS5 was also significantly lower than that in the 16 m/min ($P = 0.0109$) and 24 m/min groups ($P = 0.0288$) (Fig.5A). In the chronic exercise group, there were no significant differences in the expression levels of Sox9, Col2a1, Acan, and MMP-13 compared to the control group. However, the expression of ADAMTS5 was decreased in the 12 m/min ($P = 0.0042$), 16 m/min ($P = 0.0260$), and 24 m/min ($P = 0.006$) groups compared to the control group. Additionally, the expression in the 24 m/min group was lower than that in the 8 m/min group ($P = 0.0078$) (Fig.5B).

DISCUSSION

This study investigated the effects of acute and chronic aerobic exercise at different intensities

on articular cartilage in young healthy mice. The histologic results indicate that low-intensity exercise loads, such as those that simulate walking, increase the metabolism of synthetic cartilage without increasing the catabolism of articular cartilage. On the other hand, exercise that simulated high-intensity endurance exercise increased the expression of catabolic molecules in articular cartilage. Subchondral hyperplasia demonstrated by high-intensity exercise is a response to excessive mechanical stress. High-intensity aerobic exercise may cause excessive mechanical stress before cartilage degeneration occurs, and articular cartilage may be undergoing degeneration. The density and distribution of chondrocytes remained unchanged regardless of the intensity of aerobic exercise. Our findings suggest that aerobic exercise of any intensity affects chondrocyte metabolism, but not chondrocyte differentiation or apoptosis.

We measured the acute and chronic effects on expression of type II collagen and MMP-13 and found no changes in mRNA or protein levels. Previous studies in rodents [2,24,25] have shown that neither anabolic nor catabolic levels of type II collagen were altered, which is consistent with the findings presented here. In these studies, the protein levels of type II collagen remained unchanged despite various running conditions. Collagen type II has a low turnover rate in healthy cartilage, with a half-life of up to 100 years [26,27]. Degradation of collagen type II has been associated with damage to articular cartilage [28]. In contrast, in the healthy mice used in this experiment the type II collagen content was not altered, likely because the articular cartilage had not yet undergone degeneration. This finding is consistent with previous study. Moderate-intensity aerobic exercise without damage to the joints did not affect the results for type II collagen levels, even though it could alter the dramatic changes in collagen associated with type V and others [25]. Research has demonstrated that prolonged endurance running can cause significant changes in collagen [29,30]. This suggests that changes in type II collagen may be linked to prolonged, long-distance, and high-intensity exercise [5,30].

In this study, low-intensity exercise was associated with an increase in aggrecan protein expression. Moreover, high-intensity exercise resulted in elevated levels of the ADAMTS5 protein. These results show that aerobic exercise may significantly affect cartilage changes, which well corresponded to previous reports [2,13,30]. The trends in ADAMTS5 gene expression as an acute effect of exercise and ADAMTS5 protein expression as a chronic effect after prolonged exercise were comparable. These findings suggest that exercise-induced

changes in cartilage are primarily caused by the acute effects of a single exercise session and its repetitions. Low-intensity exercise, such as 8 m/min, may promote cartilage synthesis by inhibiting the expression of ADAMTS5. On the other hand, high-intensity exercise may result in a permanent increase in catabolic reactions. The study found that acute exercise resulted in mRNA changes that were consistent with protein levels observed after prolonged exercise. Conversely, mRNA expression after prolonged exercise was inversely proportional, with an increase in ADAMTS5 mRNA expression after high-intensity exercise. Compensatory mechanisms that maintain the balance between anabolism and catabolism are observed early in OA and disintegrate as the disease progresses. In a previous study investigating the catabolic response of cartilage based on OA arthroscopy severity, ADAMTS5 transcript levels were found to be suppressed in cartilage with the mildest arthritis [31]. The present study suggests that ADAMTS5 is similar to the compensatory mechanisms observed in the early stages of OA. At 24 m/min, a demonstration of subchondral bone proliferation accompanied this, suggesting that the down-regulation was suppressed in the early stages of OA [31].

The expression of Sox9, a significant transcription factor that regulates the fundamental expression of ECM in cartilage formation, occurs prior to the expression of type II collagen and aggrecan. However, there were no changes in either protein levels or mRNA expression in this experiment. This may be since Sox9 regulation involves multiple factors. The early inhibition of ADAMTS5 is mediated by the upregulation of Sox9, along with other related proteins that are involved in regulation and enhancement, possibly in synergy with other factors of cartilage differentiation. For example, it functions in conjunction with SOX family members Sox5 and Sox6 and is not expressed alone, and the activation of the expression of target genes (e.g., Col2a1 and Acan) during chondrogenic differentiation has been termed the chondrogenic SOX trio [8,32]. On the other hand, Sox9 may be metabolized at the time of sampling. Sox9 undergoes post-translational modifications, such as phosphorylation, and its transcriptional levels are regulated in other tissues including gonads and cartilage. In particular, Sox9 is stabilized by phosphorylation, translocated into the nucleus, and promotes transcription of its downstream molecules. Previous studies have shown that Sox9 may increase downstream signaling without altering itself [33,34]. The results of these studies suggest that downstream signaling was enhanced by an increase in phosphorylated Sox9. This phenomenon occurs

independently of changes in the mRNA or protein levels of Sox9. However, further studies are necessary to confirm this finding.

A limitation of this study is the use of a healthy young mouse model. This choice limits our ability to explore whether the effects of low-intensity exercise on cartilage synthesis are resilient to OA-inducing factors such as aging and trauma. Nevertheless, this study highlights the fundamental effects of exercise on cartilage, unaffected by pre-existing conditions or age-related degeneration. Furthermore, we did not examine if biomechanical factors like body weight changes and quadriceps muscle strength have a consistent impact on cartilage anabolism and catabolism. This omission stems from our goal to focus solely on the direct influence of exercise intensity on cartilage health. Our research lays the groundwork for future studies that might include these more complex variables. Subsequent studies will seek to expand the current findings by increasing the sample size.

Our study reflects the interaction of different aerobic exercises on anabolism and catabolism of articular cartilage. Higher-intensity aerobic exercise leads to increased expression of cartilage catabolic proteins, creating a compensatory response that maintains the anabolic homeostasis characteristic of early cartilage degeneration. Lower-intensity aerobic exercise maintains ECM proteins by inhibiting the catabolic response of cartilage to acute exercise, leading to an increase in cartilage thickness. These results suggest that low-intensity aerobic exercise such as walking may be "moderate" for articular cartilage.

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AUTHOR CONTRIBUTIONS

Conceptualization: HM. Methodology: CH, TT. Investigation: CH, TT, SI. Visualization: CH, TT, SI, JH, HJ. Supervision: HM. Writing—original draft: CH, TT, HJ. Writing—review & editing: CH, TT, SI, JH, HJ, HM. Funding acquisition: HM.

COMPETING INTEREST

The authors have declared that no conflict of interest exists.

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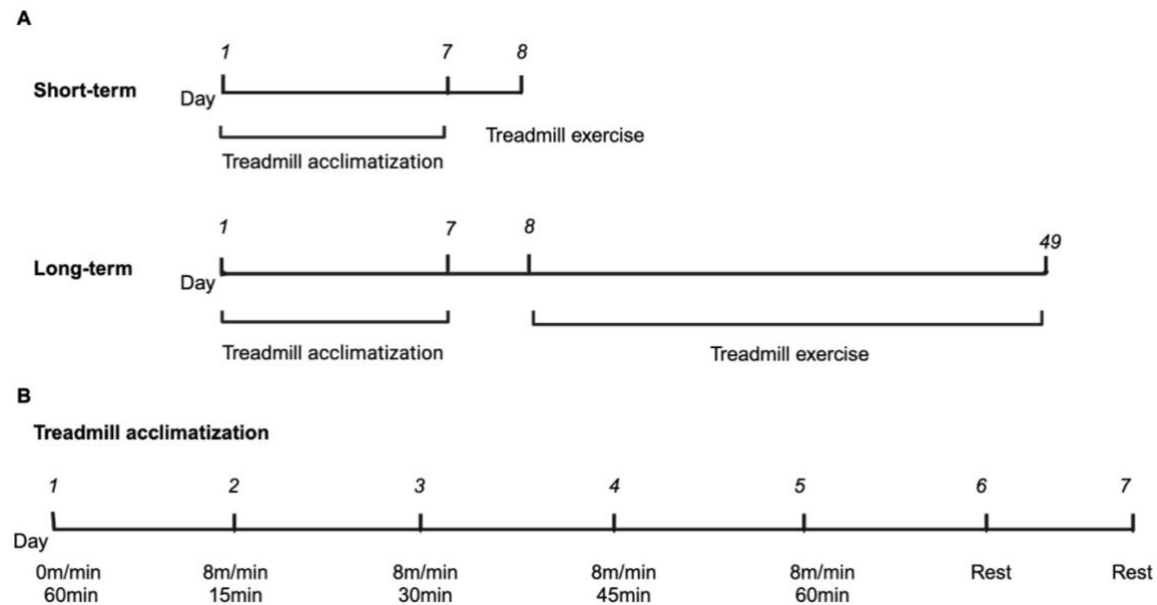
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Table1. Articular cartilage thickness and chondrocyte density in the anterior (TA) and posterior (TP) regions of the tibia ($n = 4$ mice per group).

			Control	8m/min	12m/min	16m/min	20m/min	24m/min
TA	cartilage thickness	noncalcified layer	45.2 ± 2.28	45.7 ± 4.97	43.3 ± 1.29	47.6 ± 1.20	48.9 ± 2.48	47.4 ± 1.26
		calcified layer	54.5 ± 5.54	52.5 ± 13.4	55.2 ± 5.51	44.4 ± 5.45	48.5 ± 6.36	45.2 ± 5.34
	Cartilage cell denticity	noncalcified layer	35.2 ± 3.53	35.8 ± 5.87	36.8 ± 5.17	36.7 ± 2.66	37.5 ± 6.00	32.0 ± 2.28
		calcified layer	10.0 ± 1.08	12.6 ± 2.23	11.5 ± 1.11	11.4 ± 2.22	8.9 ± 1.97	11.0 ± 4.70
TP	cartilage thickness	noncalcified layer	37.0 ± 3.82	39.5 ± 4.35	42.5 ± 2.85	39.3 ± 1.52	38.8 ± 0.66	39.9 ± 0.58
		calcified layer	65.9 ± 5.60	62.9 ± 10.5	72.8 ± 7.49	62.8 ± 7.64	61.9 ± 6.45	62.4 ± 7.55
	Cartilage cell denticity	noncalcified layer	41.1 ± 8.30	42.8 ± 9.56	49.9 ± 7.31	46.8 ± 6.57	41.0 ± 5.70	40.5 ± 7.10
		calcified layer	12.4 ± 2.65	15.8 ± 1.22	13.4 ± 4.66	14.2 ± 4.10	15.2 ± 1.86	12.8 ± 5.42

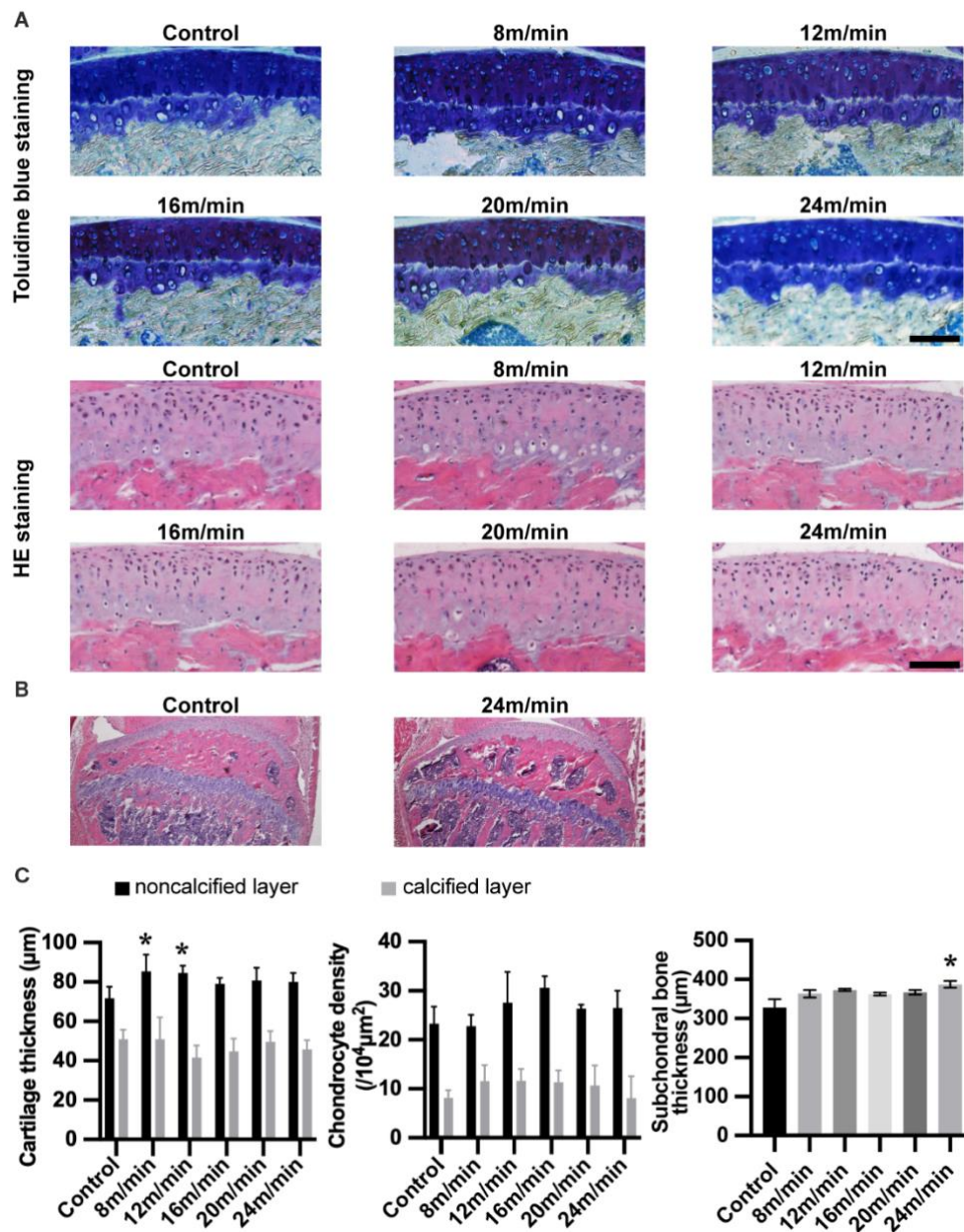
Figure legends

Figure 1. Exercise protocol and 7-day treadmill exercise acclimatization



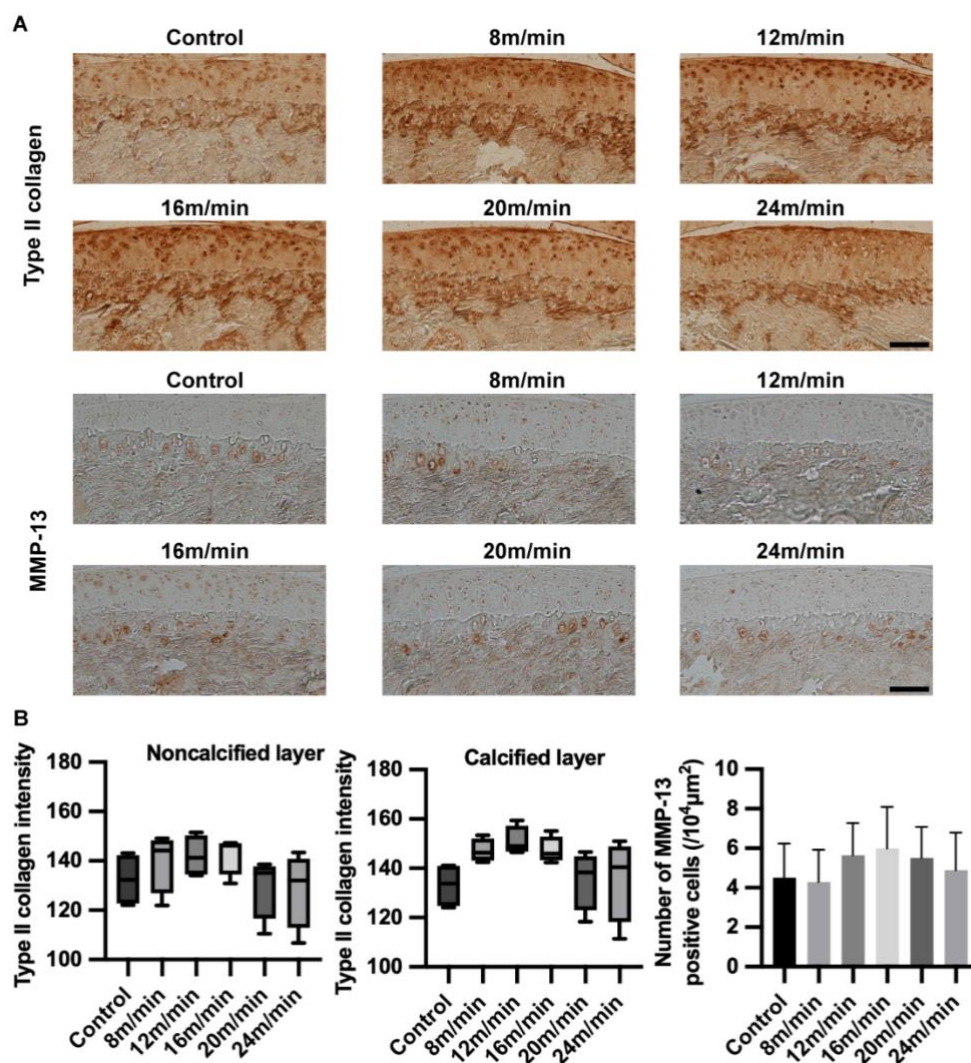
(A) Training programs for acute and chronic exercise. Both acute and chronic exercise programs commenced following a 7-day acclimatization period. The exercise programs included exercises at varying intensities of 8, 12, 16, 20, and 24 m/min. The chronic exercise extended over a period of 6 weeks. (B) A one-week acclimatization training protocol before aerobic exercise. The acclimatization protocol spanned one week, designed to gradually familiarize the mice with the treadmill. From day one to day five, the regimen progressively increased both the speed and duration of treadmill exercises: starting at 0 m/min for 60 minutes, then increasing to 8 m/min for 15, 30, 45, and eventually 60 minutes. Days 6 and 7 were designated as rest days.

Figure 2. Histologic changes in articular cartilage.



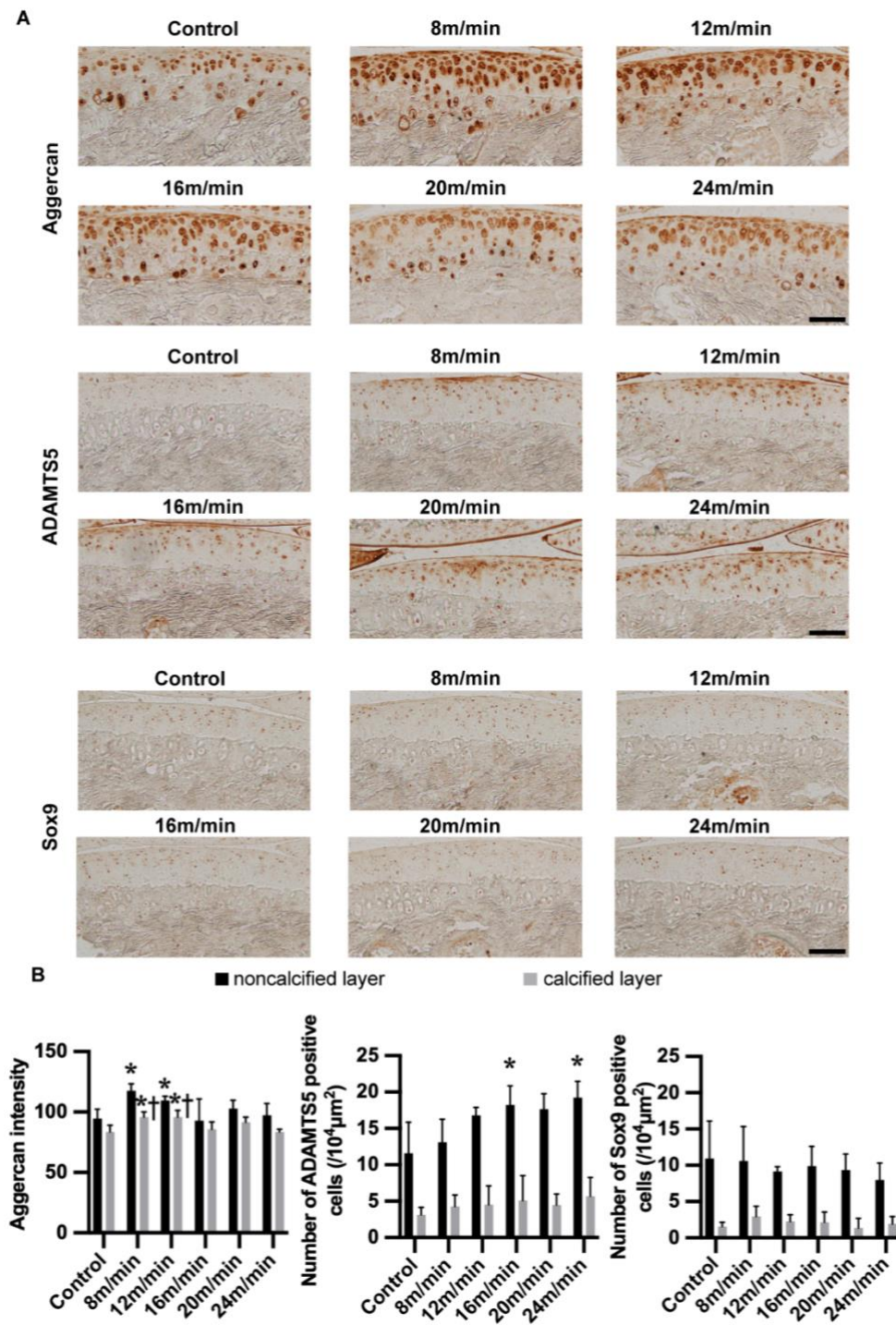
(A) Toluidine blue-stained and HE-stained images of the articular cartilage (scale bar is 100 μm). (B) Subchondral bone changes observed at a speed of 24 m/min. (C) Quantitative results of articular cartilage thickness, chondrocyte density and subchondral bone thickness in tibial cartilage. * indicates a significant increase compared to the control group ($n = 4$ mice per group).

Figure 3. Changes in type II collagen, MMP-13.



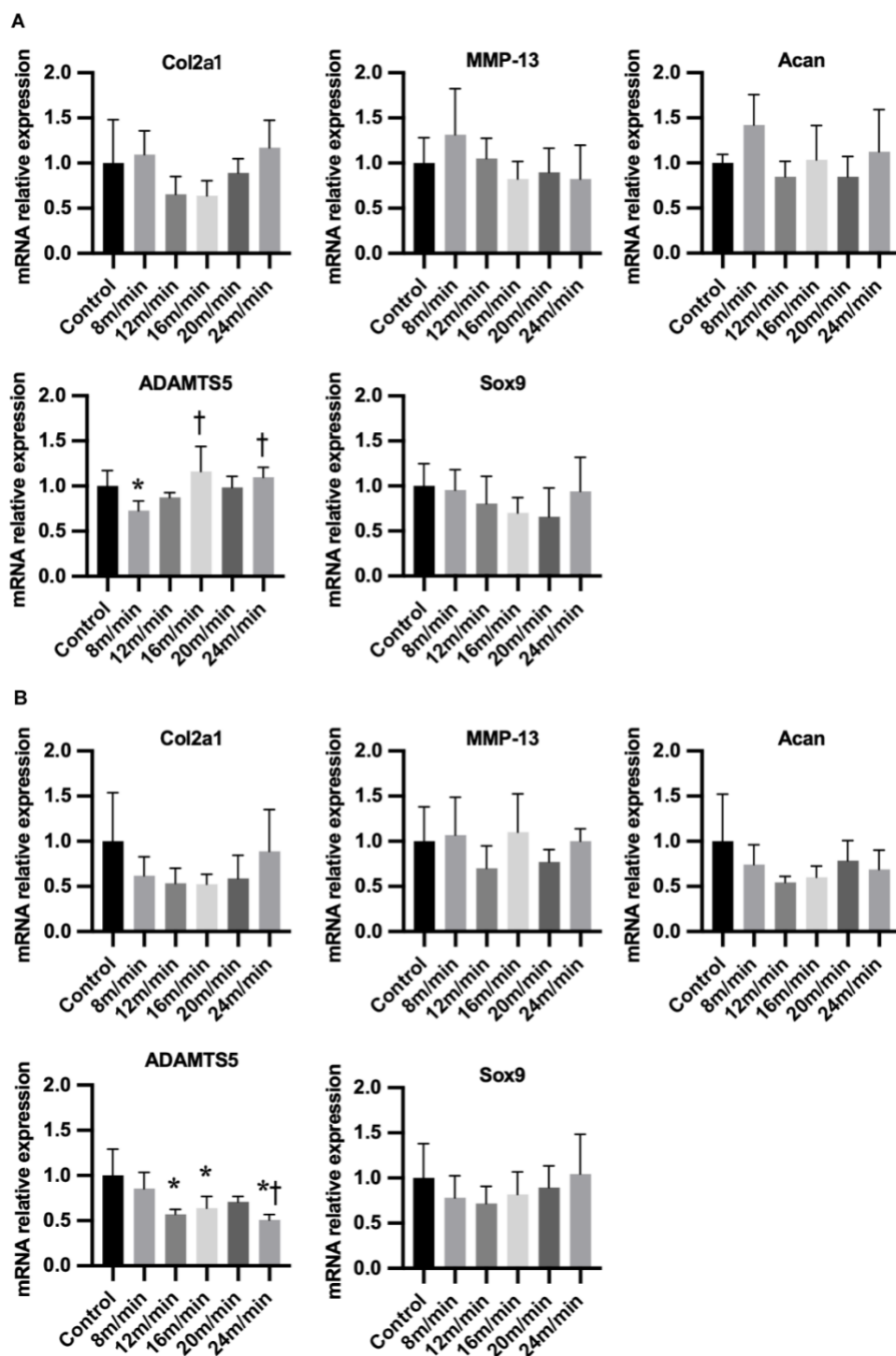
(A) Immunostaining images of type II collagen and MMP-13 in TM. Immunoreactivity of MMP-13 was detected only in the calcified layer (scale bar is 100 μm). (B) Quantitative results of type II collagen intensity and the number of MMP-13 positive cells ($n = 4$ mice per group).

Figure 4. Changes in aggrecan, ADAMTS5 and Sox9.



(A) Immunostaining images of aggrecan, ADAMTS5, Sox9 in TM (scale bar is 100 μm). (B) Quantitative results of aggrecan intensity, the number of ADAMTS5 and Sox9 positive cells in TM. * indicates a significant increase compared with the control group. † indicates a significant increase in expression compared with the 24 m/min group ($n = 4$ mice per group).

Figure 5. Changes in the expression of genes related to cartilage metabolism in acute and chronic exercise groups.



(A) The gene expression levels of type II collagen, MMP-13, aggrecan, ADAMTS5, and Sox9 were measured in the acute exercise and the control groups using 18S as an intrinsic control. (B) The gene expression levels of type II collagen, MMP-13, aggrecan, ADAMTS5, and Sox9 were measured in the chronic exercise and the control groups using 18S as an intrinsic control.

The $2^{-\Delta\Delta CT}$ values were then calculated ($n = 4$ mice per group).