

Effect of Running with Different Intensities on Lubricin Expression of Achilles Tendon in a Rat Model

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Abstract

Background: Lubricin is well known to facilitate the movement of tendon fascicles gliding and recoil against the surrounding tissues in tendons. However, little is known about its response under various mechanical loading conditions. This study was aimed to understand the effect of treadmill running with different exercise intensities on the alternations of lubricin content in rat Achilles tendon.

Methods: In this study, eighteen rats were randomly divided into three groups, strenuous treadmill running (STR), moderate treadmill running (MTR), and control (CON). Rats in two running groups were subjected to treadmill running (8 weeks) protocol. Histological observation and biochemical analysis were conducted using the collected Achilles tendons.

Results: After 8 weeks, the morphologies of collagen fibers are relatively parallel, crimping and elastic in the CON and MTR groups, but more ruptured in the STR group. The cell density in Achilles tendon sections markedly increased in MTR group than CON group, whereas considerably decreased in STR group than CON or MTR group. Additionally, compared to the CON group, the content of lubricin was dramatically increased in MTR group. However, the lubricin content in STR group was markedly decreased compared with that in CON or MTR group. Moreover, the TGF- β 1 expression was upregulated in MTR group in contrast to CON group, while significantly downregulated in STR group than that in CON or MTR group. Conversely, the expression of IL-1 was statistically downregulated in MTR group compared with CON group, but statistically upregulated in STR group than that in CON or MTR group.

Conclusions: These results suggest that moderate treadmill running might induce the increase of lubricin by the up-regulation of TGF- β 1 expression to improved lubrication, and enhance the loading transmission efficiency. Whereas, strenuous treadmill running could result in the decrease of lubricin as a result of the dramatically increased expression of IL-1 to enhance interfascicular tribology, and predispose to tendinopathy even tendon rupture.

Background

Lubricin, a mucinous glycoprotein, was initially reported to be isolated from synovial fluid and produced by synovial cells as an essential lubricant in joints to protect cartilage surfaces[1, 2]. Recent researches have demonstrated that lubricin is also found to be present in canine, caprine as well as human tendons, likewise, show anti-adhesion and lubricating properties[3–6]. Moreover, this molecule has been mainly identified within fascicles and fascicular sheaths, which facilitate the tendon movement fascicles gliding and recoil against the surrounding tissues in tendons., suggesting a lubricating role with regard to motion between the fascicles in tendons to enhance tendon-gliding in vitro and in vivo[7, 8].

The main function of tendons is to transfer mechanical force from skeletal muscles to bones to move or/and stabilize joints[9]. It has been shown that collagen fibers are regarded as predominant structural components and the main contributor to mechanical force transmission[10–11]. Collagen fibers are divided into bundles and then gathered to form the entire tendon. They are straight and parallel, but have

periodic curls[12–13]. As transmitting a mechanical force, the natural action of tendons induces gliding between collagen fibers to allow tendon to stretch and crimps become flatten. When the tendon relaxes, collagen fibers recoil elastically and crimps restore to their original shape[13]. The presence of lubricin, a lubricating molecule within fascicles and fascicular sheaths, facilitates sliding in tendons, suggesting enhancement of the loading transmission efficiency[14]. Therefore, lubricin seems to play a critical role in tendon function by facilitating tendon fascicles gliding between adjacent fascicles.

Furthermore, there is some evidence that certain mechanical and biochemical stimuli could regulate the content of lubricin in tendons. For example, Sun et al. showed that stress deprivation by suspending forepaws without weight-bearing for twenty-one days reduced lubricin synthesis in canine flexor tendon[15]. Conversely, Zhang et al. reported that extracorporeal shockwave therapy induced expression of lubricin in tendons and septa though both low-dose and high-dose for four days[16]. In addition, studies have demonstrated that transforming growth factor- β 1 (TGF- β 1) and interleukin-1 (IL-1) could alter lubricin expression in tendons as well[16].

Although there are a lot of research reports on lubricants, the effect of lubricants on tendons under various mechanical loading conditions is still unclear. The purpose of this study is to observe the effect of different exercise intensities on the change of lubricant content. The current study will improve our understanding of the role of lubricin in Achilles tendon function, and help provide a potential explanation for the disparate incidence of tendinopathy even tendon rupture in strenuous exercise.

Material And Methods

Experimental animals and training protocols.

All experimental protocols, including the treadmill running and tendon sample collection were approved by the animal ethics committee of Nanfang hospital, Southern Medical University.

Eighteen male Wistar rats, aged 12 weeks, weighing in the range of 200–250 g, were purchased from the Animal Experimental Center of Southern Medical University and randomly divided into three groups: (1) control [CON, n = 6], (2) moderate treadmill running [MTR, n = 6], and (3) strenuous treadmill running [STR, n = 6]. All animals were housed in the animal care facility on a 12:12-h light-dark cycle and were fed with chow and water ad libitum.

The running protocol was conducted as previously described [17]. Briefly, all animals were firstly accustomed to 1 week's treadmill running at a speed of 10 meters per min (30 min per day, 5 days per week). Then, the mice in the MTR and STR groups ran for 60 minutes a day, 5 days a week, for 8 weeks; respectively at a speed of 19 meters per min with 5° incline (MTR) and 27 meters per min with 10° incline (STR). On the other hand, rats in CON group were set to move freely. Experimental protocols and the use of experimental animals are implemented in accordance with the standards of the institution.

After the treadmill running experiment, the experimental animals were killed by asphyxiation with carbon dioxide and cervical dislocation. Remove the plantaris tendon and other soft tissues, and dissect the gastrocnemius and soleus muscles. The two combined tendons were cut at the distal end of the gastrocnemius soleus and the stop point of the calcaneus.[10]. For each rat, one Achilles tendon was fixed in 10% buffered formalin, and then histological examination was performed; while place the contralateral Achilles tendon at -80°C for cryopreservation and then extract mRNA.

Hematoxylin-eosin (H&E) staining.

H&E *staining* was conducted as previously described [18]. Formalin-fixed tendon is treated with alcohol and embedded in paraffin. Next, samples were cut into 4- μm -thick sections. After deparaffinization and rehydration, the sections were stained with hematoxylin-eosin. The morphologies of collagen fibrils were observed under the microscope (Axioskop 40 Pol) (20 \times objective).

Immunohistochemistry for lubricin.

Immunohistochemistry for lubricin was performed as described previously[1]. Briefly, 4-mm thick sections were deparaffinized with xylene and alcohol, followed by incubation with 3% hydrogen peroxide for 20 min to quench the endogenous peroxidase activity. After the antigen retrieval and blocking steps, the sections were incubated for 8–12 h at 4 °C with specific anti-rat lubricin (sc-98454) (Santa Cruz Biotechnology INC., CA, USA). The secondary antibodies (1: 200; all from Santa Cruz Biotechnology INC., USA) were incubated for 1 h at room temperature. Then, sections were treated with 3,3'-Diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark) and counter-stained in haematoxylin. In the controls the primary antibody was replaced with blocking solution. All incubation conditions and times were strictly controlled for guarantee of good comparability. Observe the results under the Nikon H600L microscope (Tokyo, Japan). Images were collected using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Western blotting.

Achilles tendon tissues were lysed with radioimmunoprecipitation assay lysis buffer pre-mixed with 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitors (all from SigmaAldrich; Merck KGaA). Next, the samples were oscillated by ultrasound system and centrifuged at 12,000 \times g for 15 min (4°C). The supernatant was collected, and the protein concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher scientific fc, Inc.). Subsequently, the protein samples (20 μg for each sample) were separated using 12% SDSPAGE (BioRad Laboratories, CA, USA) and electrophoretically transferred onto the polyvinylidene difluoride membranes (Thermo Fisher Scientific, MA, USA). Next, 3% bovine serum albumin was used to block the membranes, followed by incubation with anti-rat lubricin antibody (1:100; Abcam ab175404) for 12 h at 4°C. Then the membranes

were washed with 0.1% TBST buffer for three times and incubated for 1 h at room temperature with mouse antirabbit IgGHRP antibodies (1:1,000; Santa Cruz sc2357). Chemiluminescence solution (Luminata™ Crescendo Western HRP substrate; Ma, USA) and molecular imaging® ChemiDoc™ XRS system (Bio Rad Laboratory, Inc.) were used for luminescence imaging.. The relative protein expression of lubricin (normalized to GAPDH level) was assessed by densitometric quantitative analysis using ImagePro Plus software (version 6.0, Media Cybernetics, MD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Trizol reagent (TaKaRa, Dalian, China) was used to extract total RNA from the Achilles tendon tissues according to the manufacturer's protocols. The RNA was reverse transcribed into cDNA using a transcription RT kit (TaKaRa Dalian, China). Real-time quantitative PCR was performed on ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA), using the following protocol: 10 min heating at 95 °C, 95 °C for 10 sec (45 cycles), 55 °C for 15 sec and 72 °C for 30 sec. Table 1 showed the information of PCR primers (Bio Teke, Beijing, China). The expression level of the target gene was normalized to GAPDH gene level. Relative mRNA expression of lubricin in MTR or STR group standardized to CON group was calculated by $2^{-\Delta\Delta Ct}$.

Table 1
Primer sequence used in quantitative PCR

Primer	Forward	Reverse
GAPDH	5'-GGCACAGTCAAGGCTGAGAATG - 3'	5'-ATGGTGGTGAAGACGCCAGTA-3'
TGF-β1	5'- TGCGCCTGCAGAGATTCAAG - 3'	5'- TAACGCCAGGAATTGTTGCTA-3'
IL-1	5'-CTCCATGAGCTTTGTACAAGG-3'	5'-TGCTGATGTACCAGTTGGGG-3'
GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β1, transforming growth factor-β1; IL-1, interleukin-1.		

Statistical methods.

Data are presented by mean ± standard deviation. One-way analysis of variance was used for group comparison and Tukey's test was used for post hoc analysis. Using SPSS 21.0 (Chicago, IL, USA) for statistical analysis, $p < 0.05$ was statistically significant.

Results

Histological observation

Figure 1 presented the morphologies of collagen fibrils, which was stained with H&E under a polarized light microscope, from Achilles tendon sections after 8 weeks. A relatively parallel, crimping and elastic

collagen fibers were presented in the CON (Fig. 1A) and MTR (Fig. 1B) groups, and more ruptured collagen fibers were found in the STR group (Fig. 1C). There was a trend towards decreased interfascicular friction after moderate exercise, whereas increased interfascicular friction after strenuous exercise.

H&E staining

Representative images for tendon histology were shown for H&E staining in CON, MTR and STR groups (Fig. 2A-C) after 8 weeks' running tread. Histological analysis (Fig. 2D) revealed that the cell density in Achilles tendon sections obviously increased in MTR group in contrast to CON group ($p = 0.041$), whereas markedly decreased in STR group in contrast to CON ($p = 0.039$) or MTR ($p = 0.039$ and $p = 0.028$) group.

Immunohistochemistry.

Representative images of lubricin in Achilles tendon sections were showed in CON, MTR and STR groups (Fig. 3A-C) after 8 weeks. Immunohistological analysis of lubricin was also presented (Fig. 3D). At 8 weeks, lubricin content was significantly higher in MTR group (0.17 ± 0.004) than that in CON group (0.14 ± 0.003), but markedly lower in STR group (0.12 ± 0.006) than CON or MTR group.

Western blotting.

Protein levels of lubricin were obtained using western blotting, which confirmed the observations on immunohistological analysis of lubricin as well. after 8 weeks' training, lubricin protein expression levels increased statistically in MTR animals than that in CON group ($p = 0.035$; Fig. 4A); while statistically decreased in STR group in contrast with CON ($p = 0.036$) or MTR ($p = 0.021$) group (Fig. 4B).

qRT-PCR.

Figure 5 recorded the changes of mRNA gene expression of TGF- β 1 (Fig. 5A) and, IL-1 (Fig. 5B) in rat Achilles tendons. At the 8th week, the expression of TGF- β 1 was dramatically upregulated in MTR group in comparison to CON group ($p = 0.034$), whereas, dramatically downregulated in STR group in comparison to CON or MTR group ($p = 0.042$ and 0.023). Nevertheless, at the 8th week, the gene expression of IL-1 was dramatically downregulated in MTR group compared with CON group ($p = 0.037$), but dramatically upregulated in STR group in comparison to CON or MTR group ($p = 0.039$ and $p = 0.025$).

Discussion

Achilles tendon, the strongest tendon in the human body, was subjected to the largest stress from calf muscle to calcaneus[19]. Thus, it is one of the most commonly injured tendons, posing a clinical challenge to orthopedic surgery[20]. During exercise, the major function of Achilles tendon was to transmitting load along the long axis, another major function of it is to stretch when loaded to store energy, and it can later return to the system[12]. Whereby, Achilles tendon is called energy storing tendon, which can experience strains in excess of 10% during use in vivo, while positional tendon (such as the anterior tibialis tendon) is generally subjected to small strains in the region of 2–3%[14, 21]. Therefore, the more demanding the mechanical environment is, the more vulnerable the energy-storing tendons are to injury, which is called tendon disease[12]. During this natural action of stretching and recoiling of Achilles tendon, fascicles, bound together by collagen fibers which are regarded as predominant structural components and major attribution for the mechanical force transmission, glide between adjacent tendon fascicles and the surrounding tissues[22]. Energy storage tendons rely on lubrication between bundles to make beam slippage more elastic and recoverable to improve the efficiency of load transmission[14]. In contrast, the reduction of lubricin is associated with increased interfascicular friction and energetic cost, suggesting that it may be at increased risk of rupture[8]. Therefore, lubricin seems to play a critical role in tendon function.

In this study, treadmill models with different conditions were used to simulate different exercise intensity to distinguish moderate, vigorous and strenuous exercise, we observed that collagen fibers in rat Achilles tendon at 8 weeks were organized in a crimping and elastic pattern in the CON and MTR groups, in comparison to sub-rupture in STR group. Meanwhile, the alternations of lubricin content in rat Achilles tendon at 8 weeks were examined. The principal finding was that MTR led to an increased content of lubricin in Achilles tendon, whereas STR led to a decreased content of lubricin. Using a similar rat model, we had previously demonstrated that there is a marked intensity-specific effect of running on the immunolocalisation and gene expression of lubricin in cartilage [1]. In line with this previous study, the present study provides additional evidences that mechanical factors are key determinants of lubricin metabolism in vivo.

To reveal the underlying mechanisms, the mRNA gene expression of TGF- β 1 and IL-1 was examined. In this study, TGF- β 1 expression was significantly up-regulated in MTR group than that in CON group, while significantly down-regulated in STR group (compared with CON or MTR group). In agreement with this, the expression of TGF- β 1 is significantly higher after moderate exercise, while dramatically lower after strenuous exercise[13, 23]. Previous researches have demonstrated that TGF- β 1 can stimulate mitogenic responses of tendon fibroblasts and mediate lubricin synthesis in tendon[24–26]. This was consistent with our data from histological analysis in this study, which revealed that the cell density in Achilles tendon sections significantly increased in MTR group in contrast to CON or MTR group after 8 weeks. Moreover, it was reported that tenocytes are capable of expressing lubricin[3–5]. Therefore, it can be concluded that the content of lubricin is increased in Achilles tendons in response to moderate exercise by the up-regulation of TGF- β 1 expression, which improved lubrication and reduce gliding resistance and enhanced wear protection, suggesting enhancement of the loading transmission efficiency.

Additionally, it should be noted that the mRNA gene expression of IL-1 was dramatically decreased in MTR group in comparison to CON, but dramatically increased in STR group in contrast to CON or MTR group. Previous studies have indicated that repeated microinjuries caused by strenuous exercise may occur, and IL-1, a typical pro-inflammatory cytokine, is up-regulated[18, 27]. Other studies have shown that IL-1 decreased expression of lubricin by inhibiting cell proliferation. This supports our results presenting that the cell density in Achilles tendon sections was significantly decreased in STR group in contrast to CON or MTR group after 8 weeks. Thus, these findings suggest that the downregulation of lubricin content as a result of the dramatically increased expression of IL-1 in response to strenuous exercise may enhance interfascicular tribology, which predisposed to tendinopathy leading to tendon rupture[28].

However, the limitation of this study was lacking other observation time point (except for 8 weeks) and differences in lubricant expression between rodents and humans. In addition, the roles and mechanisms of other key molecules involved in tendon function need to be further explored in future.

Conclusions

In conclusion, the present work shows that the strength of the treadmill running effect is dependent on rat Achilles tendon lubricin content. The experimental data demonstrated that moderate exercise might benefit the Achilles tendon through increasing lubricin content and facilitating sliding and recoil. Nevertheless, strenuous exercise may lead to decreased lubricin content and enhance interfascicular tribology, thus predispose to tendinopathy leading to tendon rupture. However, more point-in-time and time-frame studies are needed to validate these findings.

Abbreviations

TGF- β 1: transforming growth factor- β 1; IL-1: interleukin-1; MTR: moderate treadmill running; STR: strenuous treadmill running; H&E: Hematoxylin-eosin; qRT-PCR: Quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Declarations

Ethics approval and consent to participate

All experiments were performed in accordance with the guidelines for animal care and use approved by animal ethics committee of Nanfang hospital, Southern Medical University.

Consent for publication

All authors gave consent to publish.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

We hereby declare that none of the authors has any competing interests concerning this study.

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Author contributions

GN conceived of the study. SX participated in the design and wrote most of the manuscript. CS, SD, SL, WL, and YH performed the experiments, and analyzed data. SL helped draft the manuscript. All authors read and approved the final manuscript.

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Figures

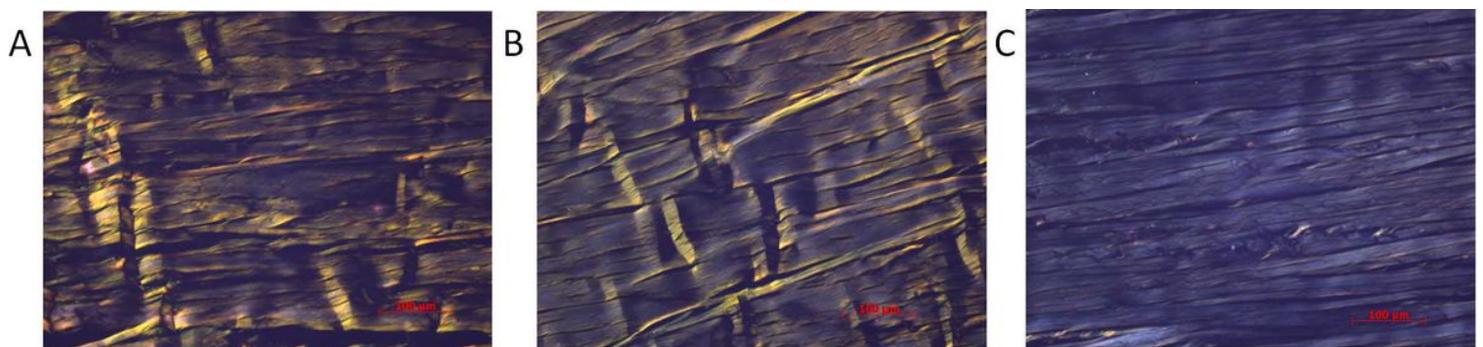


Figure 1

Morphologies of collagen fibrils from rat Achilles tendons stained with H&E under a polarized light microscope in CON (A), MTR (B) and STR (C) groups at 8 weeks. Scale bar represents 100 μm .

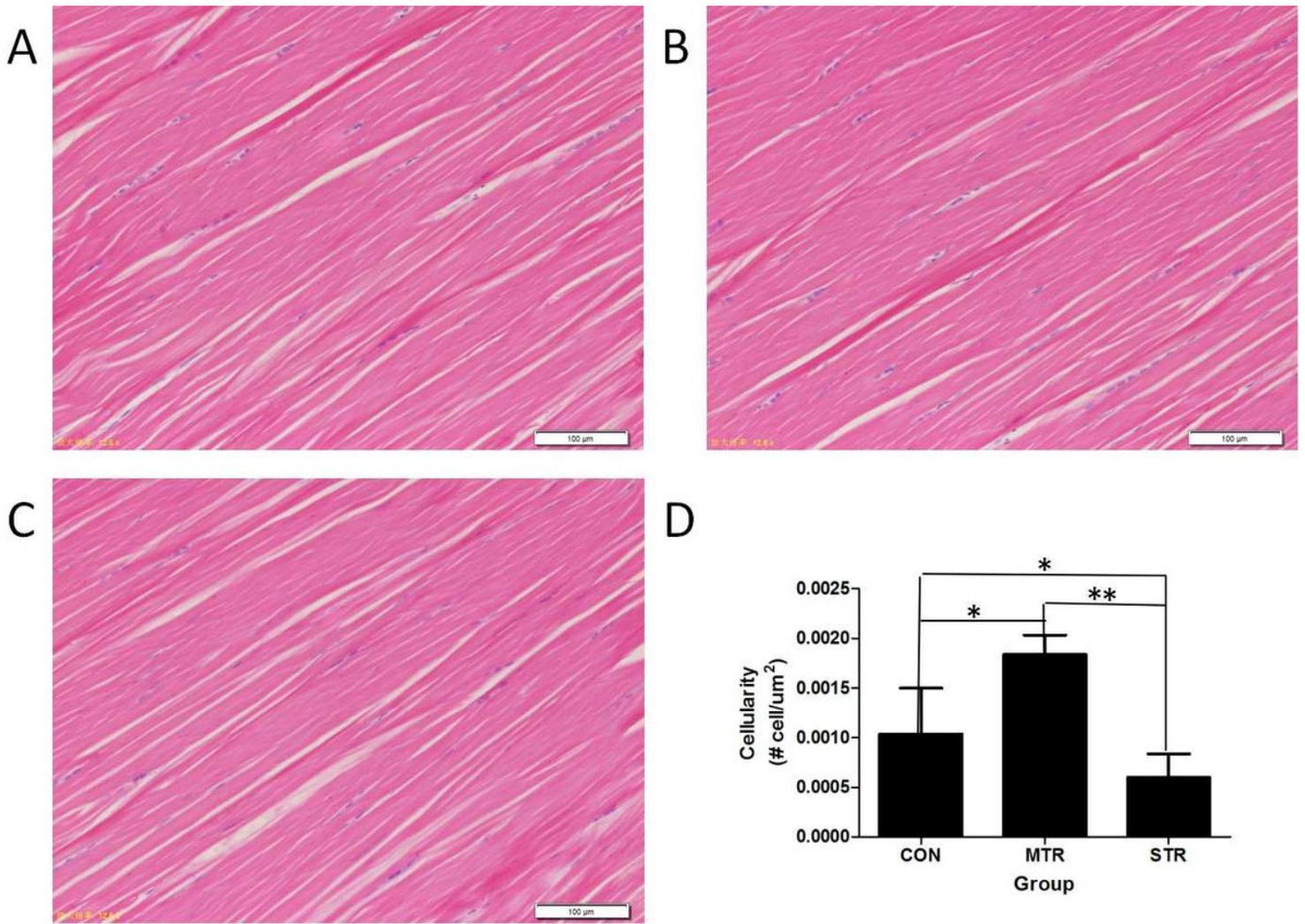


Figure 2

Representative photographs showing the H&E staining from rat Achilles tendons in CON (A), MTR (B) and STR (C) groups at 8 weeks. Figure 2D showed the cell density in each group (Data are shown as mean \pm SD; * $P < 0.05$ compared to CON group; ** $P < 0.05$ compared to MTR group). Scale bar represents 100 μm .

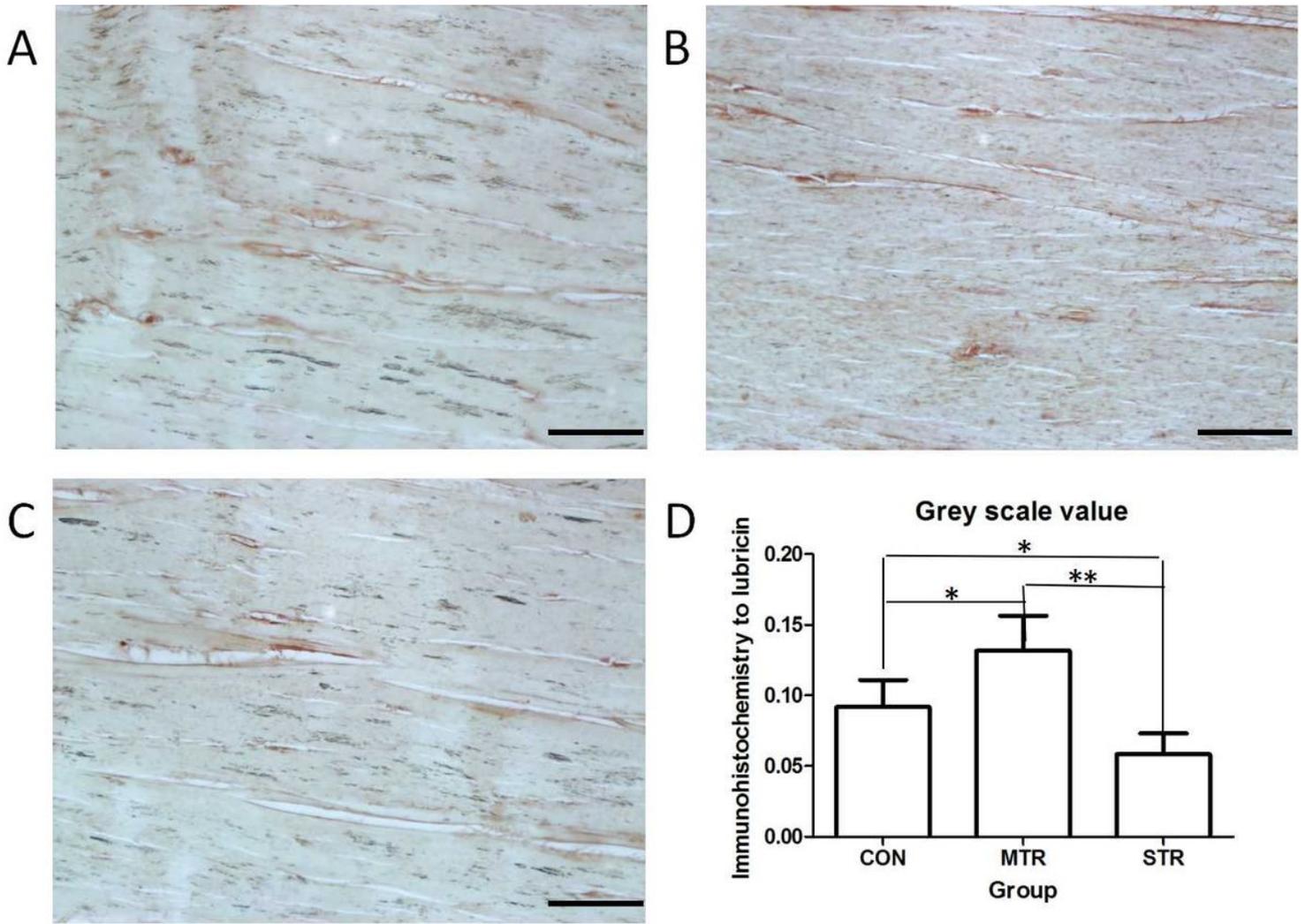


Figure 3

Representative photographs showed the immunohistochemical staining for lubricin from rat Achilles tendons in CON (A), MTR (B) and STR (C) groups at 8 weeks. Figure 3D showed immunohistological analysis of lubricin in each group (Data are shown as mean \pm SD; * $P < 0.05$ compared to CON group; ** $P < 0.05$ compared to MTR group). Scale bar represents 50 μm .

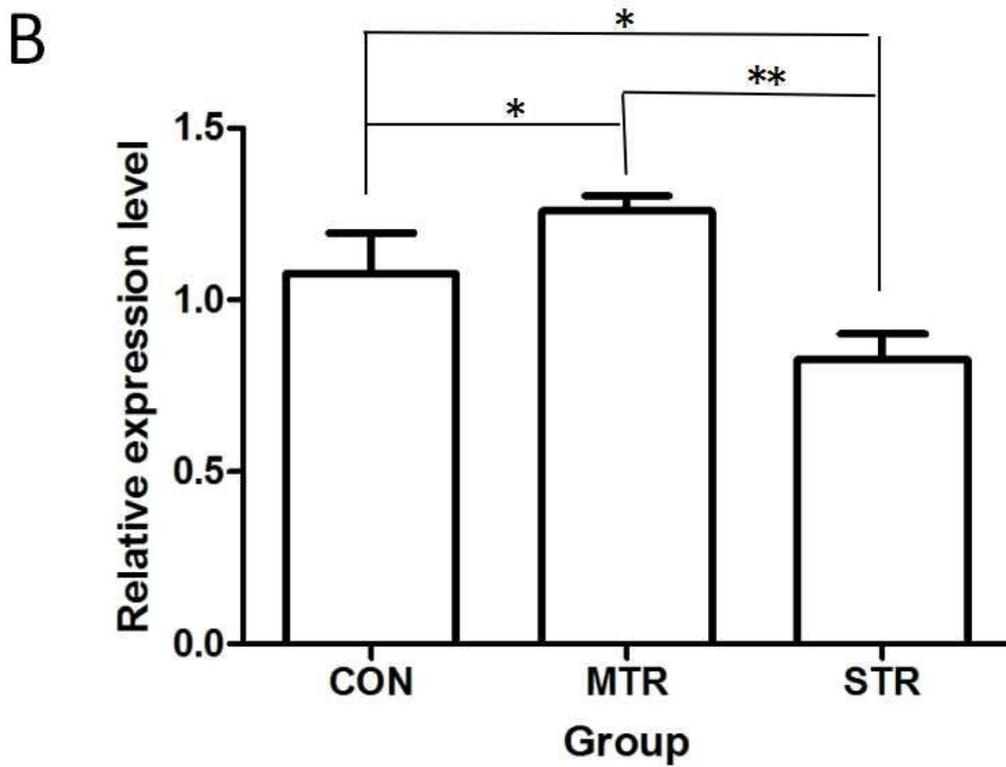
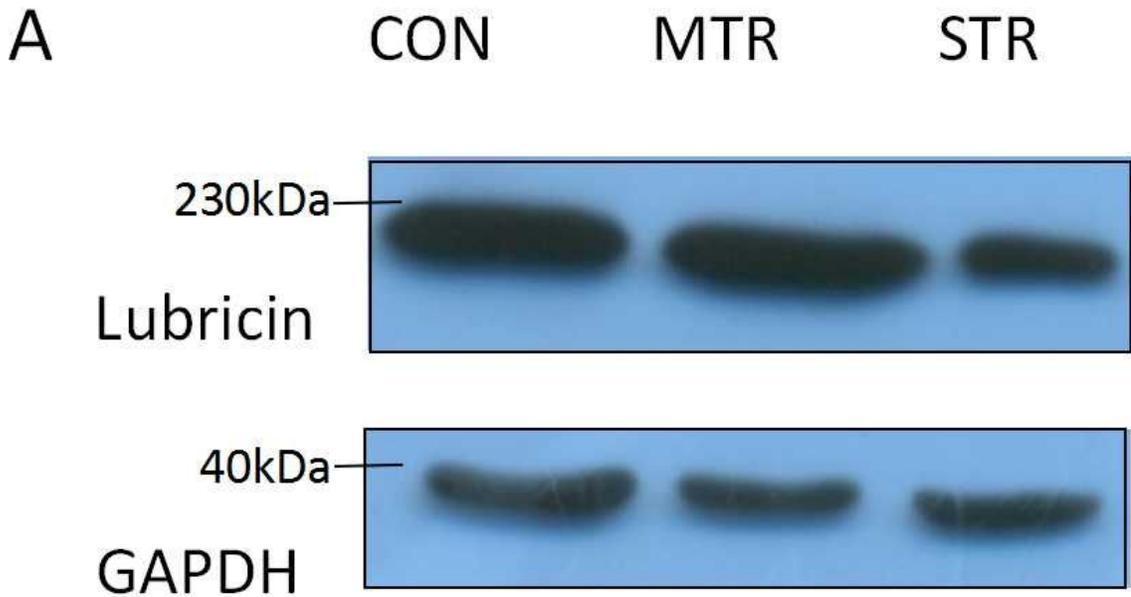


Figure 4

Representative western blot images showing protein expression levels of lubricin (A). Figure 4B showed the quantification of lubricin protein expression levels in rat Achilles tendons in each group (Data are expressed as the mean \pm standard deviation. * $P < 0.05$ compared to CON group; ** $P < 0.05$ compared to MTR group).

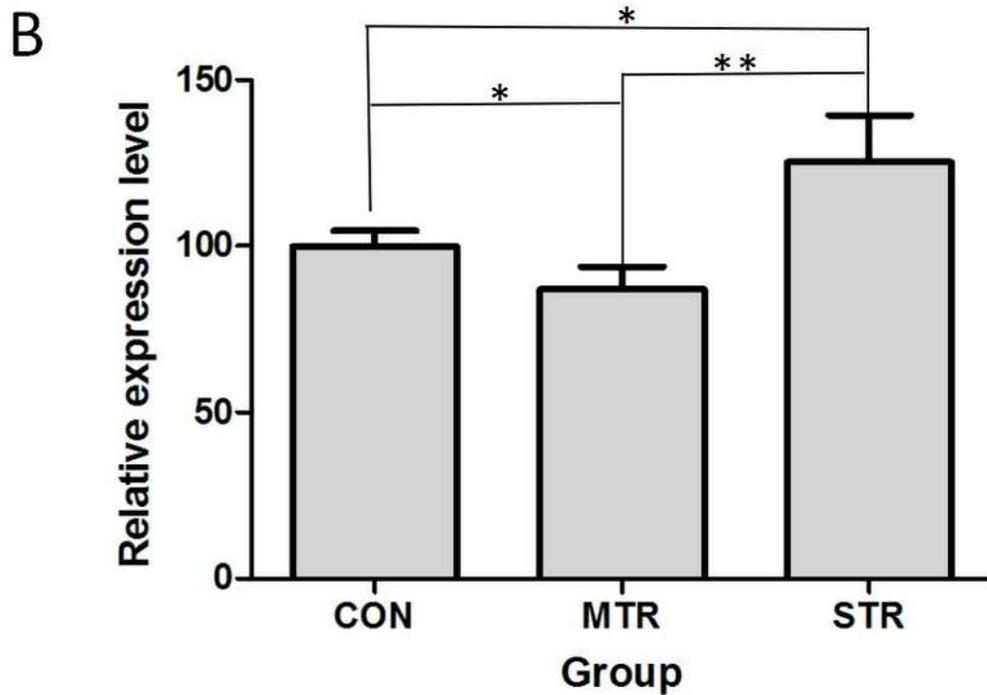
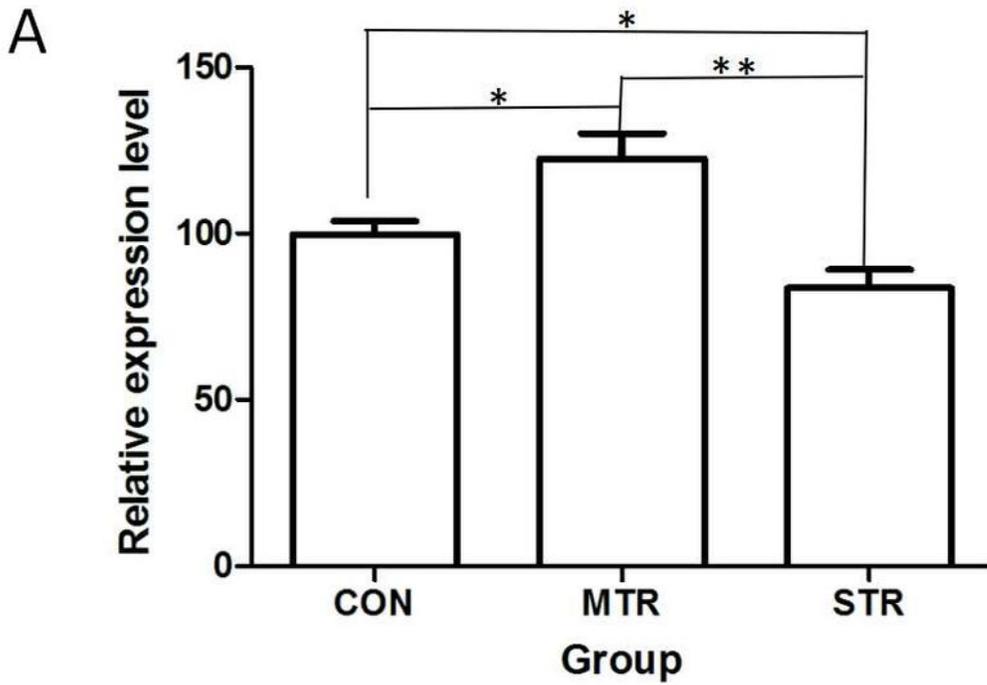


Figure 5

The mRNA expression levels of TGF- β 1 (A) and IL-1 (B) from rat Achilles tendons in each group determined by RT-PCR (Data are shown as mean \pm SD; * P<0.05 compared to CON group; ** P<0.05 compared to MTR group).

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