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Strenuous Treadmill Running Induces a Chondrocyte Phenotype in Rat Achilles Tendons

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Although tendinopathy is common, its underlying pathogenesis is poorly understood. This study aimed to investigate the possible pathogenesis of tendinopathy.

Material/Methods: In this study, a total of 24 rats were randomly and evenly divided into a control (CON) group and a strenuous treadmill running (STR) group. Animals in the STR group were subjected to a 12-week treadmill running protocol. Subsequently, all Achilles tendons were harvested to perform histological observation or biochemical analyses.

Results: Histologically, hypercellularity and round cells, as well as disorganized collagen fibrils, were presented in rat Achilles tendon sections from the STR group. Furthermore, our results showed that the expression of aggrecan, collagen type II (Col II), and Sex-Determining Region Y Box 9 (Sox 9) were markedly increased in the STR group compared with that in the CON group. Additionally, the mRNA expression of bone morphogenetic protein-2 (BMP-2) and biglycan was significantly up-regulated in the STR group in contrast to that in CON group.

Conclusions: These results suggest that a 12-week strenuous treadmill running regimen can induce chondrocyte phenotype in rat Achilles tendons through chondrogenic differentiation of tendon stem cells (TSCs) by BMP-2 signaling.

MeSH Keywords: Biglycan • Bone Morphogenetic Protein 2 • Tendinopathy

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Background

Chronic tendinopathy is an extremely common tendon disorder in athletes and the general population [1]. It is usually characterized by activity-related chronic tendon pain, local tenderness, and dysfunction [2]. The majority of scholars believe change in tendon loading as a result of repetitive tendon overuse is the primary etiological factor of tendinopathy [3]. Tendon overuse injuries have been reported to account for 30–50% of all sports-related injuries and half of all occupational illnesses in the USA [4]. Damaged tendon tissue generally heals very slowly and rarely attains the structural integrity and mechanical strength of normal, undamaged tendon [5].

Calcified tendinopathy is regarded as a special type of tendinopathy with chondrocyte phenotype and/or calcium deposits in tendon tissue [6]. Many studies have reported ectopic calcification in tendons in clinical samples and in animal models [7–10]. Clinically, forearm extensor tendons, Achilles tendons, and patella tendons are particularly susceptible to calcified tendinopathy, as well as rotator cuff and supraspinatus tendons [11]. The histopathological features have been described to include altered cell morphology, disorganized collagen arrangement, increased glycosaminoglycan (GAG) content, occasional fatty deposits, acquired chondrocyte phenotypes, and/or ectopic ossification [12,13]. Moreover, these alterations are reported to worsen clinical manifestations of tendinopathy, with an increase in the rupture rate, slower recovery times, and higher frequency of postoperative complications [14]. Nevertheless, the underlying pathogenesis of tendinopathy and calcified tendinopathy is poorly understood and treatment is usually palliative [15].

It was recently found that tendon stem cells (TSCs) are the precursors of tendon cells (tenocytes), which naturally occur in tendon tissues in various species, including humans, horses, rabbits, rats, and mice [16]. Subsequently, various studies demonstrated that these cells have several universal characteristics of stem cells, including clonogenicity, self-renewal, and multi-potent differentiation capacity [16]. It is well accepted that TSCs are essential for maintaining tendon tissue homeostasis and repair as TSCs-formed tendon-like tissue [17]. Similarly, a previous study indicated that mechanical loading regulates TSCs proliferation and differentiation [18]. For example, Zhang et al. reported that low mechanical stretching (4%) induced differentiation of TSCs into tenocytes, while large stretching (8%) promoted differentiation of TSCs into non-tenocyte lineages such as adipocytes, chondrocytes, and osteocytes [18]. Given that chondrocytes, osteoblasts, and adipocytes do not have good migratory ability, it was accepted that these non-tenocytes were from the differentiation of TSCs [16]. There is increasing evidence that erroneous differentiation of TSCs into non-tenocytes, including chondrocytes and osteoblasts, might contribute to the pathogenesis of calcified tendinopathy, due to the changes in the mechanical and biological microenvironment [17,19]. Using a collagenase-induced patellar tendon injury model, Lui et al. found chondrocyte phenotype at week 4 and ossified deposits surrounded by chondrocyte phenotype at week 12 [20]. In line with this, chondrocytes phenotype/markers and/or ectopic ossification were found in clinical samples of calcified tendinopathy [12,21].

Ectopic expression of chondro-osteogenic bone morphogenetic proteins, including bone morphogenetic protein-2 (BMP-2), was found to be higher in clinical samples and animal model tendons of ossified and unossified tendinopathy compared to normal, healthy tendons [22,23]. Early work indicated that BMP-2, which is signaled through the Smad1-Smad5-Smad-8 pathway, promotes chondrogenic and osteogenic differentiation of TSCs, and inhibits tenogenic marker expression [4]. It was also reported that TSCs are sensitive to BMP-2 stimulation [24]. Fibrocartilaginous tissue and ectopic mineralization were found after TSCs were treated with BMP-2 and subcutaneous transplantation into mice [16]. Hashimoto et al. showed that ectopic bone was formed in rabbit tendon injected with recombinant BMP-2 [24]. These findings suggest that BMP-2 might be involved in the pathogenesis of calcified tendinopathy [25]. Finally, a previous study found that biglycan might affect the sensitivity of TSCs to BMP-2 [26]. Biglycan is a class I molecule in the small leucine-rich proteoglycan family and is expressed mainly in tendon, cartilage, and bone as well as in dermis and blood vessels [27]. In tendon, it was reported to be multifunctional and widely involved in many biological processes [28]. Biglycan in extracellular matrix (ECM) can interact with type I collagen to regulate collagen fibril assembly, binding to transforming growth factor-beta (TGF-beta) to participate in modulation of cell proliferation and becoming a strong trigger of pro-inflammatory signaling [27,29]. This molecule was also reported to affect bone BMP signaling, thereby influencing the proliferation and differentiation of TSCs and participating in the pathogenesis of calcified tendinopathy [16].

Despite this progress, the precise pathogenic mechanisms of calcified tendinopathy have not been determined. In the present *in vivo* study, we mimicked excessive mechanical loading on Achilles tendons using a strenuous treadmill running regimen characterized by repetitive overuse to investigate the pathogenesis of calcified tendinopathy.

Material and Methods

Experimental animals and exercise protocols

The Animal Ethics Committee of Nanfang hospital, Southern Medical University approved all experimental protocols using

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rats, including the treadmill running and collection of tendon samples.

A total of 24 male Sprague-Dawley rats (12 weeks, weight 200–250 g) were randomly and evenly divided into 2 groups: (1) control (CON, n=12) and (2) strenuous treadmill running (STR, n=12). All animals were housed and fed chow and water ad libitum. The room was under controlled light: dark (12: 12 h) and maintained at 22±1°C.

The running regimen has been described previously [30]. Briefly, rats in the STR group were first accustomed to treadmill running for 1 week at a speed of 10 meters per min, for 30 min per day, 5 days per week. Then, they regularly ran for 12 weeks at a speed of 27 meters per min with 10° incline, for 60 min per day, 5 days per week. Animals in the CON group were allowed to move freely in cages. All experiments were conducted in accordance with the institutional guidelines for the care and use of experimental animals.

After completion of the treadmill running regimen, rats were killed by carbon dioxide asphyxiation followed by cervical dislocation. Then, both Achilles tendons were dissected. Only the mid-substance, but not the tissue at the tendon-bone junction, was carefully harvested. Thereafter, a randomly selected Achilles tendon (left or right) of each animal was frozen in liquid nitrogen and stored for mRNA expression analysis at –80°C. The contralateral Achilles tendon was fixed in 10% buffered formalin for histological observations.

Hematoxylin-eosin (H&E) staining

The formalin-fixed tendon samples were fixed with 4% neutral formaldehyde, dehydrated in ethanol, and embedded in paraffin. Then, all samples were cut into 4-μm–thick sections. Subsequently, sections were deparaffinized, rehydrated, and stained with hematoxylin-eosin.

Picrosirius red staining

Achilles tendon tissues from each group were obtained by surgical excision, fixed in buffered formalin, and embedded in paraffin. After cutting with 4-mm–thick sections, sections were deparaffinized and stained with 5% picrosirius red to highlight collagen fiber structure and improve its natural birefringence under a polarized light microscope (Axioskop 40 Pol) (20× objective).

Immunohistochemistry

Immunohistochemistry for aggrecan, collagen type II (Col II), and Sex-Determining Region Y Box 9 (Sox 9) (the chondrocyte-related factor) was performed. Briefly, formalin-fixed tendon samples were dehydrated in ethanol and embedded in paraffin. Then, 4-mm–thick sections were cut and deparaffinized with xylene and different concentrations of alcohol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 20 min at room temperature. Antigen retrieval was performed with citric acid (pH 6.0) by high-pressure method. After blocking with 5% normal bovine serum for 20 min at room temperature, the sections were incubated with specific primary antibodies at 4°C overnight. The primary antibodies were anti-rat aggrecan (1: 100 dilution, Fisher Scientific, IL, USA), anti-rat Col II (1:100 dilution, Fisher Scientific, IL, USA), and anti-rat Sox 9 (1:100 dilution, Fisher Scientific, IL, USA). The secondary antibodies (1:200; all from Santa Cruz Biotechnology, CA, USA) were incubated for 1 h at room temperature. Then, sections were developed with 3,3'-Diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark) and counter-stained in hematoxylin. Primary antibody was replaced with blocking solution in the controls. For good reproducibility and comparability, all incubation times and conditions were strictly controlled. The sections were examined under a light microscope (Nikon H600L Microscope and Image Analysis System, Tokyo, Japan). Images were captured using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

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

Achilles tendon tissues for qRT-PCR were broken into pieces with a masher. Subsequently, total RNA was extracted using Trizol reagent (TaKaRa Biotech Co., Ltd., Dalian, China) in accordance with the manufacturer's instructions. Afterwards, the RNA was reversely transcribed to cDNA using a transcription RT kit (TaKaRa Biotech Co., Ltd., Dalian, China), following the manufacturer's protocol. Real-time quantitative PCR with SYBR Green detection chemistry was performed using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of genes was normalized to the expression of β-actin gene. The PCR protocol used was as follows: 10 min heating at 95°C, followed by 45 cycles at 95°C for 10 s, 55°C for 15 s, and 72°C for 30 s. PCR primers pairs (Bio Teke Co., Ltd., Beijing, China) are shown in Table 1. Relative gene expression in the STR group compared to the CON group was calculated according to the $2^{-\Delta\Delta CT}$ formula.

Statistical methods

Results are expressed as the mean ± standard deviation. Statistical analysis was carried out using one-way analysis of variance (ANOVA) and Tukey's test for post hoc analysis. The data analysis was done using SPSS 16.0 (Chicago, IL, USA) with significance set at $P<0.05$.

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Table 1. Primer sequence used in quantitative PCR.

Primer	Forward	Reverse
Aggrecan	5'-ATCGTGGGCCGCCCTAGG CA-3'	5'-TGGCCTTAGGGTTCAGAGGGG-3'
Col II	5'-CCCCCGGCCCTGTCGGTCCC-3'	5'-CAGCAAAGGCGCACATGTCG-3'
Sox 9	5'-ACTCCGAGACGTGGACATC-3'	5'- TGTAGGTGACCTGGCCGTG-3'
biglycan	5'-TCTACATCTCCAAGAACCACCTGG-3'	5'-GCTCTGGGCTCCTACTCCTT-3'
Runx 2	5'-CGGAGCGGACGAGGCAAGAG-3'	5'-AGAGTCATCAAGCTTCTGTCTGTG-3'
BMP-2	5'-TAGTGACTTTTGGCCACGACG-3'	5'-GCTTCCGCTGTTTGTGTTTG-3'
β-actin	5'-ATCGTGGGCCGCCCTAGGCA-3'	5'-TGGCCTTAGGGTTCAGAGGGG-3'

Col II – collagen type II; Sox 9 – sex-determining region Y Box 9; Runx 2 – runt-related transcription factor 2; BMP-2 – bone morphogenetic protein-2.

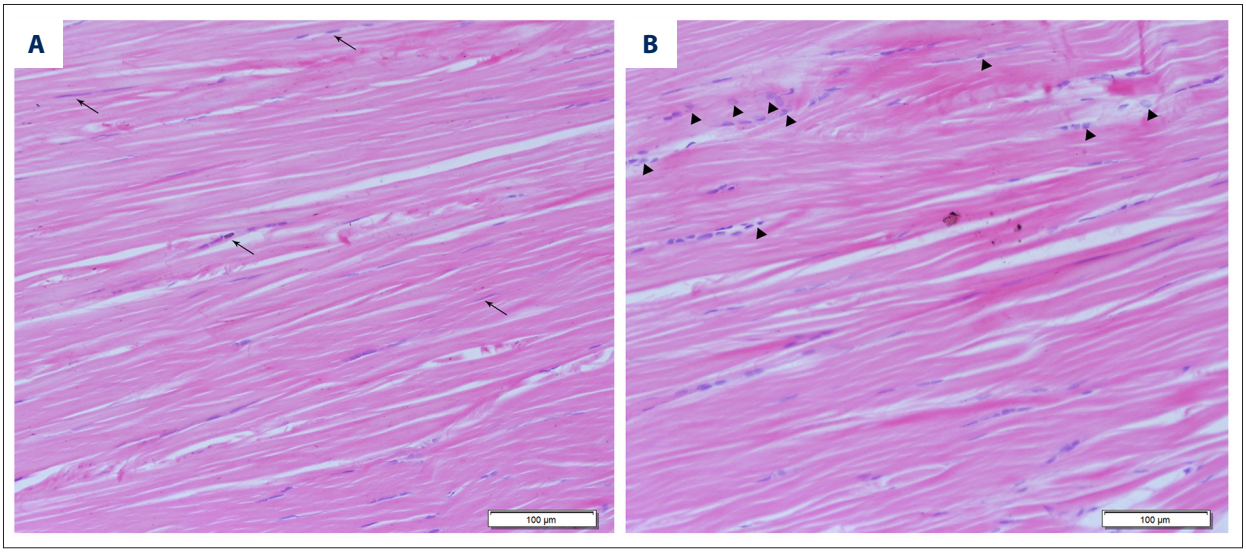

Figure 1. Representative histological sections with H&E staining from rat Achilles tendons in CON (**A**) and STR (**B**) groups. Scale bar represents 100 μm; arrows: tenocytes; triangle: chondrocyte-like cells.

Results

H&E staining

Figure 1 presents typical photographs from the 2 groups from Achilles tendon sections stained with H&E after completion of the 12-week treadmill running regimen. A relatively small number of elongated cells are shown in the CON group (Figure 1A), and more round cells were found in the STR group (Figure 1B).

Picrosirius red staining

Figure 2 shows the structural features of collagen fibrils in the 2 groups by picrosirius red staining under a polarized light microscope. Collagen birefringence was strong and collagen fibers were organized in a parallel and regular pattern in the CON group (Figure 2A), in contrast to the lower collagen birefringence and irregularly organized collagen fibrils found in the STR group (Figure 2B).

Immunohistochemistry

Figure 3 shows representative immunostaining of aggrecan (Figure 3A, 3B), Col II (Figure 3D, 3E), and Sox 9 (Figure 3G, 3H) in Achilles tendon sections in the CON (Figure 3A, 3D, 3G) and STR (Figure 3B, 3E, 3H) groups. Immunohistological analysis was performed using Image-Pro Plus 6.0 software (Figure 3C, 3F, 3I). The expression of aggrecan was significantly higher in the STR group (0.238±0.046) than in the CON group (0.175±0.023), and the expression of Col II was markedly upregulated in the STR group (0.249±0.031) compared with that in the CON group (0.141±0.018). Similarly, the expression of Sox 9 was pronouncedly increased in the STR group (0.217±0.057) in contrast to that in the CON group (0.158±0.013).

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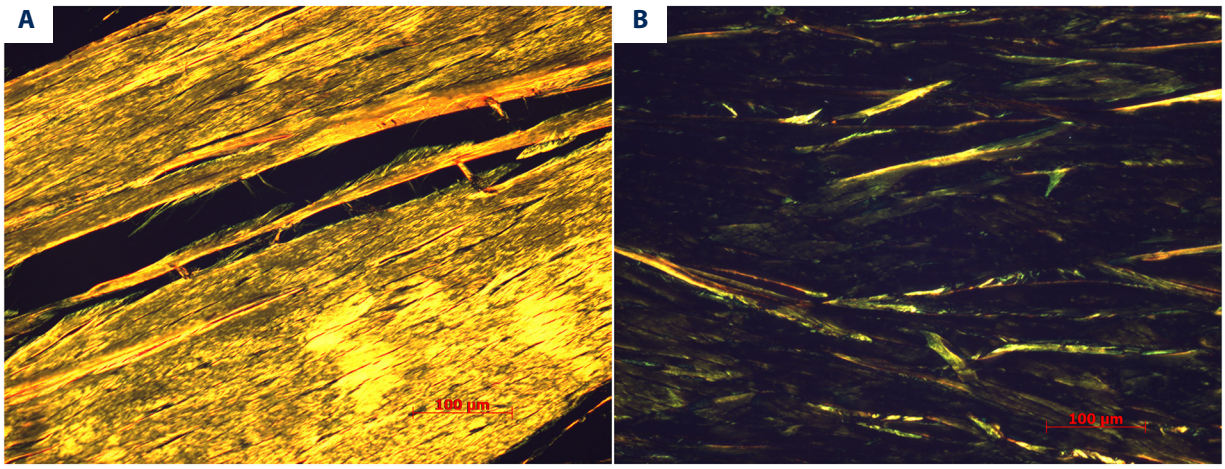

Figure 2. Representative histological sections of rat Achilles tendons in CON (**A**) and STR (**B**) groups, stained with picrosirius red using a polarized light microscope (20× objective).

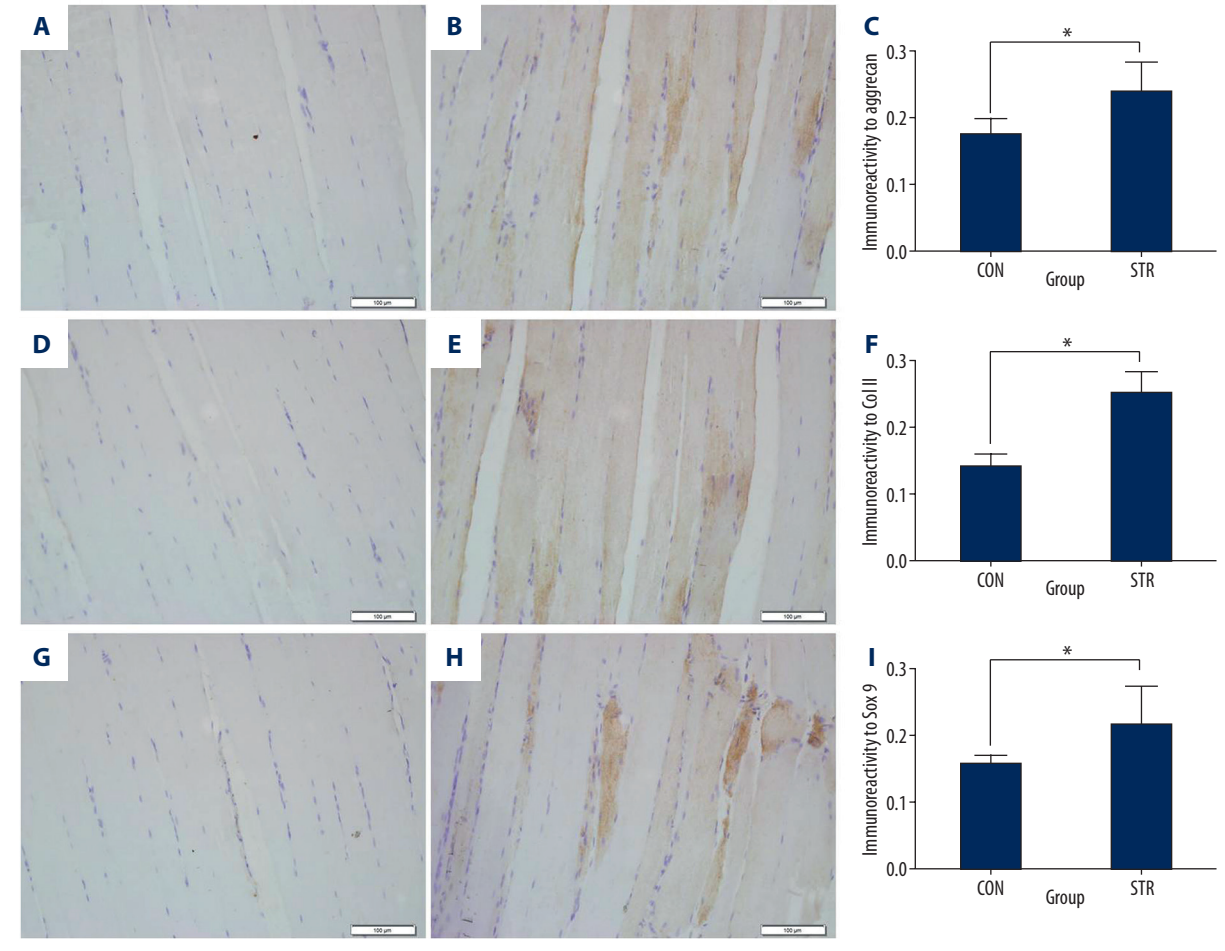

Figure 3. Representative photographs showed the immunohistochemical staining for aggrecan (**A, B**), Col II (**D, E**), and Sox 9 (**G, H**) in Achilles tendon sections in CON (**A, D, G**) and STR (**B, E, H**) groups, and statistical analysis (**C, F, I**). * P<0.05 compared to CON group. Scale bar represents 100 µm.

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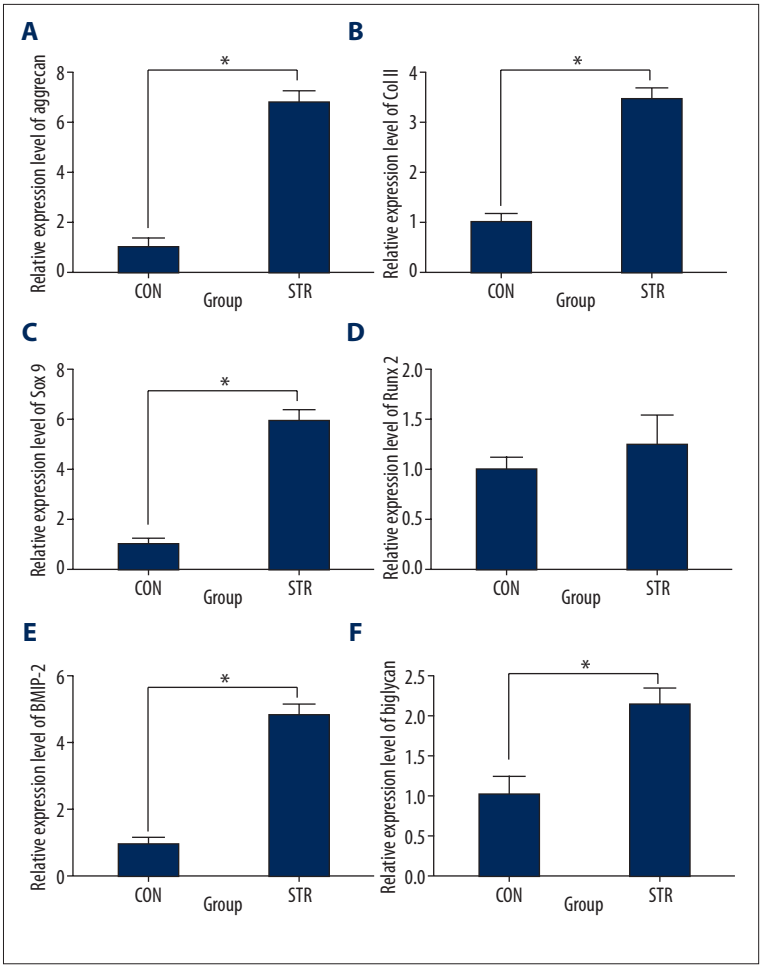

Figure 4. The mRNA expression levels of aggrecan (**A**), Col II (**B**), Sox 9 (**C**), Runx 2 (**D**), BMP-2 (**E**), and biglycan (**F**) from rat Achilles tendons in response to strenuous treadmill running after 12 weeks were determined by RT-PCR. Data are shown as mean ±SD; * P<0.05 compared to CON group.

qRT-PCR

Figure 4 shows changes in mRNA gene expression in rat Achilles tendons in the CON and STR groups. The mRNA expression of aggrecan, Col II, and Sox 9 was dramatically up-regulated in the STR group compared with that in the CON group (P=0.038, P=0.020, P=0.032, and P=0.029, respectively). In addition, a markedly increased mRNA expression of BMP-2 and biglycan was found in the STR group in comparison to that in the CON group (P=0.027). However, a less pronounced change in mRNA gene expression of runt-related transcription factor 2 (Runx 2) (known as osterix) was recorded in the STR group compared to that in the CON group (P=0.092).

Discussion

In the present study we used a treadmill running regimen at a speed of 27 m/min with 10° incline for 60 min/day for 12 weeks to model the repetitive, excessive mechanical loading placed on Achilles tendons. A previous study demonstrated that rats running on a 10° incline at 17 m/min to 20 m/min for 60 min/day for 12 weeks showed only slight adaptive changes in their Achilles tendons. However, a slight increase in speed and duration resulted in signs of tendinopathy, including hypercellularity, collagen fibril disorganization, and increased GAG content [31].

In the present study, hypercellularity and cell rounding was found in rat Achilles tendon sections from the STR group by H&E staining. It was well known that the tenocytes in tendons are elongated, whereas the chondrocytes are round [16]. Irregular collagen fibrils were observed after the 12-week regimen of strenuous treadmill running with picrosirius red staining under a polarized light microscope. Our findings show that the expression of aggrecan was markedly increased in the STR group. Aggrecan is regarded as one of the predominant large aggregating proteoglycans in tendons, containing many GAG chains [32]. Therefore, a marked increase in aggrecan provided a very likely basis for the increased GAG content in Achilles tendons. Additionally, aggrecan is commonly found in fibrocartilaginous regions or areas where the tendon inserts into bone or is wrapped around bone [33]. Similarly, Col II is highly expressed in cartilages or in fibro-cartilaginous regions of tendon, while low

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levels are found in the tensile mid-tendon [34]. Nevertheless, we found that the level of Col II was significantly increased in the STR group compared with that in the CON group. Our data suggest that the chondrocyte-like cells were acquired in rat Achilles tendons after 12 weeks of strenuous treadmill running. This was further confirmed by the dramatically up-regulated expression of Sox 9 in the STR group, which was reported as the specific marker for chondrocytes [35].

Our data from qRT-PCR indicate that the expression of BMP-2 was pronouncedly up-regulated in the STR group in comparison to that in the CON group. BMP-2 was reported to induce erroneous differentiation of TSCs and be regulated by biglycan. In the present study, the expression of biglycan was markedly increased in the STR group compared with that in the CON group, which is in contrast to results of previous studies. Using genetically engineered mice, studies indicated that genetic inactivation of biglycan causes ectopic ossification in tendon tissues [16,26]. The discrepancy might be due to use of different models. In our study, Achilles tendinopathy was mimicked with hypercellularity, abnormal-shaped cells, and irregularly organized collagen fibrils after 12 weeks of strenuous treadmill running. This is consistent with a clinical study that found an increase in the mRNA expression of biglycan in painful Achilles tendinopathy compared with that in normal tendon samples [33]. Lui et al. also reported that the expression of biglycan was up-regulated in calcified tendinopathy by collagenase injection [6]. Lui et al. suggested that decreasing stiffness of tendon due to abnormal collagen formation in tendinopathy induces ectopic chondrogenesis and/or ossification to attempt to compensate for the original decreased stiffness, and this could occur either in the absence or excess of biglycan [6]. Therefore, it can be concluded that BMP-2 is produced in Achilles tendons in response to strenuous treadmill running, regulated by biglycan, and induced the erroneous differentiation of TSCs into chondrocytes, which locally increased expression of aggrecan, Col II, and Sox 9 in tendons.

However, the present study did not find calcium deposits in Achilles tendon mid-substance. Similarly, a less pronounced change in the level of Runx 2 was observed in the STR group. This could be because ectopic ossification is rare, whereas ectopic chondrogenesis with loss of the typical elongated appearance of tenocytes and the accumulation of GAG is more common in tendinopathy [36]. On the other hand, the treadmill running time might have been insufficient, because calcification was thought to form at the end stage of tendinopathy [18]. In a study using a collagenase-induced tendon injury model, chondrocyte phenotype at week 4 and ossified deposits surrounded by chondrocyte phenotype at week 12 were observed [18]. In addition, other chondro-osteogenic BMPs such as BMP-4 and BMP-7 were also reported in clinical samples and in an animal model tendinopathy [4]. Clarification of these issues may require additional research.

Conclusions

Repetitive tendon overuse such as strenuous treadmill running resulted in collagen fibril disorganization and increased expression of BMP-2. Subsequently, BMP-2 regulated by biglycan might induce erroneous differentiation of TSCs into chondrocytes and failed healing of tendons. Finally, abnormal extracellular matrix components (e.g., GAG accumulation), round cells, and acquisition of chondrocyte phenotype were observed. These alterations could increase the stiffness but decrease the tensional load-bearing capacity of tendons, and might predispose tendons to rupture [37]. However, further studies are needed to validate our findings.

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Declaration of interest

The authors hereby declare that they have no conflicts of interest concerning this study.

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