Response of decorin to different intensity treadmill running

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Abstract. Decorin is widely understood to affect collagen fibrillogenesis. However, little is understood about its response to various mechanical loading conditions. In the present study, 36 Wistar rats were randomly divided into control (CON), moderate treadmill running (MTR) and strenuous treadmill running (STR) groups. Animals in the MTR and STR groups were subjected to a 4- or 8-week treadmill running protocol. Subsequently, all Achilles tendons were harvested to perform histological and biochemical analyses. Decorin expression was markedly increased in the MTR group compared with the CON group at 4 and 8 weeks. Conversely, decorin expression was markedly decreased in the STR group compared with the CON and MTR group at 4 and 8 weeks. Furthermore, between the two time points, decorin expression levels were significantly increased in the MTR group, whereas they were markedly decreased in the STR group. These results suggested that MTR exercise may induce increased decorin expression via a balance of MMP-2 and TIMP-2, improving tendon structure and function. However, STR exercise may result in degradation of decorin due to an imbalance of MMP-2 and TIMP-2, with a bias to MMP-2, resulting in a predisposition to tendinopathy.

Introduction

Tendons are responsible for transmitting tensile forces from skeletal muscle to bone (1). They are composed of fibroblasts and extracellular matrix (ECM) components, including type I collagen (Col I), proteoglycans (PGs) and water (2). Previous findings have demonstrated that Col I fibrils are highly aligned and significantly contribute to transmission of mechanical forces (3). Furthermore, Col I molecules are produced by fibroblasts and organized into fibrillar structures via a self-assembly process known as fibrillogenesis (4). During this process, PGs regulate the diameter, length and organization of collagen fibrils (5,6).

In the tensile region of the tendon, the predominant PG is decorin (80-90% of total PGs), followed by a small presence of biglycan (7), which are class I prototype members of the small leucine-rich proteoglycan (SLRP) family (8). Decorin was reported to be made up of a 40 kDa protein core and chondroitin/dermatan sulfate glycosaminglycans, and binds to Col I to regulate collagen fiber assembly (9,10). Furthermore, variations in decorin content has been reported to affect tendons; Reese *et al* (5) reported that during polymerization of Col I, decorin significantly increased the modulus and tensile strength of collagen gels via modification of collagen fibril organization. Conversely, knockout studies have demonstrated that mice with a decorin deficiency exhibit decreased strength and stiffness (11,12). Therefore, decorin may serve critical roles in tendon structure and function.

Additionally, it was identified that decorin is synthesized by stromal fibroblasts and degraded by matrix metalloproteinases (MMPs) (13,14). When tendons transmit mechanical force, fibroblasts detect this and convert mechanical signals into cellular biological events, including secretion of MMPs, via mechanotransduction mechanisms (15). MMPs and their specific inhibitors, tissue inhibitor of metalloproteinases (TIMPs), play major roles in the degradation and remodeling of ECM components (16). A balance of MMPs and TIMPs is required for maintaining healthy remodeling; impaired balance may result in degradation and disorder (17). Furthermore, a previous study demonstrated that MMP-2 may cleave and degrade decorin (18). Although four TIMP molecules have been identified, TIMP-2 is the most common and inhibits the activities of all MMPs, with a high affinity for MMP-2 (19).

Despite extensive research on decorin (5,7,9) thus far, how various mechanical loading conditions affect this molecule in tendons remains to be elucidated. The present study aimed to observe the effect of varying exercise intensity, characterized by distinct loading patterns during treadmill running, on alternations of decorin content at two time intervals (4 and 8 weeks) to assess decorin metabolism in the rat Achilles tendon.

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Materials and methods

Experimental animals and exercise protocols. The animal ethics committee of Nanfang Hospital, Southern Medical University (Guangzhou, China) approved all experimental protocols using rats including treadmill running and collection of tendon samples (NFYY-2012-056).

Male Wistar rats (age, 12 weeks; weight, 200-250 g; n=36) were randomly divided into three groups: Control (CON, n=12), moderate treadmill running (MTR, n=12) and strenuous treadmill running (STR, n=12). All the animals were housed in a 12-h light/dark cycle at 22±1˚C with free access to food and water.

The running protocol used has been described previously (20). The animals were accustomed to treadmill running for one week at a speed of 10 m/min for 30 min per day, 5 days per week. Subsequently, rats in the MTR and STR groups regularly ran for 4 or 8 weeks as follows: MTR: Speed of 19.3 m/min with 5˚ incline for 60 min per day, 5 days per week; STR: speed of 26.8 m/min with 10˚ incline for 60 min per day, 5 days per week. Animals in 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.

Following the treadmill running protocol, rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation. The gastrocnemius and soleus muscles were dissected free of all soft tissues including the plantaris muscle tendon unit. The two merging tendons were isolated by amputation: Proximally at the distal end of the gastrocnemius soleus muscle belly, and distally at the calcaneus insertion. One side of the Achilles tendon of each animal was frozen in liquid nitrogen and stored at -80˚C. The contralateral Achilles tendon was fixed in 10% buffered formalin for immunohistochemistry.

Immunohistochemistry for decorin and biglycan. Immunohistochemistry for decorin and biglycan was performed as described previously (21). Formalin-fixed tendon samples were dehydrated in ethanol and embedded in paraffin. Sections (4 μm) were cut and deparaffinized with xylene and varying concentrations of alcohol. Endogenous peroxidase activity was blocked by the addition of 3% hydrogen peroxide for 20 min at room temperature. Antigen retrieval was performed with citric acid (pH 6.0) by high pressure method. Following blocking with 5% normal bovine serum (Merck KGaA, Darmstadt, Germany) for 20 min at room temperature, the sections were incubated with specific primary antibodies at 4˚C overnight. The primary antibodies used were anti-rat decorin (1:100; cat. no. ab175404) and anti-rat biglycan (1:100; cat. no. ab49701), purchased from Abcam (Cambridge, MA, USA). Following this, sections were incubated with mouse anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibodies (1:200; cat. no. sc2357; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Subsequently, sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (Dako; Agilent Technologies GmbH, Waldbronn, Germany) and counter-stained in haematoxylin. Primary antibodies were 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; Nikon Corporation, Tokyo, Japan). Sections were imaged using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Achilles tendon tissues for RT-qPCR were crushed with a masher. Subsequently, total RNA was extracted using TRIzol® reagent (Takara Biotechnology Co., Ltd., Dalian, China) in accordance with the manufacturer's protocol. Following this, RNA was reverse transcribed into cDNA using a transcription RT Kit (Takara Biotechnology Co., Ltd.), following the manufacturer's protocol. qPCR with SYBR®-Green detection chemistry was performed using an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc. Waltham, MA, USA). Expression of the target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The thermocycling conditions used were as follows: Initialization for 10 min at 95˚C, followed by 45 cycles of denaturation at 95˚C for 10 sec, annealing at 55˚C for 15 sec, and extension at 72˚C for 30 sec. PCR primer sequences (BioTeke Corporation, Beijing, China) are presented in Table I. Relative gene expression of the MTR and STR groups to the CON group were calculated according to the $2^{-\Delta\Delta Cq}$ method (22).

Western blotting. Achilles tendon tissue samples were homogenized in radioimmunoprecipitation assay lysis buffer (50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.1% SDS and 1% Igepal CA-630), 0.5% sodium deoxycholate, a cocktail of protease inhibitors and 0.5 mM phenylmethylsulfonyl fluoride (all from Sigma-Aldrich; Merck KGaA). Following ultraphonic oscillation, the samples were centrifuged at 12,000 x g for 15 min at 4˚C to obtain the supernatant. Protein concentration was measured by Bicinchoninic Acid assay (Thermo Fisher Scientific, Inc.) and a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 20 μg protein was separated by 12% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Invitrogen; Thermo Fisher Scientific, Inc.) and blocked with 3% bovine serum albumin and non-fat milk. The expression of decorin was detected incubation the membranes for 12 h at 4˚C with an anti-rat decorin antibody (1:100; cat. no. ab175404; Abcam), with gentle shaking. Following washing with 0.2% TBS with Tween-20 buffer, membranes was incubated with a mouse anti-rabbit IgG-HRP secondary antibodies (1:1,000; cat. no. sc2357; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Protein detection was performed by Enhanced Chemiluminescence (Luminata™ Crescendo Western HRP Substrate; EMD Millipore, Billerica, MA, USA) using a Molecular Imager® ChemiDoc™ XRS system (Bio-Rad Laboratories, Inc.). The resulting bands were assessed by densitometric quantitative analysis of proteins using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) and normalized to GAPDH levels.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using one-way

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Primer	Forward	Reverse
GAPDH	5'-GGCACAGTCAAGGCTGAGAATG -3'	5'-ATGGTGGTGAAGACGCCAGTA-3'
Decorin	5'-ATGATTGTCATAGAACTGGGC-3'	5'-TTGTTGTTATGAAGGTAGAC-3'
Biglycan	5'-TCTACATCTCCAAGAACCACCTGG-3'	5'-GCTCTGGGCTCCTACTCCTT-3'
MMP-2	5'-GGAAGCATCAAATCGGACTG-3'	5'-GGGCGGGAGAAAGTAGCA-3'
TIMP-2	5'-CCAAAGCAGTGAGCGAGAA-3'	5'-CCCAG GGCAC AATAA AGTC-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP-2, matrix metalloproteinase-2; TIMP-2, tissue inhibitors of metalloproteinase-2.

analysis of variance followed by Tukey's post hoc test using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Immunohistochemistry. Immunostaining for decorin was performed in Achilles tendon sections of rats in CON (Fig. 1A), MTR (Fig. 1B) and STR (Fig. 1C) groups after 4 weeks, and in CON (Fig. 1D), MTR (Fig. 1E) and STR (Fig. 1F) groups after 8 weeks. At 4 weeks, decorin expression was markedly increased in the MTR group (0.21±0.009) compared with the CON group (0.16±0.011), and was markedly reduced in STR group (0.11±0.013) compared with the CON or MTR groups. At 8 weeks, a similar pattern of expression was observed. The MTR group (0.23±0.017) exhibited markedly increased expression of decorin compared with the CON group (0.17±0.008). However, the STR group (0.08±0.010) exhibited significantly reduced expression of decorin compared with the CON or MTR groups. Furthermore, in the MTR group, decorin expression was significantly increased at 8 weeks compared with at 4 weeks. Conversely, in the STR group, decorin expression was significantly reduced at 8 weeks compared with at 4 weeks (Fig. 1G).

Immunostaining for biglycan was performed in Achilles tendon sections of rats in CON (Fig. 2A), MTR (Fig. 2B) and STR (Fig. 2C) groups after 4 weeks, and in CON (Fig. 2D), MTR (Fig. 2E) and STR (Fig. 2F) groups after 8 weeks. At 4 weeks, although markedly reduced expression levels of biglycan were observed in the MTR group (0.10±0.015) compared with the CON group (0.13±0.008), expression of biglycan was markedly increased in the STR group (0.18±0.007) compared with the CON and MTR groups. At 8 weeks, the MTR group (0.07±0.009) had markedly reduced expression levels of biglycan compared with the CON group (0.11±0.012). However, the STR group (0.21±0.005) had significantly increased biglycan expression compared with the CON or MTR groups. Furthermore, in the MTR group, biglycan expression was significantly reduced at 8 weeks compared with at 4 weeks. Conversely, in the STR group, biglycan expression was significantly increased at 8 weeks compared with at 4 weeks (Fig. 2G).

RT-qPCR. mRNA expression levels of decorin (Fig. 3A), biglycan (Fig. 3B), MMP-2 (Fig. 3C) and TIMP-2 (Fig. 3D) were detected in rat Achilles tendons in the CON, MTR and STR groups after 4 and 8 weeks. At 4 weeks, the expression of decorin was significantly increased in the MTR group compared with the CON group (P=0.043), and were significantly decreased in the STR group compared with the CON and MTR groups (P=0.044 and P=0.028, respectively). Similarly, at 8 weeks, mRNA expression levels of decorin were markedly increased in the MTR group in comparison with the CON group (P=0.039), and were markedly decreased in the STR group compared with the CON and MTR groups (P=0.040 and P=0.022, respectively). Furthermore, in the MTR group, decorin expression was increased at 8 weeks compared with at 4 weeks (P=0.039). However, in the STR group, decorin expression was markedly decreased at 8 weeks compared with at 4 weeks (P=0.036). At 4 weeks, no significant differences in the mRNA expression of biglycan was recorded in MTR group compared with the CON group (P=0.081); however, it was significantly increased in the STR group compared with the CON and MTR groups (P=0.038 and P=0.021, respectively). Similarly, at 8 weeks, mRNA expression levels of biglycan were markedly decreased in the MTR group compared with the CON group (P=0.034), and were markedly increased in the STR group compared with the CON and MTR groups (P=0.037 and P=0.018, respectively). Furthermore, in the MTR group, biglycan expression was significantly decreased at 8 weeks compared with at 4 weeks (P=0.032). However, in the STR group, biglycan expression levels were increased at 8 weeks compared with at 4 weeks (P=0.035).

At 4 weeks, no significant differences were observed in the gene expression levels of MMP-2 in the MTR group compared with the CON group (P=0.113); however, they were markedly increased in the STR group compared with the CON and MTR groups (P=0.039 and P=0.037, respectively). At 8 weeks, the gene expression of MMP-2 was increased in the MTR group compared with the CON group (P=0.042), and in the STR group compared with the CON and MTR groups (P=0.024 and P=0.037, respectively). Additionally, in the MTR group, the expression levels of MMP-2 were significantly increased at 8 weeks compared with at 4 weeks (P=0.032). However, in the STR group, no significant differences were observed between 4 and 8 weeks (P=0.092).

At 4 weeks, no significant differences were observed in mRNA expression levels of TIMP-2 in the MTR group compared with the CON group (P=2.371), and in the STR group compared with the CON and MTR groups (P=0.738

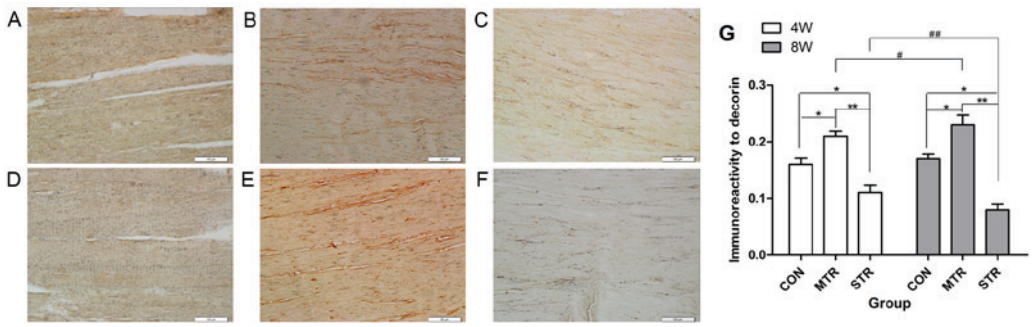

Figure 1. Immunohistochemistry for decorin. Representative images of immunohistochemical staining for decorin from rat Achilles tendons in (A) CON, (B) MTR and (C) STR groups at 4 weeks, and in (D) CON, (E) MTR and (F) STR groups at 8 weeks (scale bar=100 μm). (G) Quantification. Data are expressed as the mean ± standard deviation. *P<0.05 vs. CON; **P<0.05 vs. MTR; $^{\#}$P<0.05 vs. MTR group at 4 weeks, $^{\#\#}$P<0.05 vs. STR group at 4 weeks. CON, control; MTR, moderate treadmill running; STR, strenuous treadmill running.

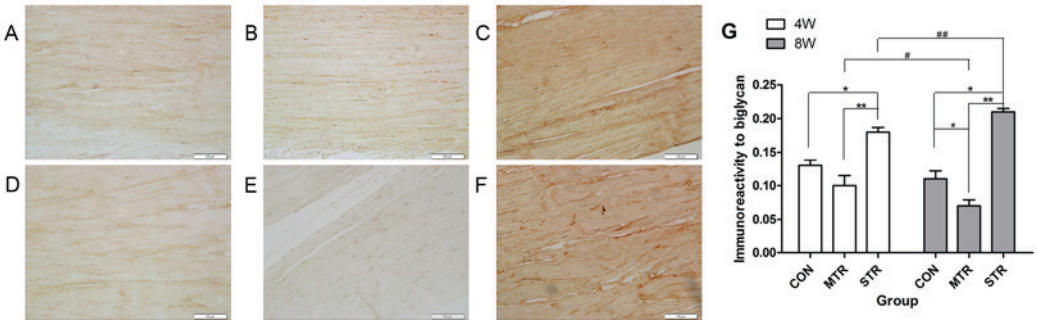

Figure 2. Immunohistochemistry for biglycan. Representative images of immunohistochemical staining for biglycan from rat Achilles tendons in (A) CON, (B) MTR and (C) STR groups at 4 weeks, and in (D) CON, (E) MTR and (F) STR groups at 8 weeks (scale bar=100 μm). (G) Quantification. Data are expressed as the mean ± standard deviation. *P<0.05 vs. CON; **P<0.05 vs. MTR; $^{\#}$P<0.05 vs. MTR group at 4 weeks, $^{\#\#}$P<0.05 vs. STR group at 4 weeks. CON, control; MTR, moderate treadmill running; STR, strenuous treadmill running.

and P=0.917, respectively). However, at 8 weeks, mRNA expression levels of TIMP-2 were increased in the MTR group compared with the CON group (P=0.034); however, were decreased in the STR group compared with the CON and MTR groups (P=0.026 and P=0.015, respectively). Additionally, in the MTR group, the expression levels of TIMP-2 were increased at 8 weeks compared with at 4 weeks (P=0.037). However, in STR group, TIMP-2 expression levels were decreased at 8 weeks compared with at 4 weeks (P=0.021).

Western blotting. Protein expression levels of decorin were detected by western blotting (Fig. 4A), and the results confirmed the observations on mRNA gene expression levels. At 4 weeks, protein expression levels for decorin were increased in the MTR group compared with the CON group (P=0.039); however, were markedly decreased in the STR group compared with the CON and MTR groups (P=0.032 and P=0.016, respectively; Fig. 4B). Similarly, at 8 weeks, decorin protein expression levels were increased in the MTR group compared with the CON group (P=0.034); however, were markedly decreased in the STR group compared with the CON and MTR group (P=0.031 and P=0.012, respectively; Fig. 4C). Decorin from the CON group was visualized as a single band of ~40 kDa, and no decorin fragments were observed in the MTR group. However, distinct decorin fragments of ~30 kDa were detected in the STR group.

Discussion

Decorin is regarded as a vital element in the tendon and regulates collagen fibril organization, thus affecting structure and function of the tendon. However, whether its expression is affected by various mechanical loading conditions remains to be elucidated. The present study examined alterations in decorin content in rat Achilles tendons at two time intervals (4 and 8 weeks), using a running treadmill model at various speeds and inclinations to represent moderate and strenuous exercise. These results indicated that at 4 and 8 weeks, expression levels of decorin were statistically increased in the MTR group compared with the CON group, whereas they were significantly decreased in the STR group compared with the CON and MTR groups. Furthermore, between the two time points, decorin expression was markedly increased in the MTR group, whereas it was significantly decreased in the STR group.

Subsequently, to evaluate the metabolism of decorin, mRNA expression levels of MMP-2 and TIMP-2 were examined. The data from the MTR group demonstrated that the balance of MMP-2 and TIMP-2 expression was maintained at 4 weeks; however, levels were altered at 8 weeks. This state of equilibrium may be conducive to the synthesis of decorin, and thus elevate the level of decorin in the ECM. Taken together, these results suggested that moderate exercise induced synthesis of decorin via the balance of MMP-2

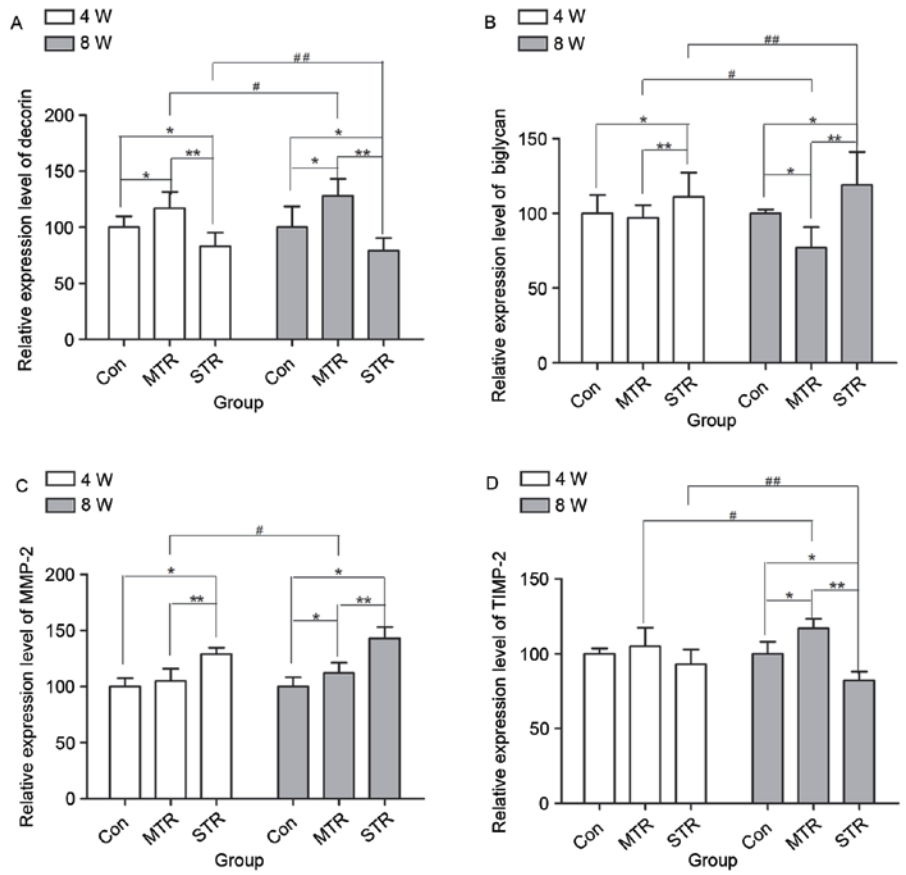

Figure 3. mRNA expression. mRNA expression levels of (A) decorin (B), biglycan, (C) MMP-2 and (D) TIMP-2 from rat Achilles tendons in response to MTR and STR after 4 or 8 weeks, as determined by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± standard deviation. *P<0.05 vs. CON; **P<0.05 vs. MTR; $^{\#}$P<0.05 vs. MTR group at 4 weeks, $^{\#\#}$P<0.05 vs. STR group at 4 weeks. CON, control; MTR, moderate treadmill running; STR, strenuous treadmill running; MMP-2, matrix metalloproteinase 2; TIMP-2, tissue inhibitor of metalloproteinases 2.

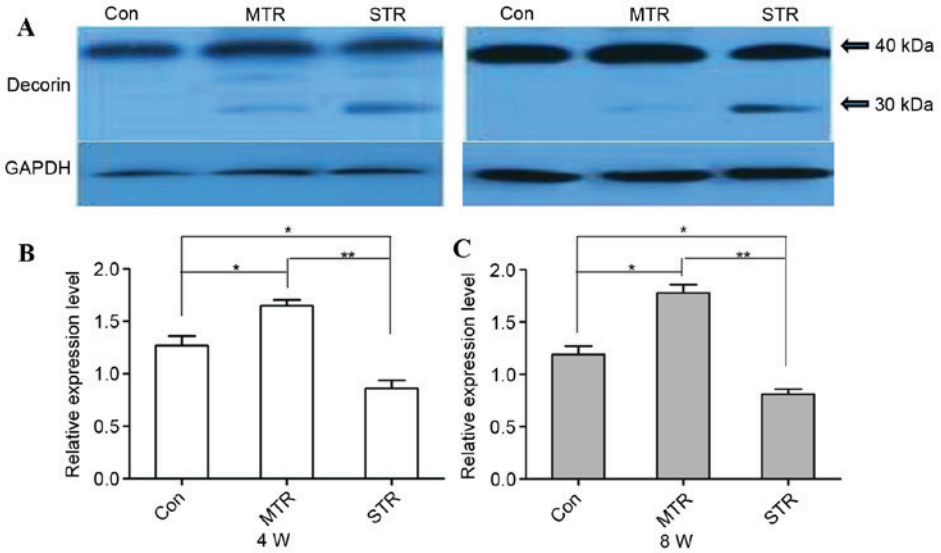

Figure 4. Protein expression. (A) Representative western blot images and quantification of decorin protein expression levels in response to MTR or STR at (B) 4 and (C) 8 weeks in rat Achilles tendons. Intact decorin core protein and fragments were identified in the STR group, whereas no low molecular weight bands were observed in the CON and MTR groups. Data are expressed as the mean ± standard deviation. *P<0.05 vs. CON; **P<0.05 vs. MTR. MTR, moderate treadmill running; STR, strenuous treadmill running.

and TIMP-2. This was consistent with previous biochemical analyses, which revealed an increase in decorin expression levels following moderate exercise (1,2). Decorin is the most abundant SLRP in the tendon and regulates the specialized assembly of Col I, which is the primary structural component and contributor to the transmission of mechanical strength (2).

This may explain why moderate exercise increased tendon stiffness and enhanced tendon tensile strength.

In the present study, observations from the STR group indicated that the balance of MMP-2 and TIMP-2 was impaired with a bias to MMP-2 at 4 weeks, and this effect increased in a time-dependent manner (from 4 to 8 weeks). This impaired balance may result in the degradation of decorin, reducing the expression levels of decorin in the ECM. In addition, the degraded fragment of decorin in the STR group was visualized by western blot analysis. This suggested that strenuous exercise, including STR, causes degradation of decorin; a key regulator of collagen fibril assembly. Previous studies have reported that collagen fibrils are coarse, irregular and haphazardly arranged with decreased strength and stiffness in the absence of decorin (11,12,23). In agreement with this, decorin content was significantly decreased in clinical samples of tendinopathy compared with healthy tendon samples (24,25). Tendinopathy is hypothesized to be involved in micro-injury and failed tendon healing due to repetitive excessive exercise (26). Therefore, decrease of decorin under strenuous exercise may lead to abnormal fibril structure and organization, decreasing tendon strength and stiffness, thus predisposing to micro-injury and tendinopathy.

In addition, biglycan has been reported to be highly homologous with decorin and bind to collagen to regulate collagen fiber assembly (6). The present study demonstrated a converse pattern of alteration in the expression levels of biglycan between the three groups at the two time intervals. A potential reason for this is that biglycan and decorin share common functions and partially compensated for each other.

In conclusion, the present study demonstrated a significant intensity-specific influence of treadmill running on decorin expression levels in rat Achilles tendons. These results suggested that moderate exercise may induce increased synthesis of decorin via balance of MMP-2 and TIMP-2, which may increase tendon stiffness and enhance tendon tensile strength, improving the structure and function of the tendon. However, strenuous exercise may result in the degradation of decorin by imbalance of MMP-2 and TIMP-2 with a bias to MMP-2, which may decrease tendon strength and stiffness, thus increasing the risk of damage and predispose to tendinopathy. Therefore, decorin may serve an important role in tendon pathophysiology. These results suggest decorin as a potential therapeutic target for the treatment of tendinopathies. However, further studies are required to validate these findings.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

GXN, SYL and SYX designed the study. SYX, SYL and LX performed the experiments. SYX, SYD and YBH collected the data. SYX and SFL analyzed the data. GXN, SYL and SYX interpreted the data. GXN, SYL, LX and SYX wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Animal Ethics committee of Nanfang Hospital, Southern Medical University (application no. NFYY-2012-056).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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