Time-Specific Effects of Acute Eccentric Exercise on Myostatin, Follistatin and Decorin in the Circulation and Skeletal Muscle in Rats

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Summary

Myostatin (MSTN), an important negative regulator of skeletal muscle, plays an important role in skeletal muscle health. In previous study, we found that the expression of MSTN was different during skeletal muscle injury repair. Therefore, we explored the expression changes of MSTN at different time points during skeletal muscle injury repair after eccentric exercise. In addition, MSTN is regulated by follistatin (FST) and decorin (DCN) in vivo, so our study examined the time-specific changes of FST, DCN and MSTN in the circulation and skeletal muscle during skeletal muscle injury repair after eccentric exercise, and to explore the reasons for the changes of MSTN in the process of exercise-induced muscle injury repair, to provide a basis for promoting muscle injury repair. The rats performed one-time eccentric exercise. Blood and skeletal muscle were collected at the corresponding time points, respectively immediate after exercise (D0), one day (D1), two days (D2), three days (D3), seven days (W1) and fourteen days (W2) after exercise (n=8). The levels of MSTN, FST, DCN in serum and mRNA and protein expression in muscle were detected. MSTN changes in the blood and changes in DCN and FST showed the opposite trend, except immediately after exercise. The change trends of mRNA and protein of gastrocnemius DCN and MSTN are inconsistent, there is post-transcriptional regulation of MSTN and DCN in gastrocnemius. Acute eccentric exercise might stimulate the secretion of DCN and FST into the circulation and inhibit MSTN. MSTN may be regulated by FST and DCN after acute eccentric exercise.

Key words

Exercise • Myostatin • Skeletal muscle • Decorin • Follistatin

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Introduction

During exercise, especially acute exercise, sports injuries often occur, among which muscle injuries are the most common. Muscle injuries account for about 35 % - 55 % of sports injuries. If muscle injuries aren't treated improperly, they are often accompanied by muscle fiber atrophy, formation of fibrous scar tissue, decline in muscle strength, function and exercise ability [1,2]. Furthermore, it may cause repeated damage. Severe muscle damage can cause a period of inactivity, which can easily lead to muscle atrophy. In the case of long-term injury, tissue repair may become abnormal, leading to fibrous scars or adipose tissue infiltration, seriously affecting its function [3].

MSTN can induce muscle atrophy and scar formation [4]. Inhibition of MSTN can increase the expression of myogenic factors in the muscle injury site, reduce the degree of fibrosis, and enhance the healing ability [5]. FST is an antagonist of MSTN. FST blocks MSTN to promote muscle fiber regeneration after skeletal muscle injury and reduce fibrosis formation in vivo, FST stimulates myoblasts to express MyoD, Myf5 and Myogenin, and promotes the expression of myogenic differentiation factors in vitro [6]. Recently, DCN is a new type of muscle cytokine that can inhibit the action of MSTN, thereby promoting muscle fiber hypertrophy

[7]. DCN is a TGF-β inhibitor, which can reduce the occurrence of intermuscular adipose tissue and is beneficial to muscle regeneration [8]. DCN secreted by myotubes during exercise can regulate muscle hypertrophy and may play a role in exercise-related skeletal muscle remodeling [7]. DCN promotes the proliferation and differentiation of C2C12 myoblasts by inhibiting the activity of MSTN *in vitro* [9]. DCN traps MSTN in the extracellular matrix and regulates its activity on myoblasts [10].

Previous studies reported that MSTN regulates skeletal muscle regeneration and repair, while DCN regulates exercise-related skeletal muscle remodeling [11,12]. FST can combine and neutralize MSTN. While the time series changes of MSTN, DCN and FST after eccentric exercise have not been reported before. Therefore, this study aims to investigate the changes in blood and skeletal muscle MSTN, DCN, and FST levels at different time points after acute eccentric exercise in rats.

Methods

Experiment animal and exercise protocols

In this study, fifty-six male Sprague-Dawley rats (~215grams, ~7 weeks old) were sourced from the Experimental Animal Center of Nanjing Medical University, Nanjing, China. The rats were housed in the animal house under controlled conditions for five days. The temperature was maintained at 25 ± 3 °C with the 12 hours light/dark cycles. The rats stayed in cages with ad libitum access to standard chow and water. They were randomly divided into two groups: control group (n=8) and exercise group (n=48). Further, the rats of exercise group were randomly divided into 6 groups after acute eccentric exercise, namely, immediate after exercise group (D0), 1 day after exercise group (D1), 2 days after exercise group (D2), 3 days after exercise group (D3), 7 days after exercise group (W1) and 14 days after exercise group (W2) with 8 rats in each group.

Acute downhill treadmill (-16° slope) was carried out on all animals in the exercise group at a speed of 20 m/min for 90 min. This exercise program increases the speed a bit based on the Armstrong exercise program [13]. Before the beginning of the experiment, in order to adapt to the exercise, the speed was accelerated gradually from rest to 20m/min in five minutes. On attaining a speed of 20m/min, the time was calculated, and the speed maintained for 90 min. After the exercise, the rats were sacrificed at the corresponding time points. Blood samples and gastrocnemius muscles were collected and stored in the refrigerator at -80 ℃. It was shown in Fig. 1. All procedures used in this study were approved by The Nanjing Sports Institute Animals Experiment Ethics Committee and met the ethical international standards [14].

Morphological and structural analysis of gastrocnemius muscle

Muscle samples were fixed in 1 % paraformaldehyde, dehydrated and embedded in acryl resin. Samples were cut into sections using anultra microtome and transferred to grids and visualized using a transmission electron microscope Libra 120 (Zeiss, Oberkochen, Germany). The electron microscope experiment was carried out at the Nanjing Medical University Analysis and Test Center.

Serum MSTN, FST and DCN analysis

Blood samples were collected and centrifuged at 3,000 g and 4 ℃ for 10 min to isolate serum. The serum was then stored at -80℃. MSTN, FST and DCN levels were determined using commercially available GDF-8 Enzyme-linked immunosorbent assay (ELISA) Kit (DGDF80, R&D Systems, USA), rat Follistatin (FS) ELISA kit (EKU08677-BM, Biomatik, USA) and DCN ELISA kit (ELR-Decorin, Ray Biotech, USA) respectively, according to manufacturer's instructions.

RNA isolation and quantitative polymerase chain reaction（qPCR）analysis

Total RNA was isolated from gastrocnemius muscle using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to manufacturer's protocol. Concentration and quality of the extracted RNA were measured using a NanoDropND-2000 Spectrophotometer. The iScriptTM cDNA Synthesis Kit (Takara Biotechnology Co., Ltd.) was using to synthesize cDNA from 1μg of total RNA from each sample according to manufacturer's protocol. Two microliters of diluted cDNA (1:20) was used in each real-time PCR. All primers were synthesized Biotechnology co., Ltd. (Invitrogen Life Technology Co., Ltd.). The primers used in the experiment were shown in Table 1. Real-time PCR was performed with Step One Plus™ Real-Time PCR System (Life Technology Co., Ltd., USA). Expression of the target gene was normalized to glycer-aldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. Primers used in the present study

mRNA	Accession No.	Primer sequence (5'–3')		Product size (bp)
myostatin	NM_019151.1	Sense	CCGTCAAGACTCCTACAA	135
		Antisense	CAATACTCTGCCAAATACC	
decorin	NM_024129.1	Sense	GGCAGTCTGGCTAATGT	194
		Antisense	ACGGCAGTGTAGGAAGT	
follistatin	NM_031554.1	Sense	CCCTCATCTTCAGAGCAGTC	118
		Antisense	TTCCCTCATAGGCTAATCCA	
GAPDH	NM_017008.4	Sense	CAAGTTCAACGGCACAG	138
		Antisense	CCAGTAGACTCCACGACAT	

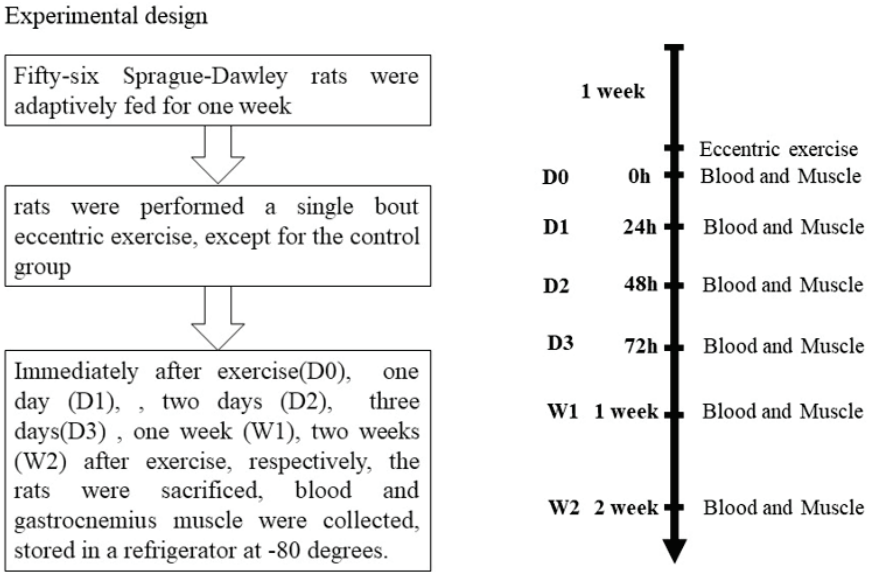

Fig. 1. Experimental design

The specificity of amplification was determined by melting curve analysis and agarose gel electrophoresis. The PCR products were sequenced to validate the identity of the amplicons. The PCR data was expressed as the fold change relative to the control group and analyzed using the $2^{-\Delta\Delta}$Ct method.

Tissue protein extraction and Western blot analysis

Total protein was extracted from gastrocnemius muscle using RIPA lysis buffer (Beyotime Biotechnology Co., Ltd., China). The protease inhibitor cocktail (Fcmacs Biotech Co., Ltd. China) was added according to the manufacturer's protocol. The protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo scientific, USA). Western blot analysis for GDF-8 (AF788, R&D Systems, USA, diluted1:1000), FST (BS9231, Bioworld Technology China, diluted 1:500) and DCN (BS6582, Bioworld Technology China, diluted 1:500) were carried out according to a previous publication [15]. Protein detection was performed by Enhanced Chemiluminescence using a ChemiDoc™ Imaging 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 (BS6945, Bioworld Technology China, diluted1:10000) levels.

Statistical analysis

The data are presented as the mean ± S.E.M. A one-way analysis of variance was used for statistical processing, and $P<0.05$ was considered to indicate a statistically significant difference. Data statistics were calculated with GraphPad Prism 5 (GraphPad Software, La Jolla, USA).

Results

Gastrocnemius muscle morphological and structural changes

After eccentric exercise, changes on the skeletal muscle fiber structure were observed, Z-line anomaly. Acute eccentric exercise damaged rat gastrocnemius muscle ultrastructure with severe injuries being observed at 48 hours and 72 hours after the exercise, while recovery was observed after one week (Fig. 2).

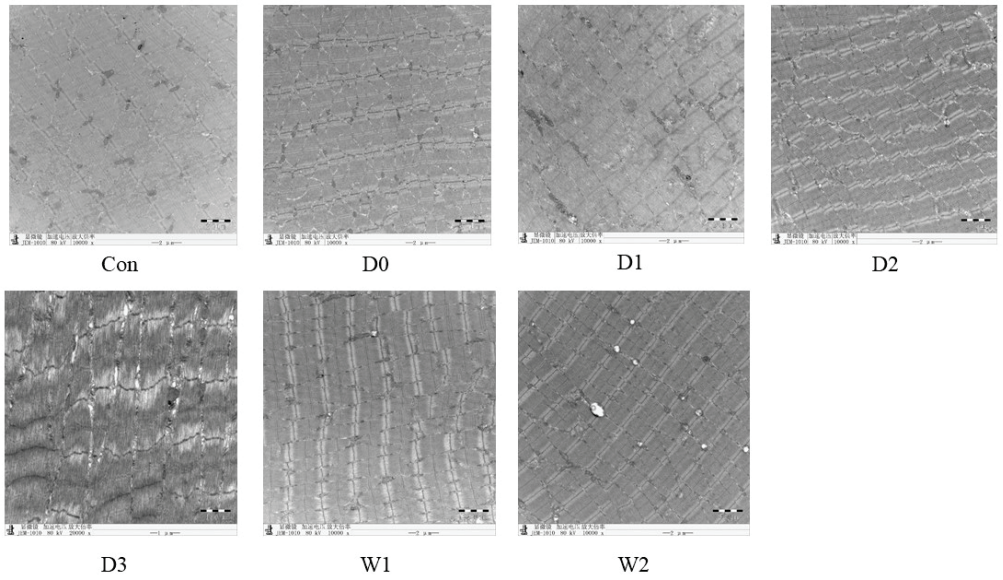

Fig. 2. Acute eccentric exercise damaged rat gastrocnemius muscle ultrastructure.

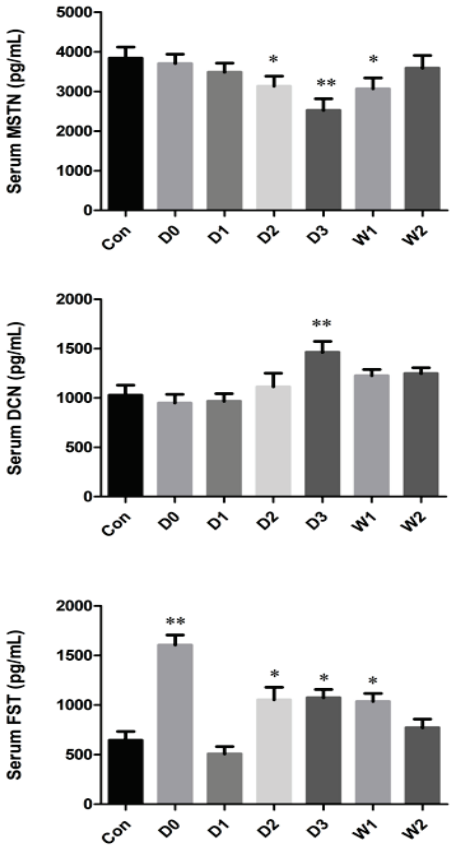

Fig. 3. Eccentric exercise stimulates the secretion of FST and DCN and suppresses the secretion of MSTN. Values are presented as a mean ± SE (n = 8). *p < 0.05, ** p < 0.01 (versus CON group)

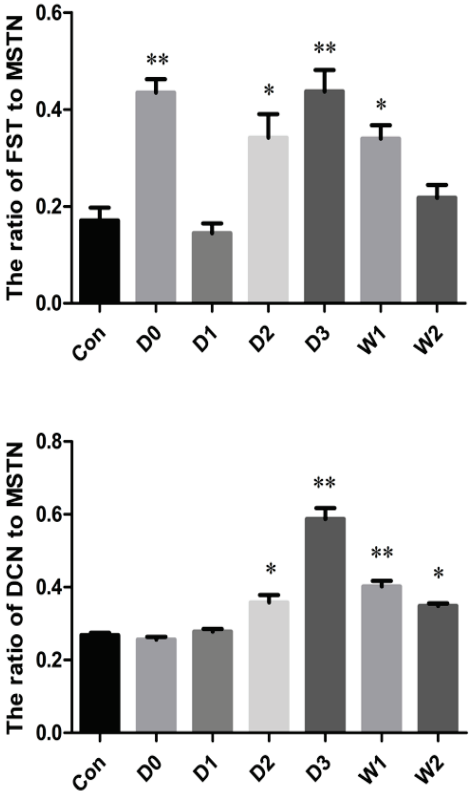

Fig. 4. Both DCN and FST suppress MSTN levels during the recovery period of eccentric exercise. Values are presented as a mean ± SE (n = 8). *p < 0.05, ** p < 0.01 (versus CON group)

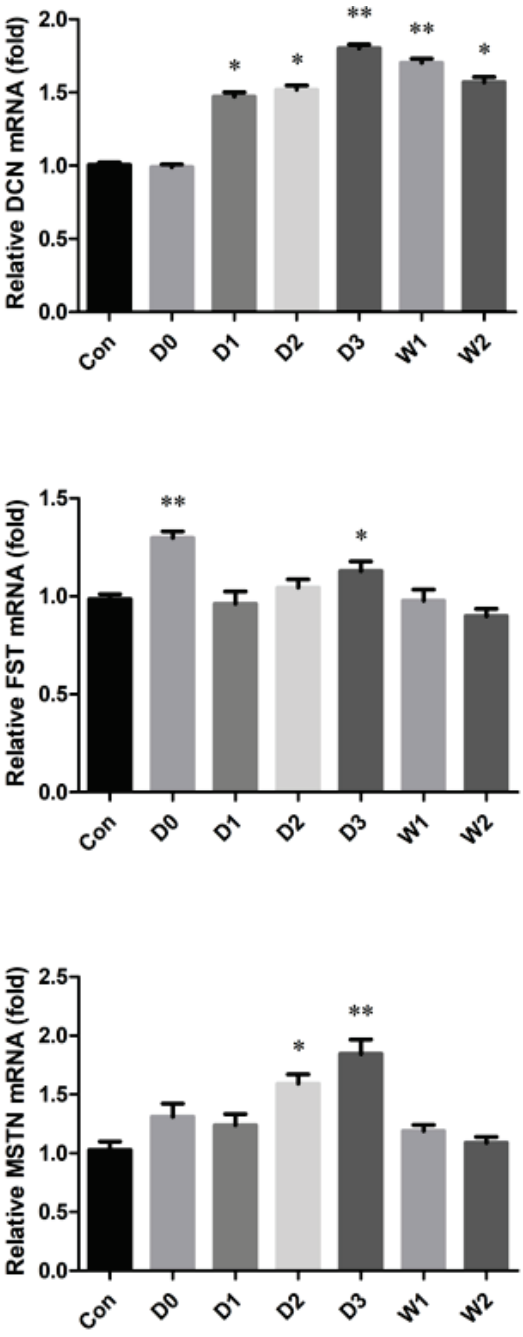

Fig. 5. The mRNA expression of MSTN, DCN and FST increased during the recovery period of eccentric exercise. Values are presented as a mean ± SE (n=8). *p < 0.05, ** p < 0.01 (versus CON group).

Serum MSTN, DCN, and FST concentrations were detected at different time points after acute eccentric exercise (Fig. 3). The levels of serum MSTN was significantly decrease in the D3 group. Contrarily, the levels of serum DCN was significantly increased in the D3 group. The concentration of serum FST was significantly increased in the D0, D2, D3 and W1 group, respectively. The ratio of FST to MSTN and the ratio of DCN to MSTN were significantly increased in 4 groups respectively, in addition to the D0 group, 3 groups were overlapped, namely D2, D3, and W1 group. In addition, the ratio of FST to MSTN was significantly increased in the D0 group, and the ratio of DCN to MSTN was significantly increased in the W2 group (Fig. 4).

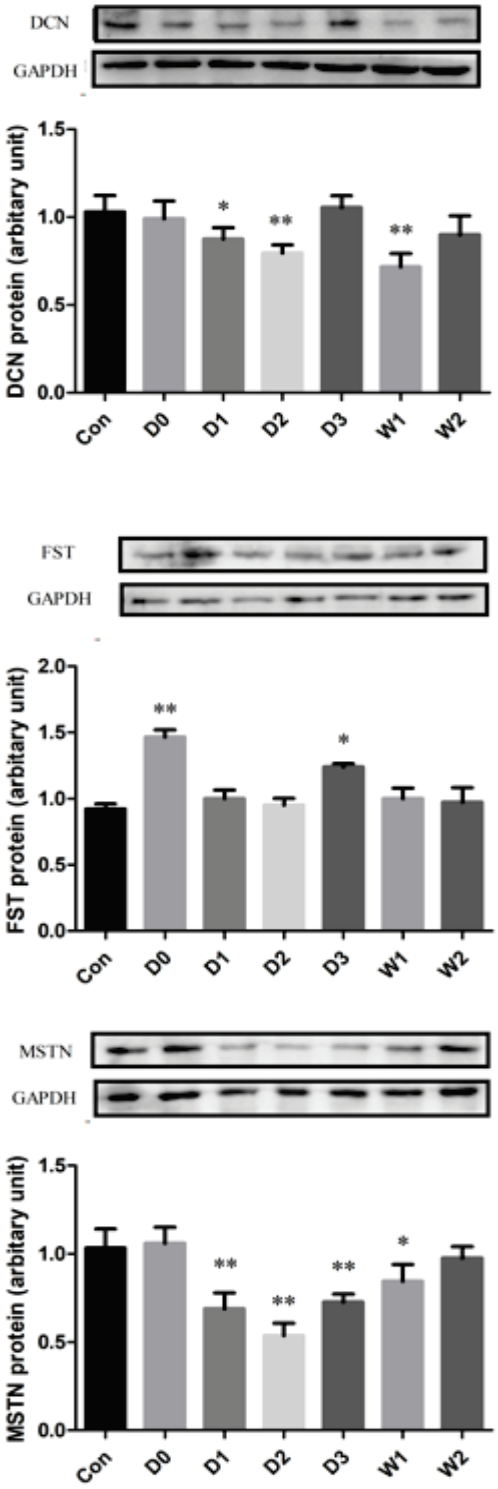

Fig. 6. The increase of FST during the recovery period of eccentric exercise inhibits the expression of MSTN. Values are presented as a mean ± SE (n = 8). *p < 0.05, ** p < 0.01 (versus CON group)

MSTN, FST and DCN mRNA levels in gastrocnemius muscle

The mRNA expression of MSTN, FST and DCN in gastrocnemius muscle were detected at different time after acute eccentric exercise (Fig. 5). The expression of DCN mRNA was significantly increased in the D1, D2, D3, W1 and W2 groups, respectively ($p<0.05$). The expression of FST mRNA was significantly increased in the D0 and D3 groups ($p<0.05$). Likewise, the expression of MSTN mRNA was increased in the D2 and D3 groups ($p<0.05$).

MSTN, FST and DCN protein levels in gastrocnemius muscle

Protein expression levels of MSTN, FST and DCN were detected by western blotting (Fig. 6), and the results of FST confirmed the observations on mRNA gene expression levels. Protein expression levels for FST was significantly increased in the D0 and D3 groups ($p<0.05$). However, protein expression levels of MSTN and DCN were opposite to their mRNA expression levels. DCN protein expression levels were decreased in D1, D2 and W1 groups ($p<0.05$). Similarly, MSTN protein expression levels were decreased in D1, D2, D3 and W1 groups ($p<0.05$).

Discussion

In this study, we investigated the impacts of acute eccentric exercise on the levels of MSTN, DCN and FST at different time points in the blood and skeletal muscle of rats. As a result, we discovered that the changing trends of serum MSTN, FST, and DCN were different at different time points after acute eccentric exercise. The changing trends of MSTN and DCN were opposite, while FST has its unique characteristics of change.

The expression level of MSTN in gastrocnemius muscle was consistent with the change level of MSTN in blood circulation. It is because that MSTN is mainly secreted by skeletal muscle. Interestingly, the change trends of MSTN mRNA and protein expression of gastrocnemius muscle were opposite, which suggested that acute eccentric exercise could affect the transcriptional regulation of MSTN in gastrocnemius muscle. It was reported that miRNAs were involved in the regulation of MSTN [16]. MiR-27a/b played an important role in promoting myoblast proliferation and preventing muscle atrophy by through negative regulation of MSTN[17]. MiR-206 and miR-1 led to the muscle hypertrophy phenotype by targeting the 3'-UTR of MSTN [17,18]. Whether miRNA is involved in the regulation of MSTN expression in skeletal muscle after acute eccentric exercise needs further study.

The change trends of FST mRNA and protein expression of gastrocnemius muscle were completely consistent, and which conformed to the change level of FST in blood circulation. Consistent with the results of our study, exercise increases the expression and secretion of FST has been reported in previous studies [19,20,21]. However, to date, the changes of FST at different time points after acute eccentric exercise are rarely reported. We demonstrated here that the expression of FST in skeletal muscle was significantly increased on the third day after acute eccentric exercise, while MSTN was significantly decreased, which suggested that FST might be involved in the repair of skeletal muscle damage after acute eccentric exercise.

The change trend of DCN protein expression level in the gastrocnemius muscle was inconsistent with that of in the blood. It might be that we only detected the expression of DCN in gastrocnemius muscle, not assay the level of DCN in other tissues. As a result, the DCN level in the blood was secreted of multiple tissues. In this study, we found that the DCN mRNA and protein levels changed incongruously at different time points, which suggested that acute eccentric exercise might affect the transcriptional regulation of DCN. Recent studies have identified miRNA binding sites such as miR-140, miR-299, miR-338 and miR-484 in the DCN gene [22]. Further studies should explore the transcriptional regulation of DCN at different time points after acute eccentric exercise.

The circulating MSTN level was first decreased and then recovered during the recovery of skeletal muscle injury after acute eccentric exercise, while the FST and DCN levels were first increased and then recovered. The trend of the two changes was opposite to that of MSTN. Studies have shown that DCN secreted into the blood from the muscles after an acute resistance exercise. The secreted DCN binds to MSTN, thereby inhibiting the negative regulation of muscle fiber hypertrophy caused by MSTN [7]. Likewise, concurrent training increased the concentration of blood FST and reduced MSTN in patients with type 2 diabetes [23]. It suggested that FST and DCN maybe inhibit MSTN. Especially on the third day after exercise, the DCN level reached the peak and the MSTN level was at the trough. In line with the results

of our study, in vivo overexpression of DCN in murine skeletal muscle promoted expression of the pro-myogenic factor by through inhibiting myostatin [7]. It suggested that DCN secreted from skeletal muscle could play a role in exercise-related restructuring processes of skeletal muscle. The change in the ratio of DCN to MSTN was consistent with the change in the ratio of FST to MSTN, except immediately after exercise and studies have reported, that a bout of swimming exercise increased blood FST levels [24]. It indicated that FST was also involved in the repair of skeletal muscle damage.

FST protein expression of gastrocnemius was significantly increased on the third day after exercise. The expression of DCN was gradually decreased after exercise, until it recovers on the third day, and then continued to decrease. While the expression of MSTN was significantly decreased on the third day after exercise. It indicated that FST and DCN could be involved in the regulation of MSTN.

However, some limitations were noted in this study, the morphological changes of skeletal muscle after acute eccentric exercise were not captured. The changes of myogenic and atrophy factors and the protein interaction between DCN and MSTN and FST and MSTN have also been ignored during the repair of skeletal muscle damage and the remodeling process of skeletal muscle were also ignored. Due to the limitations of *in vitro* models of exercise intervention, the transcriptional regulation of MSTN and DCN has not been thoroughly verified in vitro.

In conclusion, we demonstrated here that acute eccentric exercise could increase the levels of serum DCN and FST and decrease the levels of serum MSTN. The changes of serum FST and DCN at different time points after acute eccentric exercise, except immediately after exercise, were opposite to that of MSTN. The changes of FST, DCN and MSTN in the gastrocnemius muscle are similar to those of serum. However, the changes of DCN and MSTN in transcription and translation levels were different. These findings suggest that MSTN may be regulated by FST and DCN after acute eccentric exercise and hence could play a role in exercise-related restructuring processes of skeletal muscle.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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