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Original Article

Phenotypic modulation of corpus cavernosum smooth muscle cells in a hyperlipidemic rat model

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Abstract: Objective: The aim of this study was to determine the changes and underlying mechanisms of erectile organ structure and function in hyperlipidemic rats. Materials and Methods: Sixteen male rats were fed a normal diet to serve as controls, and the other sixteen rats were induced to develop hyperlipidemia with a high fat diet. After 4 months, the penile tissues were harvested and assessed for smooth muscle content. Molecular markers of phenotypic modulation were examined by qRT-PCR and western blotting. Results: Serum total cholesterol, triglyceride, and low density lipoprotein cholesterol levels were significantly higher in hyperlipidemic rats than those in control rats ($P<0.01$ for all). However, the high density lipoprotein cholesterol level was significantly reduced ($P<0.01$). Erectile function was significantly lower in hyperlipidemic rats than in control rats. There was no significant difference in the content of smooth muscle cells between the hyperlipidemic group and the control group. The expression of CCSMC phenotypic markers, such as calponin, smooth muscle myosin heavy chain 11, and myocardin was markedly lower, whereas osteopontin protein expression was significantly higher in hyperlipidemic rats than in control rats. Conclusion: CCSMCs possesses the ability to modulate the phenotype under hyperlipidemic conditions, which could have a key role in the pathogenesis of erectile dysfunction.

Keywords: Phenotypic modulation, hyperlipidemia, erectile dysfunction, smooth muscle cell

Introduction

Corpora cavernosum smooth muscle cells (CCSMCs) are known to be the most important cells in the male erection process [1]. The importance of smooth muscle relaxation in penile erection has been demonstrated in both animal and human studies.

Phenotypic modulation within CCSMCs has a critical role in the pathogenesis of erectile dysfunction (ED) [2-4]. Unlike the cardiac and skeletal muscle cells, CCSMCs can switch their phenotype from a contractile and differentiated state to a proliferative and dedifferentiated state in response to the change of local environmental stimuli such as diabetes mellitus, hypoxic conditions, and cavernous nerve damage [5]. The phenotypic modulation is characterized by enhanced proliferation, migration and extracellular matrix production combined with decreased expression of contractility associated genes such as smooth muscle α-actin (α-SMA), smooth muscle myosin heavy chain 11 (Myh11), smoothelin, calponin and desmin [5]. Our previous studies have demonstrated that the contractile phenotype in CCSMCs are necessary for maintaining normal erectile function, whereas the proliferative phenotype may contribute to the pathogenesis of ED in diabetic rats [2, 6].

Hyperlipidemia is one of the most important risk factors for the development of vasculogenic ED [7, 8]. This condition produces various functional and structural alterations in the vas-

Table 1. Description of the primer sequences

Primer	Sequence
Calponin	
Forward	5'-ACCAAGCGGCAGATCTTTGA-3'
Reverse	5'-CATCTGCAAGCTGACGTTGA-3'
Myh11	
Forward	5'-CAGTTGGACACTATGTCAGGGAAA-3'
Reverse	5'-ATGGAGACAAATGCTAATCAGCC-3'
Myocardin	
Forward	5'-GTGCCTTGTTGGAGTAAGAGTGC-3'
Reverse	5'-GTCAGTCTATGTCCCGATAATGCC-3'
OPN	
Forward	5'-GCTGAAGCCTGACCCATCT-3'
Reverse	5'-TCCCGTTGCTGTCCTGAT-3'
GAPDH	
Forward	5'-AAGCTCACTGGCATGGCCTT-3'
Reverse	5'-CGGCATGTCAGATCCACAAC-3'

culature, leading to the impairment of vascular smooth muscle cells (VSMCs) [9, 10]. In addition, the impairment was accompanied with a reduction in CCSMCs content. Although some studies have shown that hyperlipidemia can impair normal penis structure and its effects may be reversible, little is known about the precise mechanisms.

A better understanding of CCSMCs function and its phenotypic transformations can help illuminate the pathogenesis of hyperlipidemia-associated ED. To our knowledge, phenotypic modulation in CCSMCs in hyperlipidemic rats has not yet been explored. The purpose of our study was to determine the phenotypic modulation and underlying mechanisms of penile tissue in a hyperlipidemic rat model.

Materials and methods

Animals

Thirty two 3-month old male Sprague-Dawley rats (The Experimental Animal Center of Guangdong province, China) weighing 250-300 g were used in the experiments. The rats were raised using a 12:12 light cycle at 24±1°C. The rats had free access to food and drinking water. They were separated into two groups: Sixteen rats fed a diet of standard rat chow served as negative controls (control, n = 16). The remaining 16 rats (hyperlipidemic rats, n = 16) were fed a high-fat diet consisting of 1% cholesterol, 10% lard, 10% egg yolk powder, and 79% standard rat chow (Zeigler Brothers, Gardner, PA, USA) for 4 months. At 4 months, Triglycerides, total cholesterol, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in the serum were analyzed by commercial kits (Maker, Biotechnology Inc., Sichuan, China) and the chemical analyzer 7020 (Hitachi, Tokyo, Japan). All animals were handled in strict accordance with the recommendations in the ARRIVE guidelines [11]. The protocol was approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Animals were sacrificed by an anesthetic overdose intraperitoneal administration of sodium pentobarbital and then cervical dislocation was applied to rats for euthanasia.

Measurement of erectile responses

A penile erection experiment was performed using the methods described in our previous report [12]. In brief, the abdomen was opened via a repeat midline abdominal incision, the cavernous nerves were exposed and isolated, and the crus of the penis were identified. A 22-gauge needle containing 50 U/mL of heparin solution was inserted in the right penile crus to record intracavernosal pressure (ICP). The left carotid artery was exposed and cannulated with a PE-50 tube to record the mean arterial pressure (MAP). MAP and ICP were measured with pressure transducers and a data acquisition system (MP 150A-CE; Biopac, Santa Barbara, CA). The data was recorded on a computer with AcqKnowledge Data Acquisition and Analysis Software The stimulus parameters were as follows: 5 V amplitude, 15-Hz frequency, 5-ms pulse width, and 60-second duration. Erectile tissue response was determined in real time and measured as maximal ICP, MAP, and the ratio of maximal ICP and MAP (ICP/MAP). The ICP operator was blinded to the allocation of animals to different treatment groups. After quantifying erection capability, the whole penis from each rat was quickly harvested. Penile tissue samples were removed, and one was stored in liquid nitrogen, and the other was fixed in 4% paraformaldehyde for further biochemical and histological analysis.

Table 2. Comparison in body weight, total cholesterol, triglyceride and erectile function parameters between two groups (mean ± SD)

Variable	Group	
	Control	Hyperlipidemic rats
Body weight (g)	381.25±33.77*	515.16±52.73
Total cholesterol (mmol/L)	1.05±0.14*	3.25±1.64
Triglyceride (mmol/L)	0.28±0.09*	2.82±0.39
HDL-C (mmol/L)	1.35±0.12*	0.51±0.08
LDL-C (mmol/L)	0.89±0.05*	1.26±0.14
Erectile function parameters		
Maximal ICP (mmHg)	88.56±9.14*	53.13±6.57
MAP (mmHg)	117.69±6.08	115.19±9.39
ICP/MAP (%)	75.26±6.93*	46.39±6.67

*P<0.01 compared to the control group.

Masson's trichrome staining

To evaluate the smooth muscle-to-collagen ratio, specimens were stained for Masson trichrome according to a standard protocol as described previously [12]. Tissue sections (4 μm) of the middle part of the penile shaft were harvested for tissue histology study and Masson's trichrome staining. The ratio between the surface areas of smooth muscle cells and collagen was calculated. Five non-overlapping images of Masson's trichrome staining were captured from each slide at 400× magnification and semi-quantitative image analysis of Masson's trichrome staining was performed using the Image-Pro Plus 6.0 software.

Quantitative reverse transcription polymerase chain reaction analysis

Urethra and blood vessels were removed from the penis, and corpus cavernosum was obtained. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted into cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences Corp., Piscataway, NJ, USA). Using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan), quantitative reverse transcription polymerase chain reaction analysis (qRT-PCR) was performed on the Roche Light Cycler Run 5.32 Real-Time PCR System. The primer sequences are summarized in **Table 1**. The amounts of the PCR products were nonneoplasticised to GAPDH which served as internal control. Relative gene expression was determined using the ΔΔCt method.

Immunohistochemistry analysis

The smooth muscle content and fibrosis degree in the rat penile corpus cavernosum were assessed by hematoxylin and eosin (H&E), and immunohistochemical staining. Sections were deparaffinized and rehydrated, and then retrieved with heat-induced epitope retrieval. The slides were then incubated with the primary antibody (TGFβ1 [1:100], COL1A1 [1:100], and COL3A1 [1:100], all from Abcam, Cambridge, MA, USA) overnight at 4°C, rinsed 3 times in phosphate-buffered saline (PBS) for 5 min at room temperature, and incubated with a biotinylated secondary antibody (diluted 1:100); this was followed by incubation with the streptavidin-biotin peroxidase complex (diluted 1:100). Immunohistochemical detection was performed with 3,39-diaminobenzidine tetrahydrochloride following the manufacturer's instructions. Five non-overlapping images were captured from each slide at 400× magnification. Semi-quantitative image analysis was performed with the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Western blot analysis

Penises were excised, the urethra and tunica albuginea were removed, and the corpus cavernosum tissue samples were lysed, and Western blot analysis was performed, as described previously [6, 12]. The antibodies included anti-Calponin antibody (1/20000, Santa Cruz), anti-Myh11 (1/1000, Santa Cruz), anti-Myocardin (1/1000, Abcam), anti-Osteopontin (OPN) (1/1000, Abcam) and anti-GAPDH (1/1000, Abcam). Densitometry was used to measure the expression of Calponin, Myh11, Myocardin, OPN relative to GAPDH using Bio-Rad Gel Doc Software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

All experiments were performed in triplicates. Data were analyzed using SPSS 13.0 software

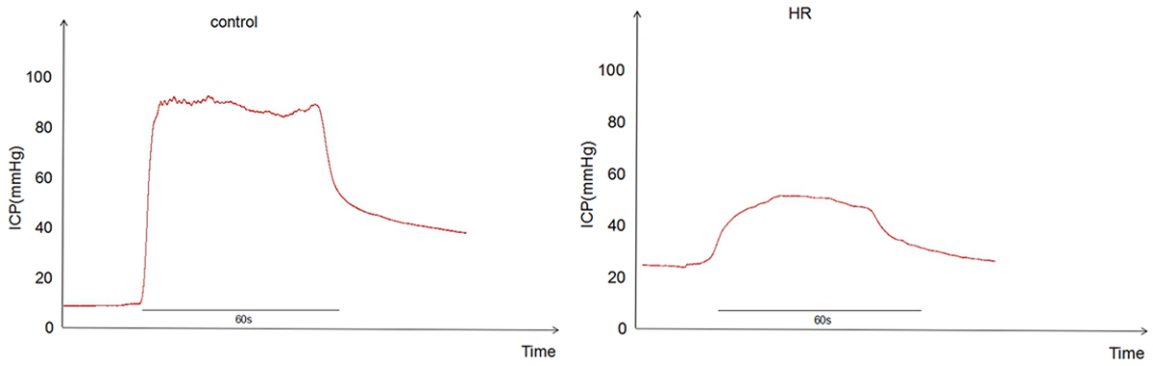

Figure 1. Erectile function was assessed by ICP. Representative graphs are presented for rats fed a normal diet and rats fed a high-fat diet. The x-axis depicted seconds and the gray bar represented 1 electrical stimulus lasting 60 seconds. The Y-axis represented changes in ICP.

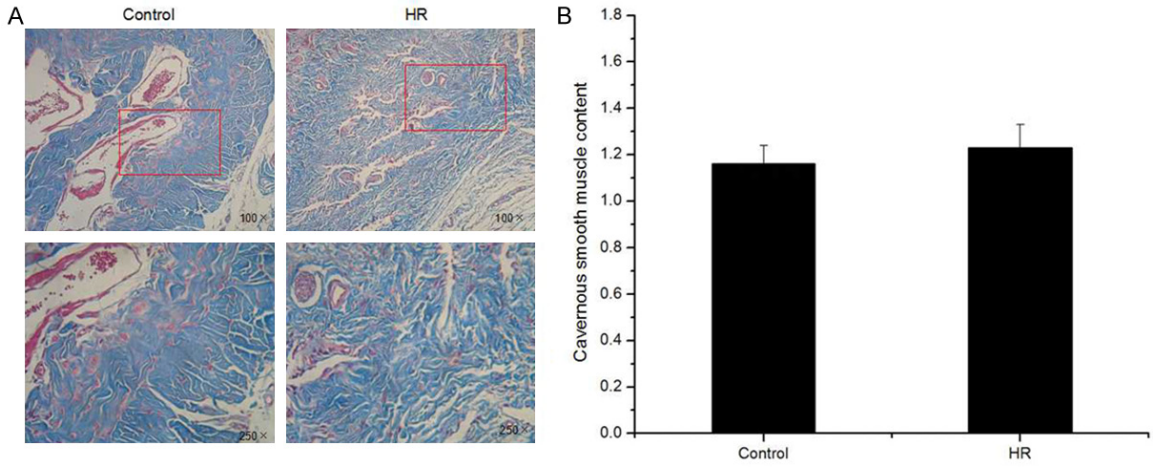

Figure 2. Masson's trichrome staining of the corpus cavernosum. A: Normal cavernous smooth muscle and collagen expression was observed in the control group. (Upper-line: 100×, down-line: 250×). B: Semiquantitative image analysis of the area fraction was calculated using Image-Pro Plus 6.0 software. HR = hyperlipidemic rats.

(SPSS Inc., Chicago, IL, USA), and continuous variables are expressed as the mean ± standard deviation. Independent sample T-test was used to analyze the differences between two groups. A two-tailed $P<0.05$ was considered statistically significant.

Results

Body weight and serum biochemistry

As shown in **Table 2**, compared with the control rats, the body weight of the hyperlipidemic rats was significantly higher ($P<0.01$); Serum total cholesterol, triglyceride and LDL-C levels were significantly higher in the hyperlipidemic rats than in rats fed a normal diet at the four month time point ($P<0.01$ and $P<0.01$, respectively). However, the HDL-C level in the hyperlipidemic group was lower than those in the control group ($P>0.01$).

Assessment of erectile response

The representative ICP measurements in response to electrostimulation of cavernous nerve are indicated in **Figure 1**. The maximal ICP and ICP/MAP ratio was clearly lower in the hyperlipidemic rats than in the control group ($P<0.001$, and $P<0.001$, respectively); MAP was not significantly different between two groups ($P = 0.379$) (**Table 1**). The measurement of erectile responses revealed that the high-fat diet led to a significant decrease in erectile function including maximal ICP and ICP/MAP ratio.

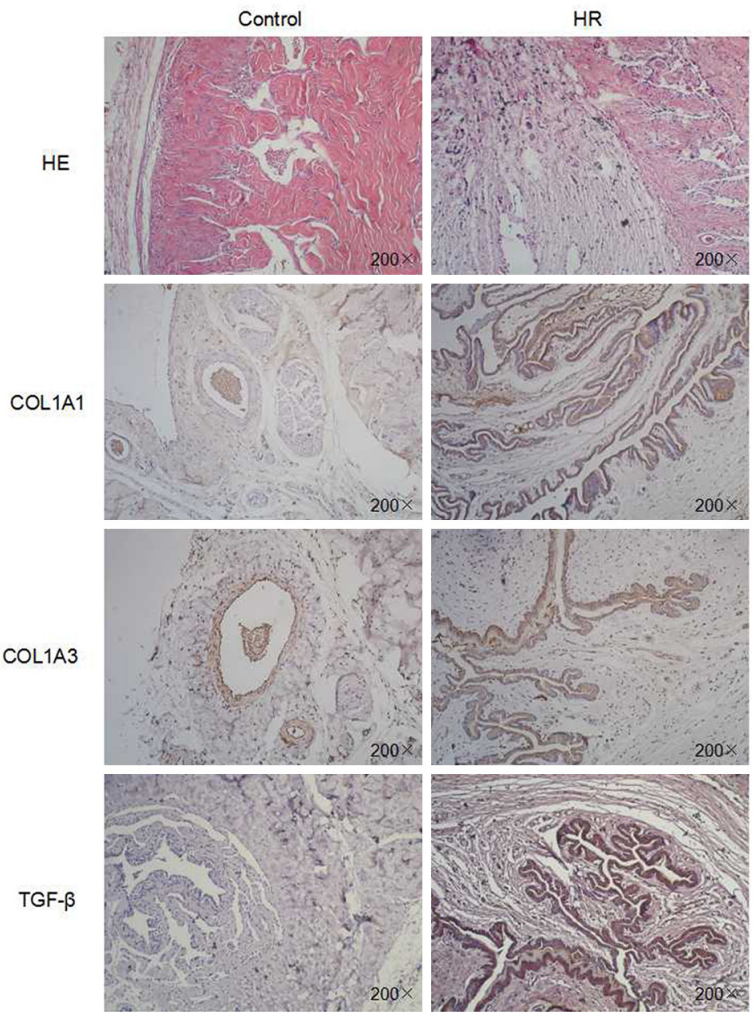

Figure 3. Representative H&E and Immunohistochemistry staining patterns. H&E staining showed hyperlipidemia induces corporal fibrosis. Representative immunohistochemical staining photomicrographs for COL1A1, COL1A3, and TGF-β proteins.

Distribution of smooth muscles in the corpus cavernosum

The areas of smooth muscle and collagen were evaluated by Masson's trichrome staining of the penile tissue from the control and hyperlipidemic rats. The smooth muscle content was higher in hyperlipidemic than in normal rats, but the difference is not significant (P = 0.146). Representative Masson's trichrome staining images for each group are shown in **Figure 2**. The result suggested that there may be no connection between the damage of erectile function and cavernous smooth muscle content.

Assessment of CCSMC morphological features

H&E and Immunohistochemistry staining were performed to examine the fibrosis degree in the corpora cavernosa. H&E staining showed that the penile corpus cavernosum in the HR group had increased fibrosis, and thinner and irregular arrangement of the smooth muscle compared with the control group. Immunohistochemistry analysis showed that the hyperlipidemic rats had higher TGF-β1, COL1A1 and COL1A3 expression than control group (**Figure 3**). Our results indicated the high-fat diet induced an inflammatory tissue response and fibrogenesis as shown by an increased expression of TGF-β1, COL1A1 and COL1A3.

qRT-PCR analysis and Western blot analysis of phenotype-associated genes in corpus cavernosum

We examined the expressions of phenotype-associated genes at the mRNA level in the cavernous tissue between two groups by qRT-PCR. Compared with the control group, the levels of calponin, Myh11, and myocardin were significantly decreased in the hyperlipidemia rats (P<0.05 for all); Expressions of differentiated-associated genes OPN were upregulated in the hyperlipidemic rats when compared with the control group (P<0.01) (**Figure 4A**).

To confirm whether the decreased mRNA of phenotype-associated genes was associated with a decrease at the protein level, western blot analysis was performed. In accordance with the expressions at the mRNA level, the levels of calponin, Myh11, and myocardin at the protein level in the cavernous tissues were decreased and OPN was increased in the hyperlipidemic rats than the control rats (P<0.05) (**Figure 4B**).

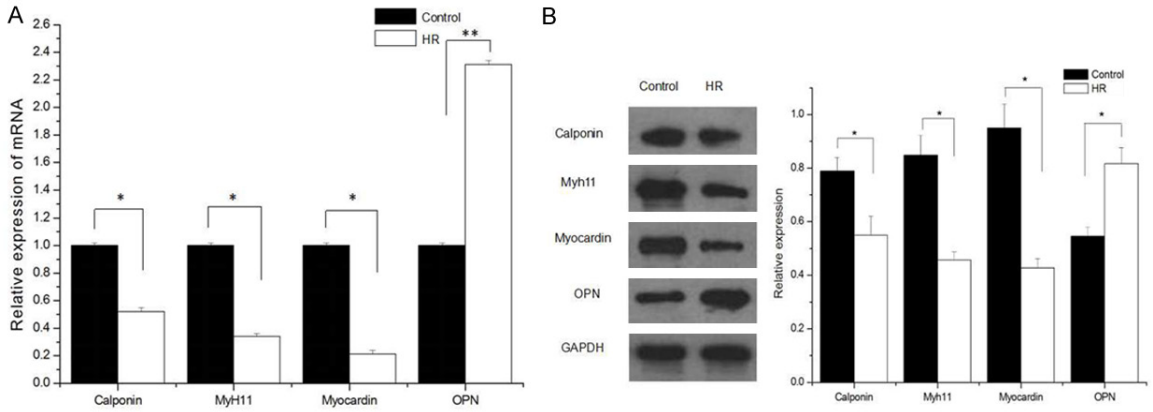

Figure 4. The expression of phenotype-associated genes at the mRNA and protein levels in each group. A: Relative gene expression was presented as the relative ratio of the product of calponin, Myh11, myocardin, and osteopontin gene to that of GAPDH mRNA. Each bar depicted means ± standard errors (n = 16 animals per group). *P<0.05 vs the control group, HR = hyperlipidemic rats. B: Western blot analysis of the levels of calponin, Myh11, myocardin, and osteopontin proteins in penile tissue of both groups (Control group: n = 16, HR group: n = 16). HR = hyperlipidemic rats. Densitometry and statistic analysis of phenotype-associated genes proteins expression (ratio to-GAPDH) in the corpus cavernosum. *P<0.05 vs the control group.

Discussion

To our knowledge, our study is the first to demonstrate that CCSMCs undergo phenotypic modulation in the hyperlipidemia rats. There is a growing body of evidence that endothelial dysfunction play a key role in the development of ED in men with hyperlipidemia [13, 14]. The presence of ED may indicate underlying vascular abnormalities, such as lipid abnormalities and the impairment of VSMCs [15, 16]. Phenotypic modulation, one of the most important characteristics within smooth muscle cells, has a critical role in the pathogenesis of a variety of diseases. Contractile and proliferative phenotypes represent two ends of a spectrum of smooth muscle cells with a continuum of states in between [17]. Additionally, smooth muscle cells contractile proteins of the corpora cavernosum, such as α-SMA, smooth muscle myosin heavy chain (SMMHC), and desmin, were downregulated in bilateral cavernous neurectomy rats, whereas synthesis of the smooth muscle cells phenotypic marker protein vimentin was significantly upregulated. However, little knowledge exists regarding the phenotypic modulation in hyperlipidemic rat CCSMCs.

In the present study, we demonstrated that compared with the control group, the erectile function parameters such as the maximal ICP and ICP/MAP were significantly lower in the hyperlipidemic rats. In addition, hyperlipidemic rats had higher expression of TGF-β1, COL1A1 and COL1A3. Four proteins (calponin, Myh11, myocardin, and osteopontin) have been used in this study to characterize CCSMCs phenotypes. A shift in phenotype from a contractile to a proliferative state leads to downregulated expression of contractility-associated markers and upregulated expression of proliferative smooth muscle cells markers. Taken together, these data demonstrated that hyperlipidemia in rats produces a phenotypic modulation of CCSMCs similar to VSMCs.

CCSMCs possessed the ability to modulate the phenotype from a contractile to a proliferative state under hyperlipidemic conditions, these structural changes can possibly explain why hyperlipidemic men are at higher risk of having ED. Similar to VSMCs, CCSMCs have contractile and proliferative phenotypes and possess the ability to modulate phenotype from a contractile to a proliferative state under local cellular stimuli. Phenotypic modulation is a hallmark of CCSMCs, and although it is well described in vascular injury and with in vitro models, the mechanism of its regulation is poorly understood. Myocardin is a potent myogenic coactivator, expressed specifically in contractile smooth and cardiac muscle tissues in adulthood [18-20]. Ackers-Johnson et al. [21] reported that myocardin negatively regulated vascular smooth muscle cells inflammatory activation and vascular disease. They proposed myo-

cardin as a guardian of the contractile, noninflammatory VSMCs phenotype. Our previous results demonstrated that the erectile responses restored by the over-expression of myocardin might be caused by upregulation of contractility-associated genes, such as SMA, SMMHC and calponin, in penile tissue [12]. Our data suggested that myocardin has biological activity in modulating CCSM cells phenotype and maintaining normal erectile function in rats. In this study, we tested the expressions of four phenotype-associated markers, including calponin, Myh11, myocardin, and OPN, and found that myocardin was significantly downregulated in cavernous tissues of hyperlipidemic rats.

Hypercholesterolemia has been frequently associated with significant changes in smooth muscle structure and function. Many studies have pointed out that hyperlipidemia caused fibrosis of smooth muscle cells, suggesting that it may play important roles in the impairment of endothelial function [22, 23]. Corporeal fibrosis is another important pathophysiologic feature leading to ED. In our study, TGF-β1, COL1A1 and COL1A3 were used to assess fibrosis in the corpus cavernosum. Our results also demonstrated that hyperlipidemia induces corporal fibrosis by up-regulation of fibrogenic cytokines. Phenotypic modulation of CCSMCS may be responsible for corporal fibrosis in hyperlipidemic mice, which can cause significant impairment in erectile function.

Hyperlipidemia have a considerable impact on erectile function in men with ED. Hyperlipidemic men are more likely to have ED than healthy men [24, 25]. It has been shown that decreased endothelial content in the corpus cavernosum might play a major role in the deterioration of erectile function in hyperlipidemic mice [26]. penile hemodynamics are substantially inferior in rats fed a high-fat diet relative to those fed a normal diet [27]. The amount of endothelial cells in the corpus cavernosum is a very important factor to evaluate the erectile function. Similar findings have also been reported in hyperlipidemic mice by Huang et al. [27] and Qiu et al. [28]. In our previous study, we found that the percentage of smooth muscle in the corpus cavernosum was decreased in all diabetic rats [12]. Furthermore, phenotypic modulation towards synthetic state from contractile state in CCSMCs was known to be associated with lower erectile function. Thus, these morphological and functional changes in the CCSMCs are probably associated with decreased contractility as the cells transit from a contractile phenotype to a proliferative synthetic phenotype.

In the present study, the principal limitations of our study included a lack of molecular mechanisms on the phenotypic modulation of CCSMCs in hyperlipidemic mice. The second limitation was the absence of cellular-based experiments. Further research will determine these underlying mechanisms. Despite this limitation, we believe that our results will be useful in ultimately revealing the mechanism involved in the regulatory effect of hyperlipidemia on penile structure and erectile function.

In conclusion, our results firstly demonstrated that CCSMCs undergo a shift in phenotypes from a contractile state to a synthetic state in hyperlipidemic rats. This phenotypic modulation will be important for the therapeutic strategy of prevention or intervention for hyperlipidemic ED patients.

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Disclosure of conflict of interest

None.

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