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Shexiang Tongxin Dropping Pill Improves Peripheral Microvascular Blood Flow via Cystathionine- γ -Lyase

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

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Background: To explore the protective effects of Shexiang Tongxin Dropping Pill (STP) in improving peripheral microvascular dysfunction in mice and to explore the involved mechanism.

Material/Methods: A peripheral microvascular dysfunction model was established by combined myocardial infarction (MI) and lipopolysaccharide (LPS) injection in mice. Then, the mice were randomized into a model group (n=10) or an STP group (n=10), which were treated with normal saline and STP, respectively. The cremaster muscle microvascular blood flow velocity and numbers of leukocytes adherent to the venular wall were evaluated before and after drug intervention. We assessed the expression of adhesion molecule CD11b and related transcript factor FOXO1 in leukocytes, cystathionine- γ -lyase (CSE) mRNA expression in the cremaster muscle, and mitochondrial DNA copy numbers.

Results: Compared with those of control mice, the cremaster microvascular blood flow velocity, cremaster CSE expression, and mitochondrial DNA copy number in mice from the model group were significantly lower and leukocyte adhesion and CD11b and FOXO1 expression were significantly higher. Intervention with STP could significantly increase the cremaster microvascular flow velocity (0.480 ± 0.010 mm/s vs. 0.075 ± 0.005 mm/s), mRNA expression of cremaster CSE, and mitochondrial DNA copy number, but it inhibited leukocyte adhesion and decreased leukocyte CD11b and FOXO1 expression.

Conclusions: STP significantly improved peripheral microcirculation, in which increased CSE expression might be the underlying mechanism.

MeSH Keywords: **Medicine, Chinese Traditional • Microvessels • Myocardial Infarction**

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Background

Ischemic cardiomyopathy is a leading cause of morbidity and mortality worldwide [1]. In the past, coronary stenosis was regarded as the main reason for myocardial ischemia. However, in recent years, microvascular dysfunction in the absence of significant artery obstruction has been established as another fundamental mechanism for myocardial ischemia and is included in the Fourth Universal Definition of Myocardial Infarction [2,3]. However, there is still no effective and validated therapy for microvascular dysfunction in clinical practice.

STP, a traditional Chinese medicine, is widely used for the treatment of cardiovascular diseases in China, such as atherosclerosis and myocardial ischemia [4]. Its major constituents have been fully reported by a previous study [5]. STP was shown to protect endothelial cells by decreasing the levels of serum endothelin-1, c-reaction protein (CRP), and increasing nitrogen oxide levels [6,7]. However, whether STP can improve microvascular dysfunction remains unknown. In this study, we established a peripheral microvascular dysfunction model in C57 mice with MI operation plus LPS, to assess the effect of STP on microcirculation and to explore the possible mechanisms.

Material and Methods

Chemicals and reagents

Shexiang Tongxin Dropping Pill was provided by the Inner Mongolia Conba Pharmaceutical Co. (Inner Mongolia, China). LPS and Rhodamine 6G were purchased from Sigma-Aldrich (USA). 4',6-diamidino-2-phenylindole (DAPI) was from Solarbio. Isoflurane was purchased from RWD (Shenzhen, China). Rabbit anti-cystathionine- γ -lyase (CSE) polyclonal antibody was from Absin (Shanghai, China). β -actin (13E5) rabbit mAb was from Cell Signaling Technology (CST). Rat anti-mouse CD11b was from BD Bioscience (San Diego, CA, USA). Primers were from Ruijie Biotechnology Company (Shanghai, China).

The entire experimental protocol used in the study was carefully checked and approved by the Animal Care and Use Committee of the Second Military Medical University. All surgery was performed under anesthesia, and all possible efforts were made to minimize suffering, in compliance with the ARRIVE guidelines on animal research.

Establishment of peripheral microvascular dysfunction model

Thirty male C57 mice (20 ± 2 g) were randomized into a control group ($n=10$) and a model group ($n=20$). All mice were anesthetized with inhaled isoflurane, as previously described [8], and

then the model group mice underwent myocardial infarction (MI) surgery by ligating the left anterior descending branch and the control group underwent a sham operation. Twenty-four hours later, standard lead II electrocardiogram (ECG) was used to determine the presence of MI after surgery. Mice without obvious ST segment elevation were excluded. One week after MI surgery, mice in the control group received an intraperitoneal injection of normal saline (NS), while mice in the model group were intraperitoneally injected with LPS (2 mg/kg). Ultrasound was used to evaluate the heart function. Then, the model mice were randomly allocated into a model group ($n=10$) or an STP group ($n=10$), which were treated with NS or STP (1 mg/kg), respectively. The area of MI was assessed by Evans Blue coupled with TTC staining.

Cremaster microcirculation assessment

Mice were anesthetized with pentobarbital (40 mg/kg). Rhodamine 6G (5 mg/kg) was injected via the jugular vein. Intravital microscopy (BX51, Olympus, Japan) of the cremaster muscle was used to record the cremaster venular blood flow and leukocyte adhesions in venules [9–12]. Then, the images and videos of microvascular blood flow were recorded using an OLYMPUS DP71. Venules 200 μ m in length and ranging from 20 μ m to 40 μ m in diameter were selected for analysis. The velocity of microvascular blood flow in cremaster and leukocyte adhesion in venules at 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h after intervention was determined with ImagePro Plus 6.0 software (Media Cybernetic, USA).

Flow cytometric analysis

Blood was collected via the great saphenous vein of mice. ACK lysis buffer was used for erythrocyte removal, and a centrifuge was used to separate plasma and leukocytes. Plasma was discarded and leukocytes were resuspended in 20 μ l NS. Then, leukocytes were incubated with appropriate antibodies, as previously described [13,14]. Rat anti-mouse CD11b was used at a dilution of 1: 12. Flow cytometry was performed on a DXP8 Calibur device upgraded by Cytek after staining with appropriate antibodies, and data were analyzed using FlowJo 7.6 software (USA).

Real-time PCR analysis

All mice were euthanized at the end of the experiment. Whole blood, cremaster, aorta, and heart were collected for gene expression detection. TRIzol reagent (Takara, Japan) was used to extract total RNA, and A260/A280 ratio was used to spectrophotometrically evaluate the purity of RNA. PrimeScript[®] RT Master Mix kits were used to conduct reverse transcription to obtain cDNA. Then, SYBR[®] PremixEx Taq[™] kits were used to

Table 1. The sequence of primers used for reverse transcription.

Gene	Forward	Reverse
β -actin	5'-GGTCATCACTATTGGCAACG-3'	5'-ACGGATGTCAACGTCACACT-3'
CSE	5'-TTCCTGCCTAGTTCCAGCAT-3'	5'-GGAAGTCCTGCTTAAATGTGGTG-3'
Foxo1	5'-GGGTCTGTCTCCCTTCCTC-3'	5'-TCAGTGGCATTACAGCAGGTA-3'
HK 2	5'-GCCAGCCTCTCCTGATTTAGTGT-3'	5'-GGGAACACAAAAGACCTTCTGG-3'
ND 1	5'-CTAGCAGAAACAAACCGGGC-3'	5'-CCGGCTGCGTATTCTACGTT-3'
16S	5'-CCGCAAGGAAAGATGAAAGAC-3'	5'-TCGTTTGGTTTCGGGGTTTC-3'

prepare a 10- μ l reaction system to measure relative expression levels of CSE and FOXO1 mRNA. Data were normalized to β -actin. DNA isolated from leukocytes and the mitochondrial versus nuclear DNA copy numbers were assessed as previously described [15]. All primers were obtained from Primer Bank and are listed in Table 1.

Western blot analysis

Creaster tissue was collected for quantitative analysis of protein. Protein was extracted by RIPA lysis buffer (MultiSciences). Protein concentrations were determined by Takara Bradford Protein Assay Kit (Takara, Japan). The dilution ratio of rabbit anti-CSE polyclonal antibody was 1: 300, and the dilution ratio of β -actin (13E5) rabbit mAb was 1: 1000. Horse radish peroxidase (HRP)-labeled secondary antibody was used at 1: 5000 dilution. All antibodies were diluted by TBST, and 5% skim milk powder was used as the confining liquid. Data were analyzed by ImageJ 1.6 software [16,17].

Assessment of leukocytes adhesion *in vitro*

Peripheral blood leukocytes were isolated from 1 mL whole heparinized blood, and then they were placed on the 96-well plates coated with rat tail collagen. After 10 μ L DAPI was added to every well, 96-well plates were put in the incubator for 30 min. Then, cells were washed by NS twice to remove debris and dead cells. The status and fluorescence intensity of leukocytes were recorded by use of a fluorescence microscope. ImagePro Plus 6.0 software was used to analyze the number of adhesive leukocytes.

Statistical analysis

Data are presented as mean \pm standard deviation. GraphPad Prism 5.01 (La Jolla, CA, USA) and SPSS 25.0 (IBM, Inc., Armonk, NY, USA) were used to analyze difference between 2 groups by paired or unpaired *t* tests. Two-sided $P < 0.05$ was considered statistically significant.

Results

MI operation plus LPS induced cremaster microvascular dysfunction

Compared with sham mice, the ST segment of lead II ECG was significantly elevated in model mice treated with MI operation plus LPS injection (Figure 1A). Moreover, Evans Blue combined TTC staining indicated that mice in the MI+LPS group had myocardial infarction successfully established (Figure 1C). The left ventricular end-diastolic volume of model mice was enlarged (0.39 ± 0.08 cm vs. 0.31 ± 0.06 cm), while the left ventricular ejection fraction ($30.34 \pm 7.42\%$ vs. $53.67 \pm 6.23\%$) (Figure 1B) and cremaster microvascular velocity were significantly decreased (Figure 1D).

Effects of STP on improving cremaster microvascular blood flow

The cremaster microvascular blood flow velocity at different time points in these 3 groups is depicted in Figure 2. STP significantly improved the cremaster microvascular blood flow velocity, from 0.075 ± 0.005 mm/s to 0.480 ± 0.010 mm/s, and the effect usually occurred about 15 min later after STP intervention. Moreover, the duration of blood flow velocity improvement lasted for more than 6 h (Supplementary Figure 1)

Effects of STP on reducing leukocytes adhesions

In model mice, MI plus LPS induced leukocytes to adhere to the small venous wall *in vivo* (Figure 3A, 3B) and *in vitro* (Figure 3C, 3D). The number of adherent leukocytes in model mice was about 2.5 times higher than in the control group. STP significantly reduced the number of leukocytes adhered to the vascular wall. Compared with the control group, the relative expression of CD11b on leukocytes in model mice was increased by 3-fold. However, STP reduced leukocytes adhesions and restored the expression of CD11b (Figure 3E, 3F).

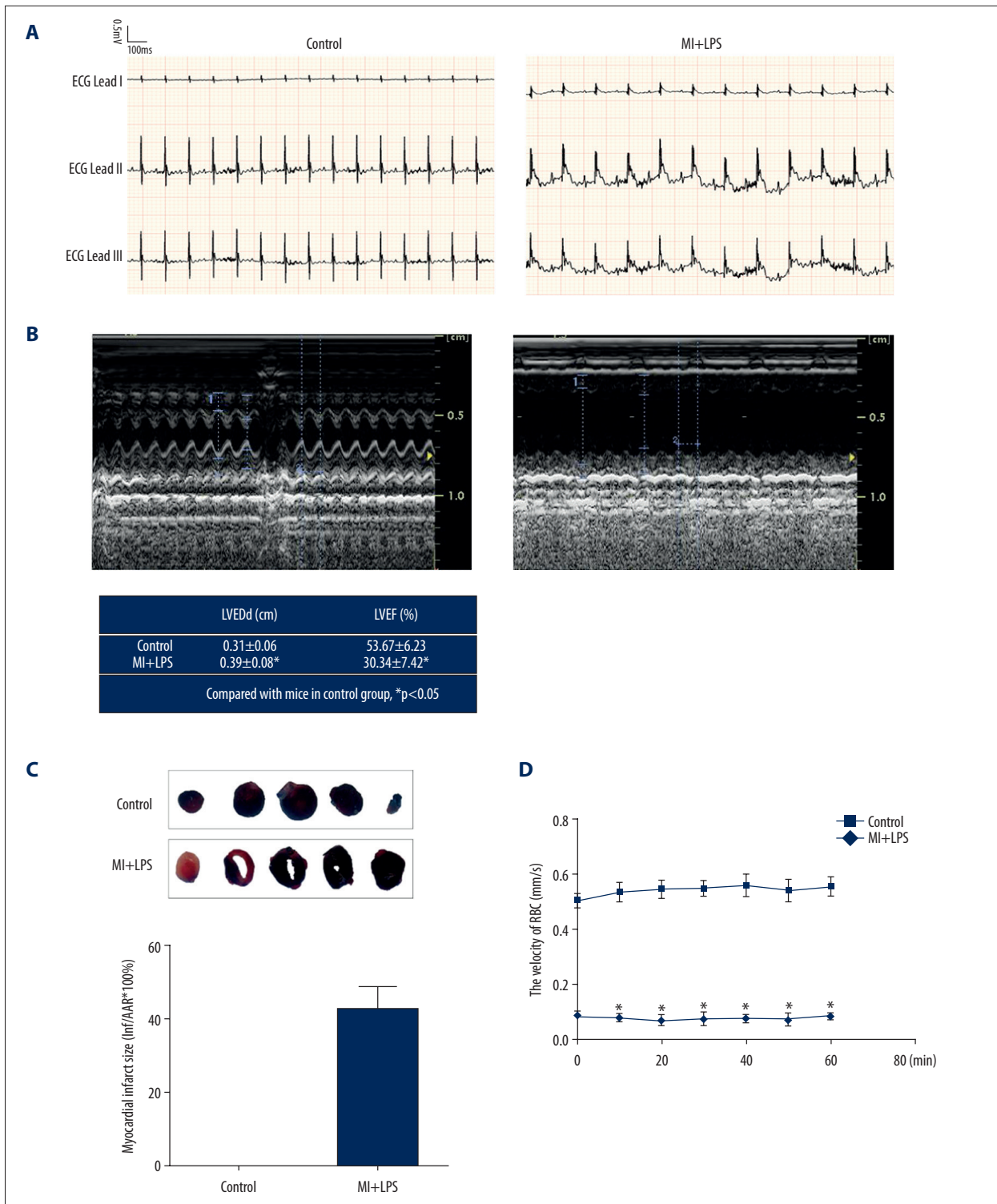


Figure 1. Acute myocardial infarction combined with LPS induced cremaster microcirculation dysfunction. Control: Control group; MI+LPS: MI plus LPS group. Data are mean ±SD from 10 mice. * p<0.05, vs. Control group. **(A)** The electrocardiogram was performed 30 min after LPS was injected. The ST segments of I, II, and III lead electrocardiograms were significantly elevated in the MI+LPS group. **(B)** The diameter at the end of left ventricular diastolic and left ventricular output were measured by ultrasound 30 min after LPS was injected. **(C)** At the end of the experiment, Evans Blue combined TTC staining was performed. **(D)** The cremaster microcirculation blood flow velocity was measured 30 min after LPS was injected.

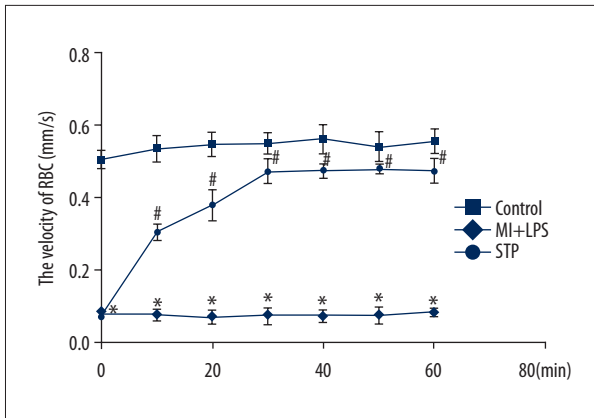
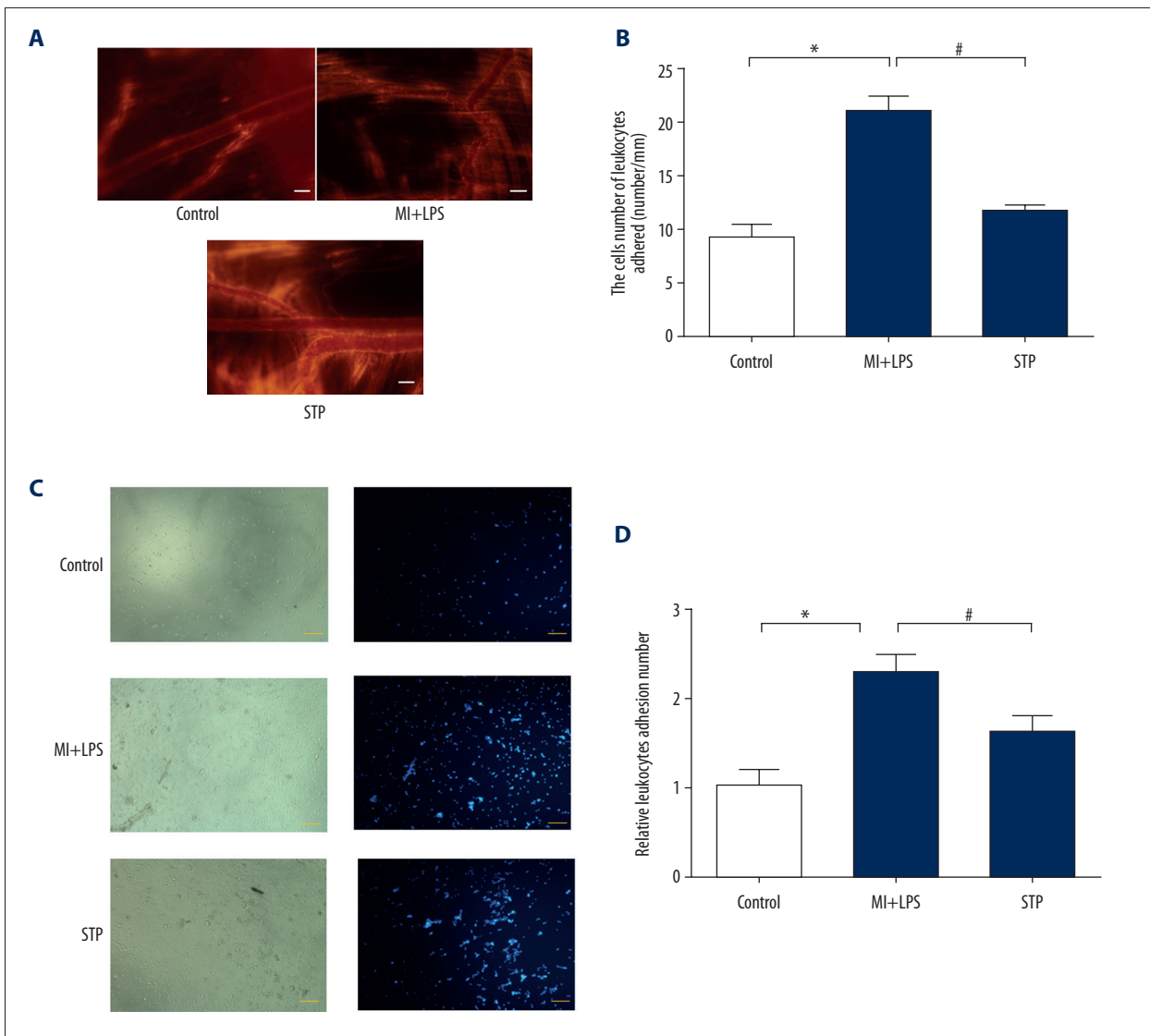


Figure 2. Effect of STP in improving cremaster microvascular dysfunction. Control: Control group; MI+LPS: MI plus LPS group; STP: STP group. Data are mean \pm SD from 10 mice. * $p < 0.05$, vs. Control group, # $p < 0.05$, vs. MI+LPS group. STP significantly improved the slow flow of cremaster microvascular, and the effect appeared 15 min after medicine was administered.



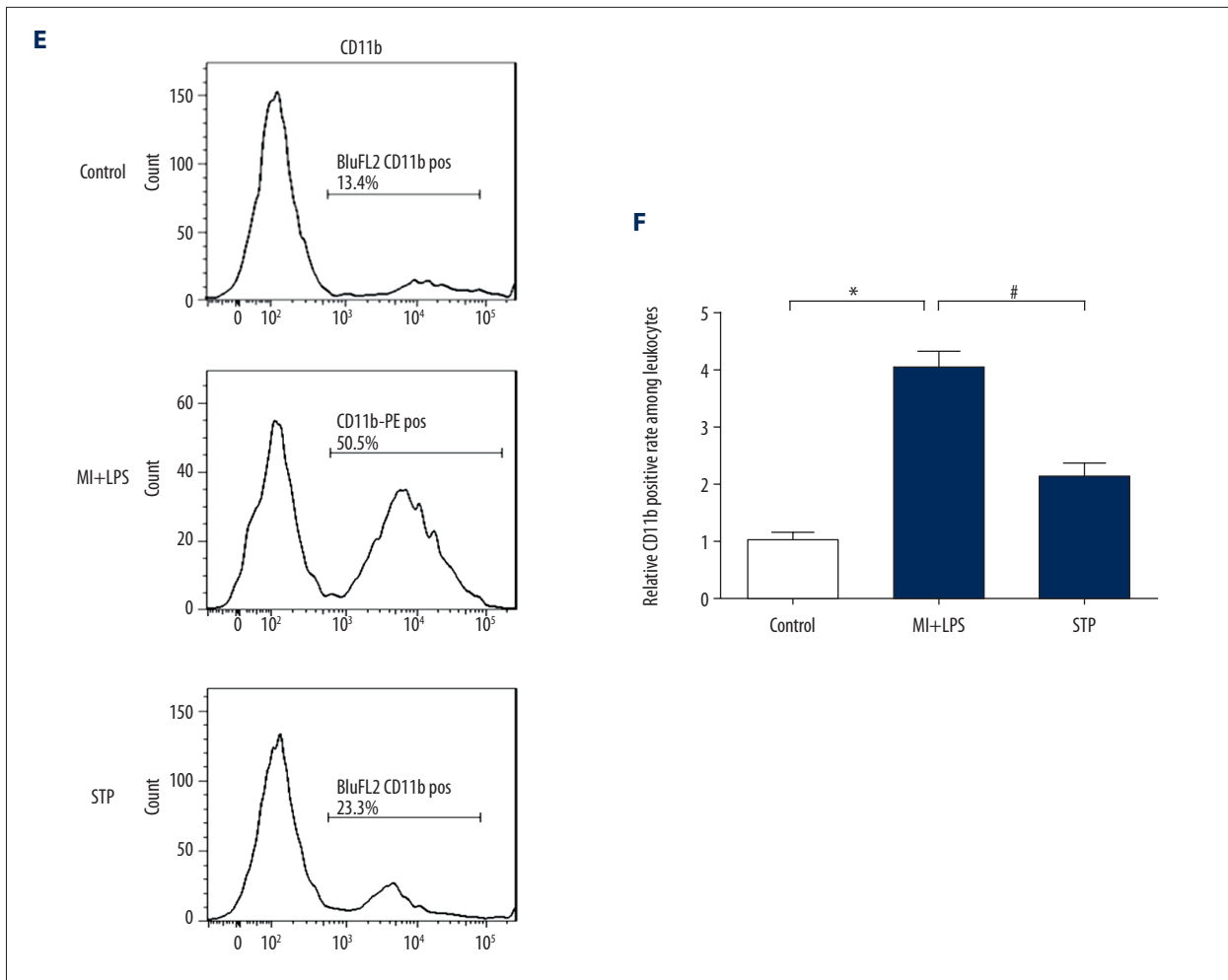


Figure 3. Effect of STP in reducing the number of adhered leukocytes and expression level of CD11b. Control: Control group; MI+LPS: MI plus LPS group; STP: STP group. Data are mean \pm SD from 10 mice. * $p < 0.05$, vs. Control group, # $p < 0.05$, vs. MI+LPS group. (A, B) Rhodamine 6G was injected via jugular. The number of white cells adhered to venular wall *in vivo* were observed by BX51 microscope. (C, D) White cells were stained by DAPI. STP also decreased the number of white cells that adhered to the 96-well plates. (E, F) The expression level of CD11b was measured by flow cytometric analysis.

Effects of STP on the expression of CSE and FOXO1

In model mice, the expression of CSE in cremaster tissue was significantly decreased (Figure 4A–4C) and FOXO1 expression in leukocytes was increased by 2.2-fold (Figure 4D). STP up-regulated the expression of CSE and downregulated the expression of FOXO1 (Figure 4D). We also found that CSE was mainly expressed in heart and cremaster tissues, and was about 5 times higher in leukocytes and aorta (Figure 4E).

STP's Protective effect on mitochondria

Compared with the control group, the relative expression of ND1 and 16S DNA in leukocytes was downregulated by nearly 70% by MI plus LPS, which indicates reduced mitochondrial DNA copy numbers, and STP reversed this phenomenon (Figure 5).

Discussion

Microvascular dysfunction is an important cause of myocardial ischemia, but it is difficult and expensive to directly monitor coronary microvascular dysfunction. Studies have shown that coronary microvascular dysfunction is associated with peripheral small vessel abnormalities of kidney, retina, and brain [18–20]. Conversely, peripheral microvascular function can be as a model to evaluate systemic microvascular function [21]. Cremaster muscle microcirculation is representative of the peripheral microvascular bed, which is widely used to visualize *in vivo* blood cells interacting with the endothelium and within the vessels [22]. The hemodynamic parameters of cremaster microcirculation such as shear stress, flow rate, and vasodilatation/vasoconstriction can also be determined by intravital microscopy. Furthermore, response to multiple drugs

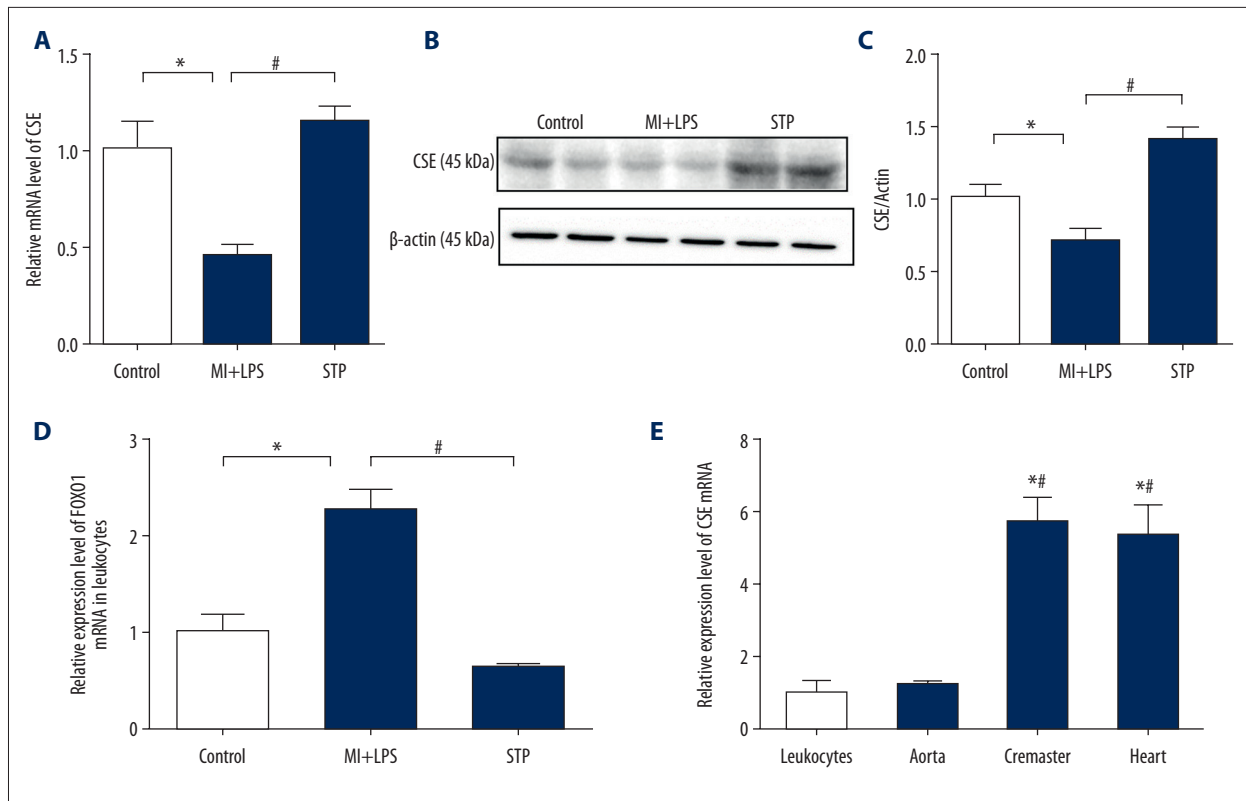


Figure 4. Effect of STP in regulating the expression of CSE in cremaster muscle. Control: Control group; MI+LPS: MI plus LPS group; STP: STP group. Data are mean \pm SD from 10 mice. * $p < 0.05$, vs. Control group, # $p < 0.05$, vs. MI+LPS group. (A) The expression level of CSE mRNA in the cremaster tissue was measured by RT-PCR and (B, C) The expression level of CSE protein in the cremaster tissue was measured by Western blot. Data were analyzed by ImageJ 1.6 software. (D) The relative expression level of FOXO1 mRNA in leukocytes was measured by RT-PCR. (E) The relative expression level of CSE in leukocytes, aorta, cremaster, and heart were measured by RT-PCR.

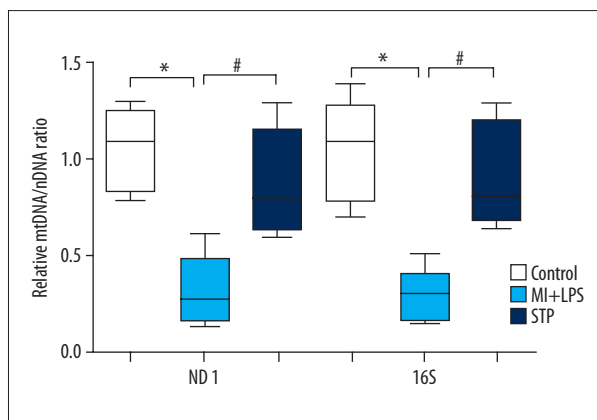


Figure 5. Effect of STP upregulating the ratio of mtDNA/nDNA. Control: Control group; MI+LPS: MI plus LPS group; STP: STP group. Data are mean \pm SD from 10 mice. ND1 and 16S were expressed in the mitochondria. STP significantly improved the mtDNA/nDNA.

and mechanisms underlying blood cell interactions within the cremaster microvascular system can be studied in a realistic scenario.

In the present study, we successfully established a mouse model of peripheral microvascular dysfunction with MI surgery and LPS injection. We found that acute myocardial infarction produces

sustained proinflammatory endothelial activation in remote arteries, including arteries in cremaster tissues [11]. In addition, LPS can also induce microcirculatory disturbances in mice [10]. Therefore, the combination of MI surgery and LPS can help establish a mouse model of peripheral microvascular dysfunction. The slow blood flow in cremaster tissue and increased leukocyte adhesions were observed in the model mice. After STP

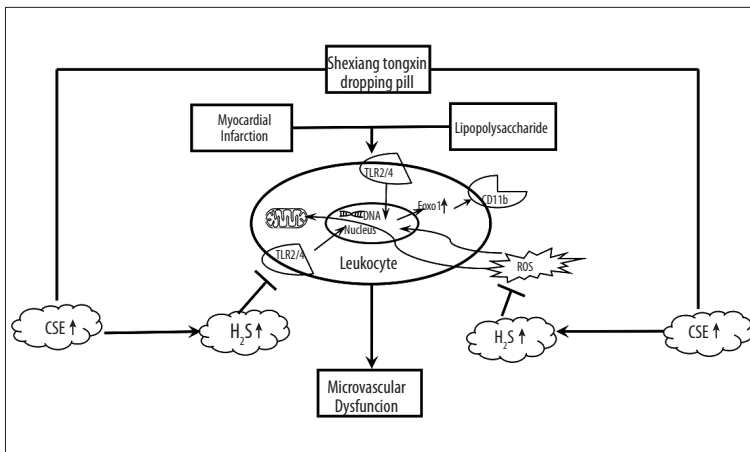


Figure 6. Likely mechanisms of STP's protective effects against peripheral microvascular dysfunction.

intervention, the immediate microvascular blood flow in cremaster tissue was significantly improved. Moreover, leukocyte adhesion was attenuated, as reflected by the downregulation of FOXO1 and adhesion molecule CD11b expression in leukocytes. CSE expression in cremaster tissue was significantly increased and mitochondrial DNA copy numbers were restored.

STP is a Chinese patent medicine used to treat ischemic cardiovascular diseases through ameliorating sputum-heat and blood stasis. Previously, Zhang et al. [23] showed STP protects the vascular endothelium of rats by increasing NO and decreasing hypersensitive CRP and endothelin levels. Wang et al. [24] found STP can play a role in improving coronary microvascular function and can be used to treat coronary slow flow, which is a disease of coronary microvascular dysfunction. Our study explored the mechanisms of STP in improving microvascular function. We found increased expression of CSE in cremaster tissue by RT-PCR and Western blot analysis. CSE is a key tissue-specific enzyme that produces hydrogen sulfide (H_2S), and a number of studies have reported that H_2S has anti-inflammation and anti-oxidation effects, inhibits apoptosis, and improves endothelial dysfunction [25–27]. Our results suggest that STP can improve microcirculation through increased CSE and H_2S in these 2 pathways (Figure 6). First, H_2S can inhibit TLR2 (Toll-like receptors-2) and/or TLR4 (Toll-like receptors-4), which downregulates the expression of FOXO1 and decreases the production of leukocyte adhesion molecule CD11b [28]. Following this, leukocyte adhesion in blood vessels is controlled. Thus, the process of microvascular thrombosis is terminated and microcirculation is improved. Second, H_2S can

inhibit reactive oxygen species (ROS), which is a main cause of mitochondrial injury [29]. STP exhibited multi-targeting effects on inflammation and oxidative stress [30,31]. Therefore, STP protects cells from mitochondrial injury and increases the mitochondrial DNA copy numbers.

Our study has certain limitations. First, due to lack of the necessary laboratory apparatus, the hydrogen sulfide concentration in tissues was not tested. Second, the coronary microvascular function in the heart was not evaluated, and the associations between peripheral microvascular function and coronary microvascular function remain to be identified. Third, we did not investigate the microvascular structural changes in retina, kidney, or other organs after prolonged use of STP. Further studies are warranted to explore the short-term and long-term pharmaceutical effects of STP on coronary microcirculation and other microvasculature.

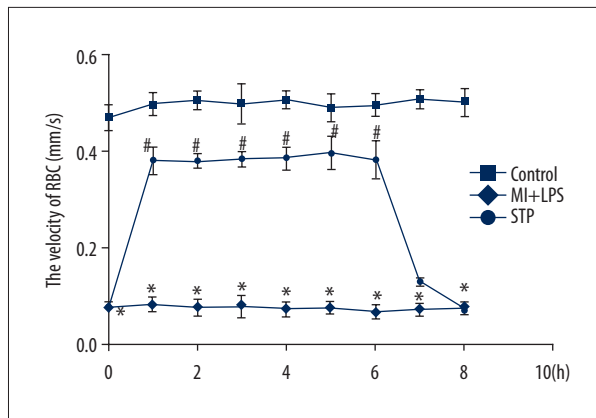
Conclusions

A peripheral microvascular dysfunction mouse model can be successfully established by MI surgery and LPS. STP can improve peripheral microvascular blood flow by upregulating the expression of CSE.

Conflicts of interest

None.

Supplementary Figure



Supplementary Figure 1. Effect of STP on improving cremaster microvascular dysfunction. Control: Control group; MI+LPS: MI plus LPS group; STP: STP group. Data are mean \pm SD from 10 mice. * $p < 0.05$, vs. Control group, # $p < 0.05$, vs. MI+LPS group. The improvement of the blood flow velocity of the cremaster muscle can last more than 6 h. The effect of STP is sustainable, not transient.

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