BASIC SCIENCE

Tetrathiomolybdate Partially Alleviates Erectile Dysfunction of Type 1 Diabetic Rats Through Affecting Ceruloplasmin/eNOS and Inhibiting Corporal Fibrosis and Systemic Inflammation

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ABSTRACT

Introduction: Patients with erectile dysfunction induced by diabetes mellitus (DMED) show a poor effect rate for oral phosphodiesterase type 5 inhibitors (PDE5is). Therefore, the new therapeutic strategy is necessary in patients with DMED.

Aim: To investigate whether Tetrathiomolybdate (TM) supplementation could ameliorate DMED by activation of eNOS.

Methods: Twenty-four diabetic rats were induced by intraperitoneal injection of streptozotocin (STZ) and the other 6 normal rats constituted the control group. Eight weeks later, the erectile function of rats was assessed with an apomorphine test. Only some rats with DMED were treated with TM orally every day for 4 weeks; the other rats remained in the same condition for 4 weeks. After 1 week washout, the erectile function of rats in each group was evaluated. Then, the serum concentration of IL-6 and histologic changes of corpus cavernosum were measured.

Main Outcome Measure: Erectile function was measured after DMED rats treated with TM. The cavernosum level of Ceruloplasmin (Cp), eNOS, endothelial cell content, corporal fibrosis, apoptosis rate and the serum level of IL-6 were also assayed.

Results: Erectile function in the DMED group was significantly impaired compared with the control group and was partly, but significantly, improved in the DMED+TM group. The DMED group showed upregulation of Cp and inhibition of eNOS, but the inhibition was partly reversed in the DMED+TM group. The DMED group showed serious corporal fibrosis. However, TM supplementation partly increased the ratio of smooth muscle to collagen, decreased the ratio of apoptosis. What's more, gavage administration of TM profoundly decreased the serum level of IL-6 in DMED rats.

Conclusion: TM supplementation inhibits endothelial dysfunction, corporal fibrosis, and systemic inflammation, ultimately leading to partial improvement of DMED in rats. **Yin Y, Peng J, Zhou J, et al., Tetrathiomolybdate Partially Alleviates Erectile Dysfunction of Type 1 Diabetic Rats Through Affecting Ceruloplasmin/eNOS and Inhibiting Corporal Fibrosis and Systemic Inflammation. Sex Med 2022;10:100455.**

Key Words: Tetrathiomolybdate; Diabetes Mellitus; Erectile Dysfunction; Ceruloplasmin; eNOS

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INTRODUCTION

Erectile dysfunction (ED) is a common complication in diabetic men and has a negative impact on the quality of life. Approximately half of patients with diabetes mellitus (DM) will eventually develop ED (DMED).[1] The main features of ED in patients with diabetes are penile neurovascular dysfunction and fibrosis, which cause poor response to phosphodiesterase type 5 inhibitors (PDE5is).[2,3]

Endothelial dysfunction, which led to decrease of endothelial nitric-oxide synthase (eNOS), was reported in DMED.[4] eNOS is a major source of nitricoxide (NO), which promotes the production of cyclic guanosine monophosphate (cGMP) in cavernosal smooth muscle cells (SMC), resulting in the relaxation of smooth muscle and subsequent penile erection.[4–6] However, PDE5is can neither increase cGMP level if the endogenous NO is restricted, nor reverse pathological changes of corpus cavernosum.[7] Therefore, the new therapeutic strategy is necessary in patients with DMED, especially those nonresponsive patients to PDE5is.

Ceruloplasmin (Cp) is one of the major copper-binding proteins in the blood. There are 2 subtypes of Cp, one of which is a secretory type (Sec-Cp) and the other is a membrane glycosylphosphatidylinositol-anchored protein (GPI-Cp).[8,9] Chris J. Sullivan used the GeneChip arrays to examine alterations in penile gene expression in streptozotocin-induced DMED rats and controls, and found the level of Cp in the corpus cavernosum of DMED rats was significantly increased.[10] Cp impairs the endothelium-dependent vasorelaxation of the aorta.[11] Later research found that Cp could inhibit eNOS activation in cultured endothelial cells,[12] and had a strong, dose-dependent inhibitory effect on the agonist-induced increase of cGMP levels. What's more, Cp is also a NO oxidase and nitrite synthase, which could oxidize NO to NO+, with subsequent hydration to nitrite.[13]Thus, Cp appears potentially meaningful target for the treatment of DMED, and inhibition of Cp may activate eNOS/NO/cGMP pathway in corpus cavernosum.

Tetrathiomolybdate (TM), as a specific and effective copper chelator, can complex with copper and inhibit the oxidase activity of Cp.[14] The expression of Cp could also be inhibited by TM.[15] What's more, TM could lessen inflammatory responses induced by lipopolysaccharide[16] and alleviate fibrosis caused by obstruction and drug.[17,18]

Dysregulation of eNOS, inflammatory response[19] and fibrosis of corpus cavernosum are the characteristics of DMED. Can TM treatment partially improve erectile function by regulation of eNOS pathway and alleviating the above pathological features? In this study, we investigated the effects of TM in a DMED rat model. Our results showed that TM administration ameliorated ED in rats with DM, which may become a new therapy for DMED.

MATERIALS AND METHODS

Drugs

Streptozotocin (STZ) was acquired from Solarbio life science (Beijing, China), apomorphine (APO) and TM were purchased from Sigma-Aldrich (St Louis, MO).

Animal Treatment

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the Third Xiangya Hospital, Central South University, Hunan, China. Thirty 8-week-old male Sprague−Dawley rats were used for this study. Of these, 24 DM rats were induced by intraperitoneally with STZ (60 mg/kg), and the others injected with citrate phosphate buffer, used as the control group (Control, n = 6). At 72 hours after STZ injection, DM was confirmed by blood glucose levels, which were measured with Accu-Check glucose monitor (Roche Diagnostics, Mannheim, Germany) and only rats with fasting glucose levels higher than 16.7 mmol/L were selected as DM.[20]

8 weeks later, all rats with DM had survived. An APO test was used to assess the DMED status. Of all the DM rats, 14 with APO-negative results were used in subsequent experiments. The rats were randomly divided into 2 groups. One group with DMED was treated with gavage administration TM 10mg/kg[16,21] (dissolved in doubly distilled water) every day for 4 weeks (DMED+TM, n = 7), and the other group was treated with phosphate buffer solution (PBS) on the same schedule (DMED +PBS, n = 7).

Measurement of Erectile Function

After 1 week washout, the erectile function of rats in each group was evaluated. All rats were anesthetized by injection with pentobarbital (40 mg/kg). Then, the carotid artery was exposed and cannulated with a PE-50 tube which was connected to a pressure transducer to continuously monitor arterial pressure. A 25-gauge needle containing 100 IU/mL heparin solution was inserted into the right penile crura and the other end of the tube was also connected to a pressure transducer to monitor intracavernous pressure (ICP). The cavernous nerve was electrically stimulated at 1 V, 20 Hz, with a pulse width of 5 ms for 1 minute and a 5-minute interval before subsequent stimulation. The ratios of maximal ICP (MaxICP) to mean arterial pressure (MAP) were calculated to evaluate erectile function in vivo.

Measurement of Serum Concentration of IL-6

After the ICP test was completed, clinical needles and vacuum tubes were used to collect blood through the inferior vena cava. The serum was separated immediately and stored at −80°C. The levels of IL-6 were assessed using ELISA kits from Meimian

Biotechnology (Jiangsu, China) according to the manufacturer's instructions.

Histologic Examinations

H&E staining. Slides containing tissue sections were deparaffinized using a dry oven at 60°C for 30 minutes and in 2 changes of xylene for 10 minutes, and then rehydrated in a series of decreasing concentrations of alcohol. Finally, the slides were washed in tap water. After that, Harris's hematoxylin reagent was used to stain for 8 minutes. Then, the slides were destained in 0.5% acid-alcohol for 3 seconds and washed with running water. They were counterstained with 0.5% eosin for another 1 minute. After dehydration with different concentrations of alcohol and xylene, the slides were observed with microscope for histopathologic examination.[22]

Masson Trichrome Staining. Tissue sections of the middle part of the penis were stained according to Masson kit instructions (Solarbio, Beijing, China). The collagen tissues (stained blue) of the penile sponge and smooth muscle tissues (stained red) were observed under a light microscope. The cavernous smooth muscle/collagen ratio was analyzed using ImageJ software.

Immunofluorescence. The tissues were cryoprotected in sucrose, frozen and sectioned at 8 μm in a cryostat. Slides were exposed to 0.3% Triton X-100 (Solarbio Life Science, Beijing, China) for 10 minutes. Normal 10% goat blocking fluid (Solarbio Life Science, Beijing, China) was applied to the slides for 30 minutes. Different antibodies were applied to the slides and incubated overnight at 4°C. After the hybridization of secondary antibodies, DAPI stained for the cell nucleus. The slides were observed using a fluorescence microscope (OLYMPUS, Tokyo, Japan). Antibodies against Cp were obtained from Abclonal Biotechnology (Wuhan, China); CD31 antibody was purchased from Bioworld Technology (Nanjing, China); eNOS antibody was obtained from Abcam Biotechnology (Cambridge, UK).

Immunohistochemistry. The locations and expressions of target proteins α-SMA were investigated by immunohistochemical staining within 5-mm tissue slides. The slides were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the slides in Tris/EDTA buffer (pH 9.0) before heating for 10 minutes. Then the slides were treated with endogenous peroxidase blocker for 10 minutes and normal goat serum (10%) was utilized for 30 minutes to block nonspecific binding sites. Subsequently, the slides were incubated with α-SMA antibody (Affinity, Cincinnati, OH, USA) at 4°C overnight. The primary antibody was visualized under the light microscope using the DAB method according to the kit instructions (Maixin Biotechnology, Fuzhou, China).

TUNEL Staining. Apoptosis of the penile tissue of each group was detected using a TUNEL apoptosis detection kit (KeyGEN BioTECH, Nanjing, China) following manufacturer's instructions. Each sample was randomly selected from 4 fields of view. Cells with green staining were counted as apoptotic, and the ratio of apoptotic cells to the total number of cells in the field of view yielded the rate of apoptosis.

Statistical Analysis. Results were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) and expressed as mean ± standard deviation (SD). Unpaired 2-tailed Student's *t*-test was used to determine differences between 2 different groups and one-way ANOVA for more than 2 groups. Differences among groups were considered significant at a P value less than 0.05 and represented as follows: $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

RESULTS

Basal Metabolic Variables

There was no significant difference in initial levels of fasting blood glucose and body weight among the 3 groups. At the end of 13th week after diabetes induction, rats in the DM group had significantly higher fasting glucose concentrations and lower body weight compared with those in the control group($P < .0001$), which suggested that the rat model of type 1 DM was successful. However, the final blood glucose levels and final body weights between the DMED + PBS and DMED + TM groups had no significant difference (Table 1).

Table 1. Basal metabolic variables

Variable	Control	DMED+PBS	DMED+TM
Initial weight (g)	251.24 ± 3.64.94	247.58 ± 6.87	253.65 ± 4.75
Final weight (g)	564.47 ± 47.10	234.36 ± 40.46[#]	250.14 ± 35.25[#]
Initial fasting glucose (mmol/L)	5.74 ± 0.46	5.82 ± 0.59	5.95 ± 0.62
Final fasting glucose (mmol/L)	5.89 ± 0.57	25.33 ± 4.12[#]	26.73 ± 3.75[#]

Initial weight and initial fasting glucose levels in the 3 groups of rats after 7 days of adaptive feeding; final weight and final fasting glucose in the rats after 7 days of washout at 13 weeks. Data are presented as mean ± SD.
[#]$P < .0001$ compared with the control group.

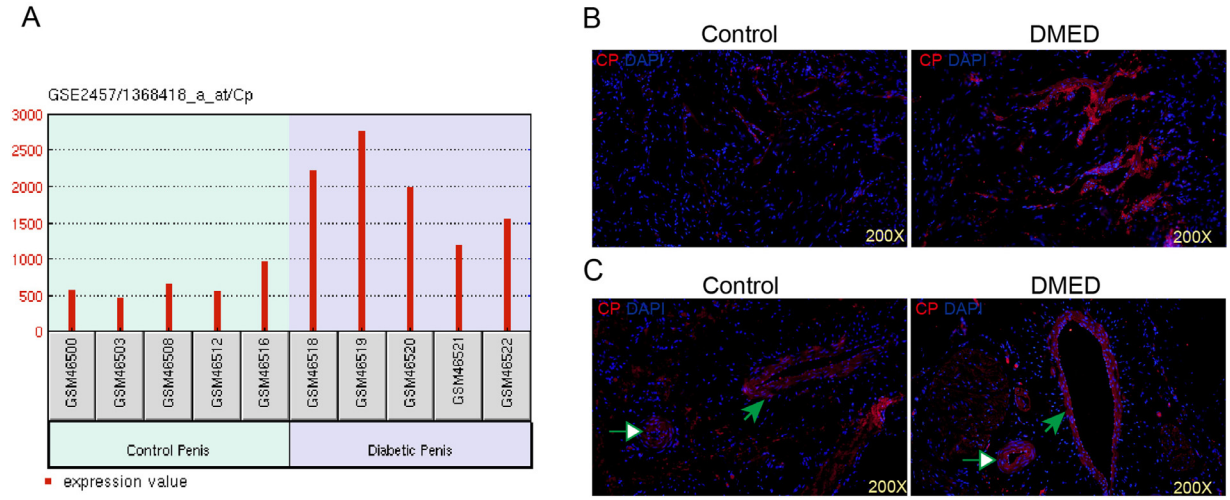

Figure 1. Upregulation of Cp in DMED rat penis. (A) The expression profile graph for Cp. Each red bar in the graph represents the expression measurement extracted from the value column of the original submitter-supplied sample record (GEO database, analyzed by GEO2R). (B) representative immunofluorescence of Cp in the DMED and control cavernosum; C Representative immunofluorescence of Cp in penile dorsal vein (solid arrow) and dorsal artery (hollow arrow).

Cp is Highly Upregulated in the Penis of DMED Rats

Chris J. Sullivan found and confirmed that Cp increased in the corpus cavernosum of DMED rats and localized in the cavernous sinusoids. Besides, Cp labeled the endothelium and smooth muscle layers of the cavernosal sinusoids.[10] We browsed GEO database and further analyzed the microarray results by GEO2R. As shown in Figure 1A, the expression of Cp was obviously upregulated in DMED penis. Furthermore, immunofluorescence was used to verify Cp protein contents. Same as previous study, Cp was highly expressed in DMED corpus cavernosum (Figure 1B). Cp staining was also observed in the penile dorsal vein and artery (Figure 1C).

TM Improves Erectile Function of DMED Rats

The erectile function was assessed by MaxICP and MaxICP/MAP. The results showed that both MaxICP and MaxICP/MAP revealed a significant decrease in DMED rats compared to normal control rats ($P < .01$). However, the Max ICP and MaxICP/MAP was significantly improved in rats treated with TM compared to PBS group (Figure 2).

TM Increased eNOS Levels in Corpus Cavernosum of DMED Rats

To determine the relationship between Cp and eNOS after TM treatment, double labeling of cavernous tissue with antibodies against Cp and eNOS was performed. The expression of Cp content in corpus cavernosum was significantly increased in the DMED group compared with the control group. Gavage administration of TM profoundly decreased the level of Cp in DMED rats. The expression of eNOS in the penis of the control group was strongly positive, while expression in the TM groups was

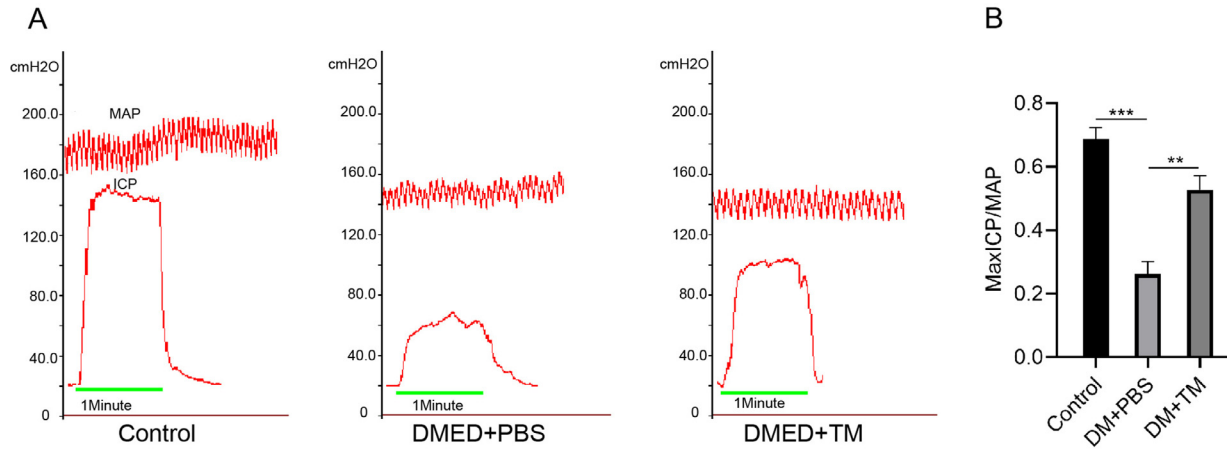

Figure 2. Erectile function of each group. (A) Representative images of ICP and MAP in response to electrical stimulation of the cavernosal nerve; (B) Results of the ratio of MaxICP to MAP in each group.

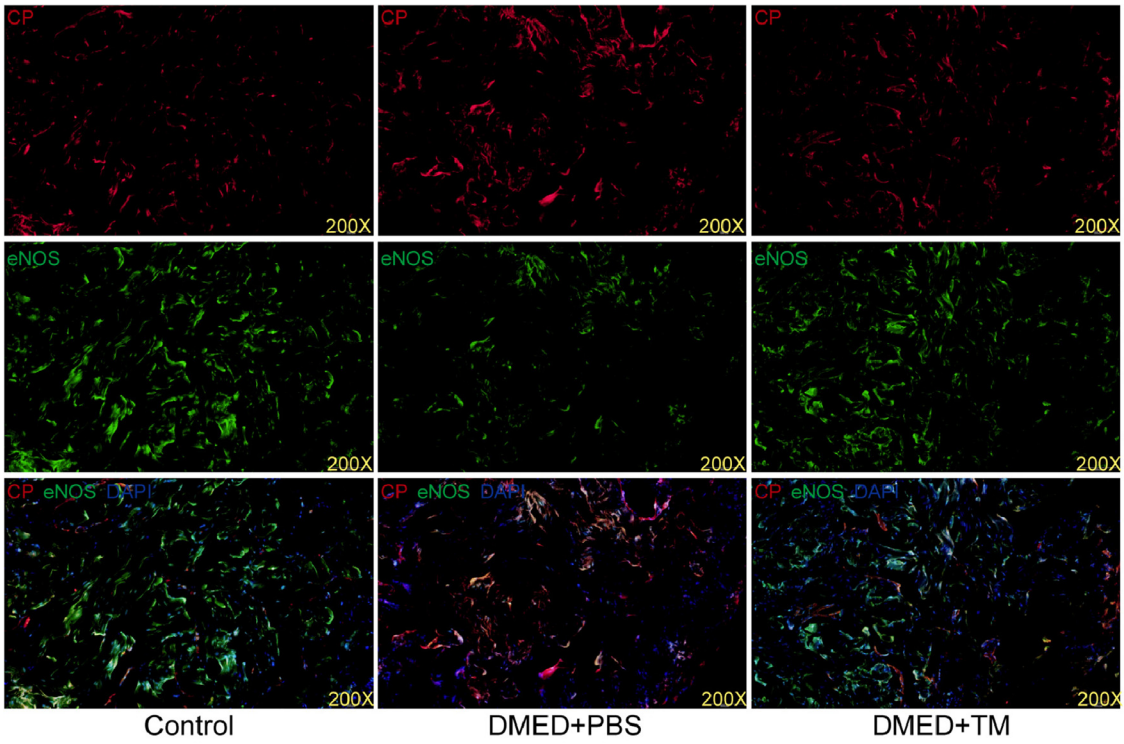

Figure 3. TM treatment suppressed Cp and increased eNOS levels Cp (red) and eNOS (green) immunostaining in corpus cavernosum from age-matched control and DMED rats.

slightly weaker than in the control group, but significantly higher than the PBS group (Figure 3).

Effect of TM on Endothelium in Corpus Cavernosum

Cavernosal endothelial dysfunction is recognized as a hallmark of the disease pathology. We found the endothelial cell content was severely decreased in PBS-treated DMED rats compared with the control group, whereas the level of endothelial cell was profoundly preserved by TM intragastric administration (Figure 4).

Effect of TM on Smooth Muscle Contents in Corpus Cavernosum

The morphological changes and smooth muscle (SM)-to-collagen ratios of the different groups were detected with H&E and Masson's trichrome staining, as shown in Figure 5A, B and D. TM treatment reduced morphological changes caused by DM and significantly increased the SM-to-collagen ratio compared with the PBS group. Moreover, Immunohistochemical staining with α-SMA antibody showed a significant reduction of smooth muscle contents in DMED rats, vs normal control (Figure 5C). However, TM treatment could partly improve the result.

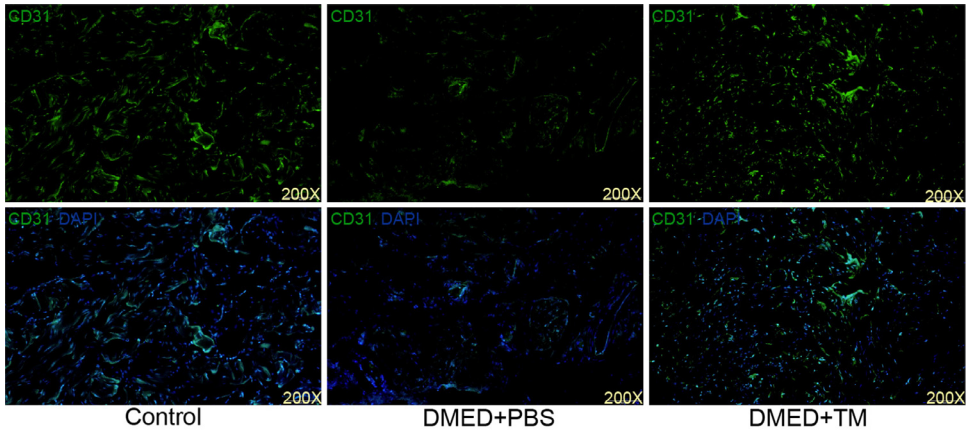

Figure 4. Effect of TM on endothelium in corpus cavernosum. Representative images of immunofluorescence staining of CD31 in the corpus cavernosum from age-matched control and DMED rats.

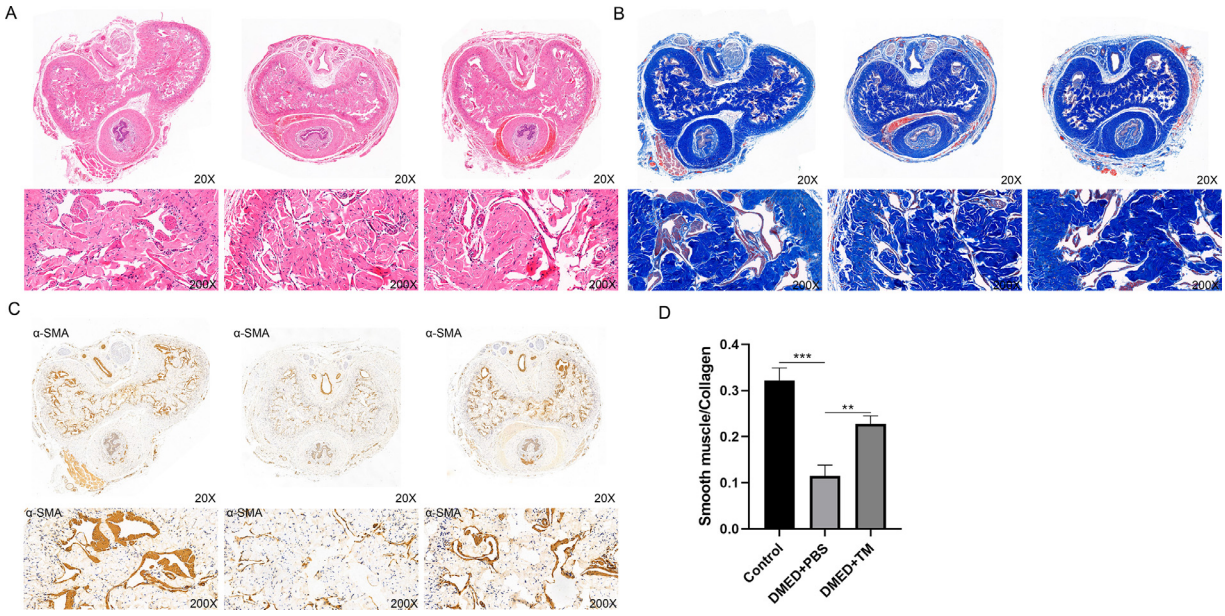

Figure 5. Effect of TM on smooth muscle contents (A) H&E staining. Thinner smooth muscle layer was found in all diabetic rats, with more severe changes in the PBS group; (B) Masson trichrome staining. Smooth muscle manifested as red, connective tissue was blue. (C) Representative images of immunohistochemistry staining of α-SMA in the corpus cavernosum from age-matched control and DMED rats. (D) The ratio of smooth muscle to collagen.

TM Decreased Apoptosis in DMED Rats

A large number of studies have revealed that long-term high glucose can induce cell apoptosis in the corpus cavernosum. We measured the apoptosis level in penile tissue samples by TUNEL (Figure 6). In the PBS group, there were a dramatically larger percentage of apoptosis cells than the control and TM groups. This indicates that TM can reduce the level of apoptosis in the corpus cavernosum of DMED rats.

Effect of TM on IL-6 Level in Serum of DMED Rats

Blood level of inflammatory IL-6 was significantly increased in ED patients and correlated negatively with sexual performance.[23] Inhibition of IL-6 attenuated ED following cavernous nerve dissection.[24] However, TM significantly inhibited LPS-induced IL-6 gene transcription.[16] As shown in Figure 7, IL-6 was increased in the blood of DMED rat, and TM treatment could obviously decrease the level. The result shows that TM may alleviate the inflammatory response in DM rats.

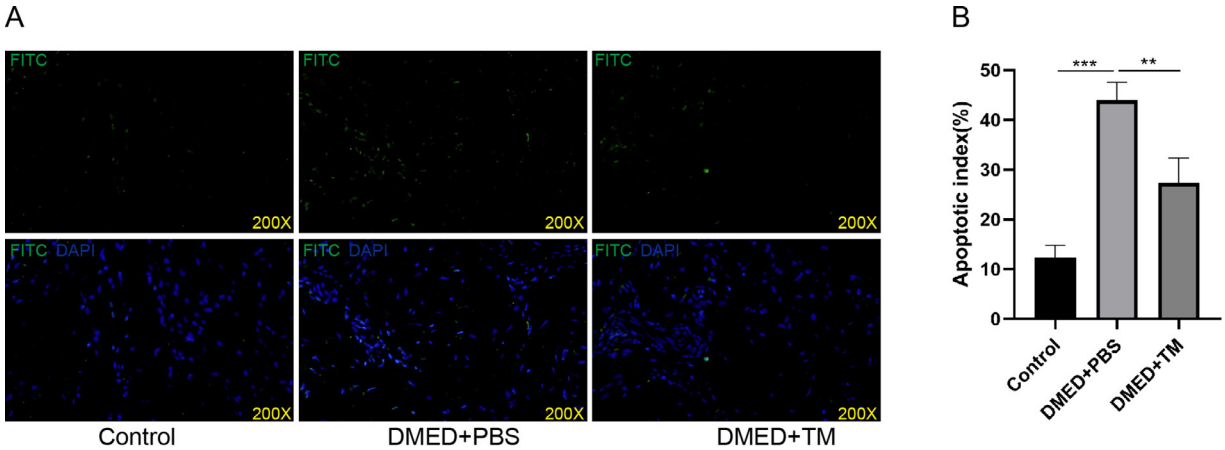

Figure 6. TM decreased apoptosis in DMED rats. (A) Representative images of TUNEL staining in the corpus cavernosum from age-matched control and DMED rats; (B) The apoptotic index, the percentage of apoptotic cells (stained green) of all cells, to quantify cavernous apoptosis level.

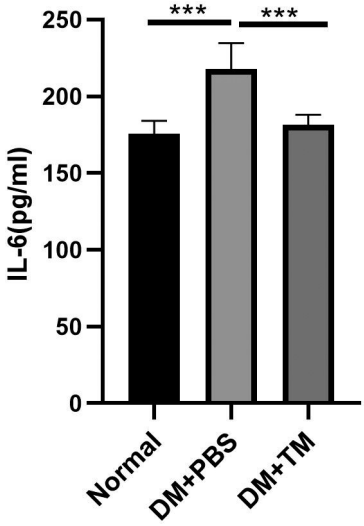

Figure 7. Effect of TM on IL-6 level in serum of DMED rats. Treatment with TM alters the serum levels of IL-6 in DMED rats.

DISCUSSION

DM is an important risk factor for ED.[1] However, ED in the diabetic population is significantly more difficult to manage and oral medications are associated with less efficacy.[2,25] The development of new, noninvasive, but effective medical treatments will lead to even more options for the treatment of this difficult disease. In our study, MaxICP/MAP was significantly increased in DMED rats with TM supplementation. This result shows that TM would ameliorate DMED.

A previous study shown that both isoforms of Cp were upregulated in corpus cavernosum of DMED rats. Cp was often present on the luminal side of the cavernosal sinusoids and labeled the endothelium and smooth muscle layers.[10] This is consistent with our result. The expression of Cp could be positively regulated by IL-6,[26,27] which was increased in the serum of DMED rats. Maybe inflammation induced by DM facilitates the expression of Cp in the corpus cavernosum. Some attentions have focused on the role that Cp may have in the function of the vascular system in health and disease. Increase in serum Cp levels is correlated with a decrease of serum NO levels in type 2 diabetes.[28] Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis.[13] Therefore, the high level of Cp may oxidize NO to nitrite, leading to the inactivation of the NO signaling pathway in penis. Besides, Cp impairs endothelium-dependent relaxation of rabbit aorta.[11] And later research confirmed that Cp can inhibit eNOS.[12] These results indicate that the increase of Cp in corpus cavernosum of diabetic rats may be involve in the pathological process of ED. TM, which has a specific effect on Cp, was used to determine its effects on DMED.

The eNOS/NO/cGMP pathway has been verified to be a key factor of erectile function.[29] Our results shown that TM treatment could reduce the level of Cp while increasing eNOS, which is a very important enzyme for EC to produce NO. To confirm whether TM accelerated the level of eNOS by protecting EC, we detected the endothelial marker CD31 to assess the EC content in the corpus cavernosum. It was found that TM treatment can significantly increase the content of EC in DMED rats. Mukhopadhyay CK et al found Cp could enhance SMC- and EC- mediated low density lipoprotein (LDL) oxidation by a superoxide-dependent mechanism.[30] Furthermore, oxidized LDL can promote the apoptosis of EC and SMC.[31,32] A large number of apoptotic cells appeared in the penile tissue of DMED rats. Maybe excessive Cp promotes LDL oxidation in penile EC and SMC, which ultimately leads to cell apoptosis and decreases the contents of the both cells. Therefore, inhibition of Cp may restrain apoptosis in corpus cavernosum of DMED rats. However, whether the changes of eNOS are associated with LDL oxidation needs further research.

Corporal fibrosis, which contributes to the poor response of DMED to PDE5is, is a typical pathological feature of DMED. High level of apoptosis index and loss of SMC are the representative features of corporal fibrosis.[33] Our study revealed that TM administration could partly prevent the loss of SMC and obviously augment SMC-to-collagen ratio. Apoptosis index, another characteristic of corporal fibrosis, was measure by TUNEL. Our results shown that TM administration could alleviate the higher apoptosis level in rats with DMED. These results showed TM inhibited the pathologic process of corporal fibrosis. Song et al found TM attenuated BDL-induced cholestatic liver injury and fibrosis in mice, in part by inhibiting TNF-α and TGF-β1 secretion.[18] TM therapy protects against bleomycin-induced pulmonary fibrosis in mice by inhibition of TNF-α.[17,34] TGF-β1, which is a key factor to promote corporal fibrosis, may also be a target of TM in corpus cavernosum of DMED rats.

Many evidences have supported the association between inflammatory milieau and ED in men with metabolic diseases.[19] Anti-inflammatory agents could be considered as a therapeutic strategy in the treatment of ED.[35] TM has been found to contribute to alleviate lipopolysaccharide-induced inflammatory responses in vivo[16] and lessen atherosclerotic lesion progress, through reducing vascular and intercellular adhesion molecule-1 (VCAM-1 and ICAM-1, respectively), monocyte chemotactic protein-1 (MCP-1), IL-6, and tumor necrosis factor (TNF)-α.[36] The drug also relieved arthritis against the increase in IL-2, IL-1ß, and TNF-α levels.[37] Our result revealed that TM significantly decreased serum IL-6 in DMED rats. Consequently, the reduction of IL-6 may also downregulate the expression of Cp. Therefore, TM reduces the level of Cp, and improves DMED partly by alleviating the systemic inflammation.

In summary, these results showed TM administration activated the eNOS pathway, increased content of EC, inhibited corporal fibrosis and alleviated systemic inflammation, thus partly alleviating DMED.

The absence of cellular-based experiments was one limitation of the present study. TM is a copper chelating agent, but this study did not study its chelation function. Besides, TM was not approved for the treatment of patients. Therefore, further studies should be performed to clarify the pharmacological functions of TM.

CONCLUSION

Our study revealed that TM treatment alleviated corporal fibrosis and systemic inflammation and protected endothelial function, finally resulting in partial improvement of erectile function in DMED rats. These results provided a basis for that TM supplementation might be a possible therapy for patients with DMED.

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STATEMENT OF AUTHORSHIP

Conceptualization, YH.Y, GM.Y and YX.T; Data curation, YH.Y, J.Z, JX.P and GM.Y, Formal analysis, YH.Y, J.Z, JX.P, HF.C, DY.P, Y.G, GM.Y and YX.T, Funding acquisition, YX.T, Methodology, YH.Y, HF.C, DJ.L and Y.G, Project administration, GM.Y and YX.T, Supervision, GM.Y and YX.T, Validation, YH.Y, DY.P and DJ.L; Writing – Original Draft, YH.Y, DY.P, DJ.L, GM.Y and YX.T, Writing – Review & Editing, YH.Y, GM.Y and YX.T.

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