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ORIGINAL ARTICLE

Schisandrin A Ameliorates Erectile Dysfunction and Regulates RhoA/ROCK1 Pathway in Rats with Streptozotocin-induced Type 1 Diabetes

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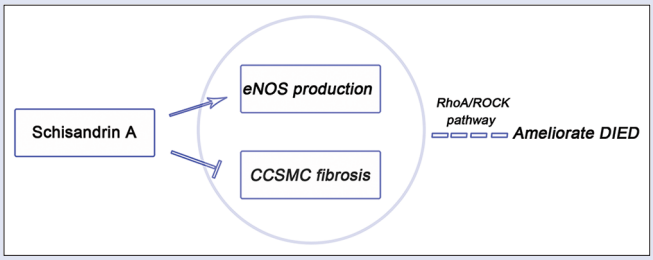
ABSTRACT

Introduction: Erectile dysfunction (ED) is a common complication of diabetes mellitus (DM) that severely affects the patient's quality of life. Schisandrin A, maybe a novel therapeutic option for patients with diabetes mellitus-induced erectile dysfunction (DIED). **Materials and Methods:** After induction by streptozotocin administration, rats were divided into four groups: Normal control (NC), DIED, low dosage (5 mg/kg/d), middle dosage (10 mg/kg/d), and high dosage (20 mg/kg/d) of schisandrin A treated DIED group. All groups were treated with normal saline or the relevant drug for 8 weeks. Body weight, erectile rate, intracavernosal pressure (ICP), mean arterial pressure (MAP), and serum glucose concentration were measured. The nitrate reductase method and nitric oxide synthase activity assay detected nitric oxide (NO) and endothelial nitric oxide synthase (eNOS) levels. Pulldown assay detected GTP-RhoA activity. Western blotting detected alpha-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1), Collagen III, Collagen IV, RhoA, ROCK1, ROCK2, phospho-myosin phosphatase target subunit 1 (p-MYPT-1), MYPT-1, phospho-eNOS (p-eNOS), and eNOS expressions. **Results:** Compared with the NC group, schisandrin A alleviated the ED rate of DIED rats, and increased ICP and ICP/MAP in DIED + SA group. Schisandrin A increased NO level and activated p-eNOS in penile tissues of DIED group. The expression of α-SMA increased, whereas, TGF-β1, Collagen III, Collagen IV decreased in DIED + SA group compared to DIED group. Schisandrin A inhibited the levels of GTP-RhoA, RhoA, ROCK1, ROCK2, and p-MYPT1 in penile tissues from rats of DIED. Schisandrin A in the high dose group (20 mg/kg/d) had a better effective effect on DIED. **Conclusion:** Schisandrin A ameliorated erectile dysfunction in rats with DIED by promoting eNOS production, inhibiting fibrosis, and inhibiting the RhoA/ROCK1 pathway.

Key words: Diabetes mellitus, erectile dysfunction, RhoA/ROCK pathway, schisandrin A, type 1 diabetes

SUMMARY

- Schisandrin A alleviated the erectile rate of rats related to DIED and increased ICP and ICP/MAP in DIED + SA group compared with the NC group. Schisandrin A promoted NO level and activated phosphorylation of eNOS in penile tissues of the DIED group. The expression of β-SMA increased, whereas, TGF-β1, Collagen III, Collagen IV decreased in DIED + SA group compared to DIED group. In addition, schisandrin A inhibited the levels of GTP-RhoA, RhoA, ROCK1, ROCK2, and phosphorylation of MYPT1 in penile tissues from rats of DIED. Schisandrin A ameliorated ED in rats with DIED by promoting eNOS production, inhibiting fibrosis, and inhibiting the RhoA/ROCK1 pathway.



Abbreviations used: CCSMCs: Corpus cavernosum smooth muscle cells; DIED: Diabetes mellitus induced erectile dysfunction; ED: Erectile dysfunction; ICP: Intracavernosal pressure; MAP: Mean arterial pressure; SD: Sprague-Dawley.

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INTRODUCTION

Erectile dysfunction (ED) is a common complication in patients with diabetes mellitus. The total prevalence of diabetic ED is about 57.7%, which is 3.5 times that of non-diabetic patients.[1] Diabetes mellitus (DM) is an independent predictor of ED, and the incidence of diabetes mellitus-induced erectile dysfunction (DIED) is rising every year.

It is well known that penile erection is regulated by nerves and vascular activity, which is dependent on cavernous neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) synthesis, as well as nitric oxide (NO) release. nNOS and eNOS play a major role in the initiation or maintenance of penile erection, respectively. The released NO diffuses freely into the smooth muscle cells (SMCs) of the corpus cavernosum, which stimulate the guanylate cyclase to increase the production of cyclic guanosine monophosphate (cGMP) leading to penile erection.[2] However, the first-line drug treatment for ED, i.e., phosphodiesterase type 5 inhibitor (PDE5i, sildenafil citrate) targeting cGMP exhibited little benefit in patients with DIED.[3] Thus, exploring

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new therapeutic drugs is of great significance in ameliorating ED in diabetes patients.

Schisandrin A, a bioactive lignan found, is one of many components in *Schisandra chinensis*.[4] Previous studies have revealed that Schisandrin A has multiple pharmacological functions, such as anti-inflammatory, antioxidant, immune regulation,[5] anti-tumor, and other effects.[6] Particularly, from the perspective of endothelial cells, schisandrin A resulted in increased NO level, decreased MDA level, and decreased ROS production to rescue hypoxia/reoxygenation damage.[7] Further, Fructus Schisandrae increased cGMP concentration, promoted eNOS expression, and relaxed the rabbit corpus cavernosum smooth muscle cells (CCSMCs).[8] Another study carried out by Kim showed that schisandrin A has a relaxant effect on sildenafil citrate-induced rabbit penile corpus cavernosum.[9] However, the roles and mechanisms of schisandrin A on rats with DIED are still unclear.

Dysregulation of eNOS and fibrosis of the corpus cavernosum are the main characteristics of DIED. However, the roles and mechanisms of schisandrin A in the treatment of DIED remain unclear. In the present study, we found that schisandrin A promoted eNOS expression and inhibited the fibrosis of CCSMCs to ameliorate DM-induced ED through RhoA/ROCK signal pathway, which may provide a new therapy insight for DIED.

MATERIALS AND METHODS

Chemicals and reagents

Streptozotocin, Sigma US Schisandrin A, National Institutes for Food and Drug Control of China RhoA activity detection kit, Cytoskeleton US Nitric Oxide Synthase Kit, Genmed scientificsinc US RhoA activity pulldown assay kit, Cytoskeleton US Chemiluminescence reagent, Beyotime China Protein assay kit, Beyotime China enhanced chemiluminescence reagent, Beyotime China α-SMA, Cat No.55135-1-AP, Proteintech China TGF-β1, Cat No.21898-1-AP, Proteintech China Collagen III, Cat No.ab6310, Abcam US Collagen IV, Cat No.ab256353, Abcam US RhoA, Cat No. 10749-1-AP, Proteintech China ROCK1, Cat No. 21850-1-AP, Proteintech China ROCK2, Cat No. 66633-1-Ig, Proteintech China p-MYPT-1, Cat No. AF5445, Affinity China MYPT-1, Cat No.AF5444, Affinity China p-eNOS, Cat No.AF3247, Affinity China eNOS, Cat No.AF0096, Affinity China ALT, AST, ALP, SR, Na, K, Ca, Blood glucose, Roche US were used in the study.

Animals and grouping

Before the experiment, a total of 24 adult male Sprague-Dawley (SD) rats (8-week-old, 200 ± 20 g) with normal erectile function were purchased from Charles River Laboratories and fed in the laboratory animal center of the second hospital of Hebei Medical University, China. Of these, 16 rats were injected intraperitoneally with streptozotocin (STZ, 65 mg/kg), and the others were injected with vehicle (NC group, 1% citrate phosphate buffer). Blood glucose levels were measured with fast blood glucose meter (Roche, GER) 3 days later. Rats with a fasting glucose concentration >16.7 mmol/L were considered diabetic. Apomorphine (APO, Sigma-Aldrich, USA) was used to determine DIED in rats with DM 8 weeks later. Select DIED rats and feed them with schisandrin A (5, 10, or 20 mg/kg/d) by gastric perfusion once a day for 8 weeks.

Erection assessment *in vivo*

After 8 weeks of STZ injection, DIED rats were selected according to the method of Heaton *et al*.[10] The rat's weight was measured and placed in a dim and quiet environment. The APO (100 μg/kg) was injected under the skin of the rat's neck. The camera was used to observe the rat in a dim and quiet environment for 30 min and the erected penile was recorded. Rats without penile erection within 30 min were judged to be DIED rats. An erection was counted when the glans was congested and exposed, the foreskin was receded, and the penis was enlarged.. Rats that did not develop penile erections within 30 min were judged to be DIED rats to perform further experiments. The rest of the rats without ED suffered from euthanasia.

ICP/MAP examination

Rats were anesthetized by chloral hydrate (0.3 ml/100 g). Then, we fully exposed the left carotid artery of rats and inserted a thin plastic cannula connecting the PE-50 tube to record MAP using the PowerLab (AD Instruments, AUS) data acquisition and analysis system. Another PE-50 tube was connected between the root of the corpus cavernosum using the PowerLab data acquisition and analysis system. Then, the bipolar electrode was hooked to the penile cavernous nerve at a distance of about 1 mm to record basic ICP. The cavernous nerve was electrically stimulated at 5 V at a frequency of 25 Hz and a pulse width of 2 ms for 1 min at intervals of at least 3 min. Then, we recorded the highest ICP and the highest ICP/MAP as an index to evaluate erectile function.

eNOS activity

eNOS activity was measured by Nitric Oxide Synthase Kit according to the protocol. The total activity of eNOS was calculated according to the standard curve.

RhoA activity

A Pulldown assay kit for RhoA activity was used to detect RhoA activity according to the protocol.[11] 500 μg of total protein was added to pull down the protein binding to Rhotekin. Then, we used an anti-RhoA antibody (Dilution ratio 1:500) to detect RhoA activity. The whole process was carried out in 4°C Odyssey FC imaging system (Li-COR Biosciences, USA), and Image J software was used for luminescence and to analyze the gray value of the band. The ratio of GTP-RhoA gray value/β-actin gray value was used to express RhoA activity.

Western blot

Penile tissues were lysed in cell lysis buffer for at least 30 min on ice. Then, the concentration by bicinchoninic acid was measured using the protein assay kit. 50 μg of total protein was added to perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto poly (vinylidene fluoride) (PVDF) membranes (Roche, USA). After blocking in 5% milk and incubating in primary antibodies, the membranes were washed. Then, membranes were incubated with secondary antibodies and treated with an enhanced chemiluminescence reagent. Odyssey FC imaging system (Li-COR Biosciences, USA) and Image J software were employed to obtain luminescence and to analyze the gray value of the band.

Ethical statement

This study was approved by the Animal Care and Use Committee of Hebei Medical University (No. 2021-AE037).

Statistical analysis

All the data were expressed as the mean ± SD, and SPSS (v21.0, IBM, US) statistical software was used to analyze the data. The one-way analysis of variance and SNK-q test were used to compare the data between groups. A comparison of the two groups was performed using the *t*-tests. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Metabolic parameters

Among the three groups, no significant differences were observed for initial body weight and serum fasting glucose concentration. Three days after the STZ injection, DIED and DIED + SA groups recovered with higher serum glucose and lower body weights. Eight weeks later, body weight in DIED + SA-L, DIED + SA-M, and DIED + SA-H groups was significantly lowered, while significantly higher fasting glucose concentrations were observed compared with the initial body weight and serum fasting glucose concentration in DIED [Table 1]. No significant differences were observed in body weights and serum fasting glucose concentrations between DIED group and DIED + SA group [Table 1].

Schisandrin A ameliorated ED in rats with DIED

After eight weeks since drug administration, the erectile rate in DIED + SA-M and DIED + SA-H groups increased significantly compared with DIED group [Table 2]. Further, we also measured ICP, MCP, and ICP/MAP in these five groups. Before electrical stimulation, no significant differences were observed between the groups of baselines ICP, MAP, and ICP/MAP. After electrical stimulation, ICP and ICP/MAP were increased in DIED + SA-L, DIED + SA-M, and DIED + SA-H groups compared with DIED groups [Figure 1 and Table 3]. Nevertheless, we

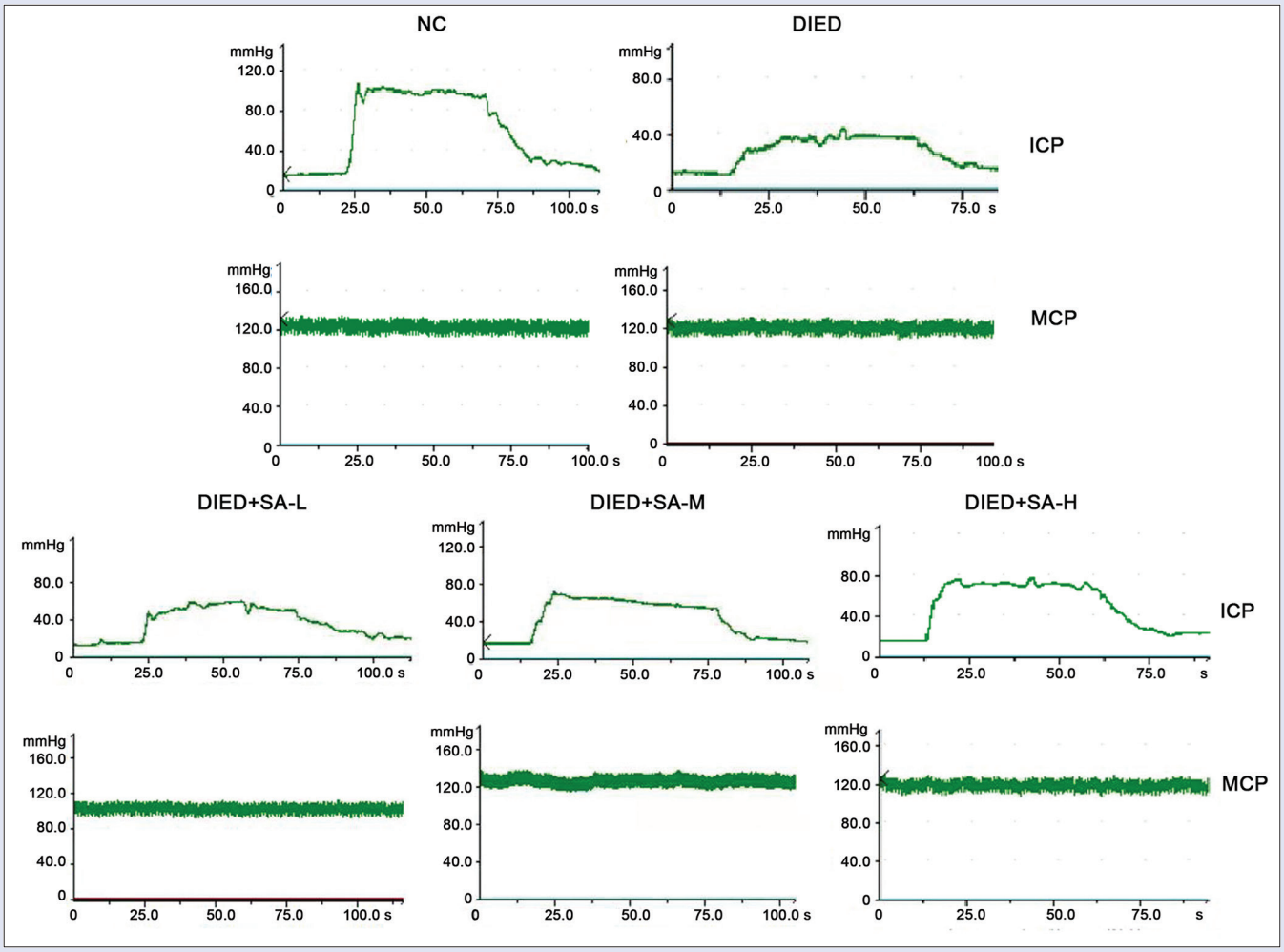

Figure 1: The ICP and MAP with the following stimulation parameters: 5 V, 25 Hz, 2 ms, and 1 min in three groups (NC, *n* = 8; DIED, *n* = 8; DIED+AS, *n* = 8)

Table 1: Body weights and blood glucose levels of experiment rats in different groups (±s)

Groups	Blood glucose (mmol/L)			Body weight (g)		
	0 week	4 weeks	8 weeks	0 week	4 weeks	8 weeks
NC	5.58±0.51	5.43±0.56	5.48±0.44	479.00±12.31	481.33±20.30	488.00±16.79
DIED	24.93±2.58*	24.33±3.15*	24.65±3.57*	282.00±22.17*	270.33±19.97*	258.83±22.92*
DIED + SA-L	24.33±2.97*	24.65±3.75*	24.65±3.29*	274.63±23.54*	263.13±24.12*	251.25±27.13*
DIED + SA-M	23.56±2.66*	24.36±3.85*	23.91±3.54*	281.17±12.44*	272.00±17.29*	264.17±16.05*
DIED + SA-H	23.77±3.11*	23.57±3.29*	23.70±3.64*	278.71±13.28*	271.86±22.28*	264.00±29.88*

NC is the normal control group, DIED is the group of diabetes-induced erection dysfunction, DIED + SA-L is DIED with schisandrin (5 mg/kg/d) group, DIED + SA-M is DIED with schisandrin (10 mg/kg/d) group, DIED + SA-H is DIED with schisandrin (20 mg/kg/d) group; Versus group NC,*$P<0.05$; Except for NC group, there were no significant differences in the levels of weight and blood sugar among other groups in a different time period ($P>0.05$)

did not observe significant differences in serum ALT, AST, ALP, SR, Na, K, and Ca levels in the serum of these five groups of rats [Tables 4 and 5]. These results indicated that schisandrin A could ameliorate ED in rats with diabetes mellitus.

Schisandrin A promoted eNOS and NO levels in penile tissues of DIED rats

The levels of eNOS and NO decreased significantly in the penile tissues of DIED group compared with those in the normal control (NC) group.

Table 2: Erectile function after intragastric administration in different groups (±s)

Groups	Number of rats with penile erection	Total number of rats	Penile erection ratio
NC	6	6	100%
DIED	0	6	0.0%
DIED + SA-L	0	8	0.0%
DIED + SA-M	1	6	16.6%
DIED + SA-H	2	7	28.5%

NC is normal control group, DIED is the group of diabetes-induced erection dysfunction, DIED + SA-L is DIED with schisandrin (5 mg/kg/d) group, DIED + SA-M is DIED with schisandrin (10 mg/kg/d) group, DIED + SA-H is DIED with schisandrin (20 mg/kg/d) group

Compared with DIED group, the phosphorylation of eNOS and the level of NO increased in DIED + SA-L, DIED + SA-M, and DIED + SA-H groups [Figure 2a-c]. The high dose of schisandrin A displayed is most effective. These results indicated that schisandrin A could release ED in rats with DM by increasing eNOS and NO levels.

Schisandrin A inhibited cavernous corpus fibrosis

The fibrosis of smooth muscle and collagen deposition induced ED. α-SMA and TGF-β1 levels were detected using western blot. The results showed that the expression of α-SMA was increased in the penile tissues of schisandrin A treated groups compared with those in DIED groups [Figure 3a and b]. The TGF-β1 expression decreased in schisandrin A treated groups [Figure 3a and c]. We also found that Collagen III and Collagen IV were decreased in schisandrin A treated groups compared with DIED group [Figure 3a, d, and e]. These results indicated that schisandrin A inhibited smooth muscle cell fibrosis and collagen deposition in the penile tissues of rats with DIED.

Schisandrin A inhibited RhoA/ROCK signal pathway

To detect RhoA activity, we used a Pulldown assay and western blot. The results showed that GTP-RhoA decreased significantly in the penile of Schisandrin A treated groups compared with those of the

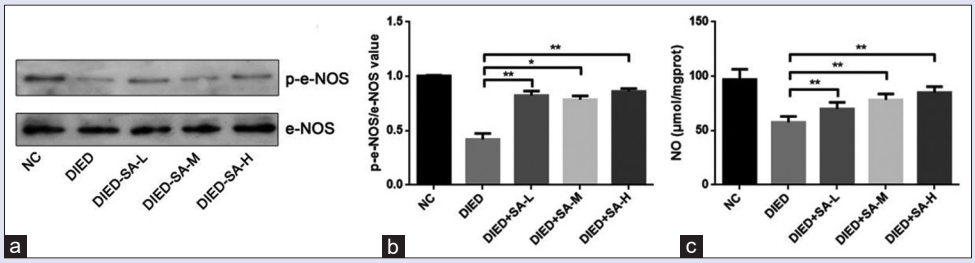

Figure 2: eNOS, NO, and p-eNOS levels in penile tissues of the three groups (NC, *n* = 8; DIED, *n* = 8; DMED + AS, *n* = 8). (ab) The expression of p-eNOS and eNOS in different groups. (c) The levels of NO in different groups

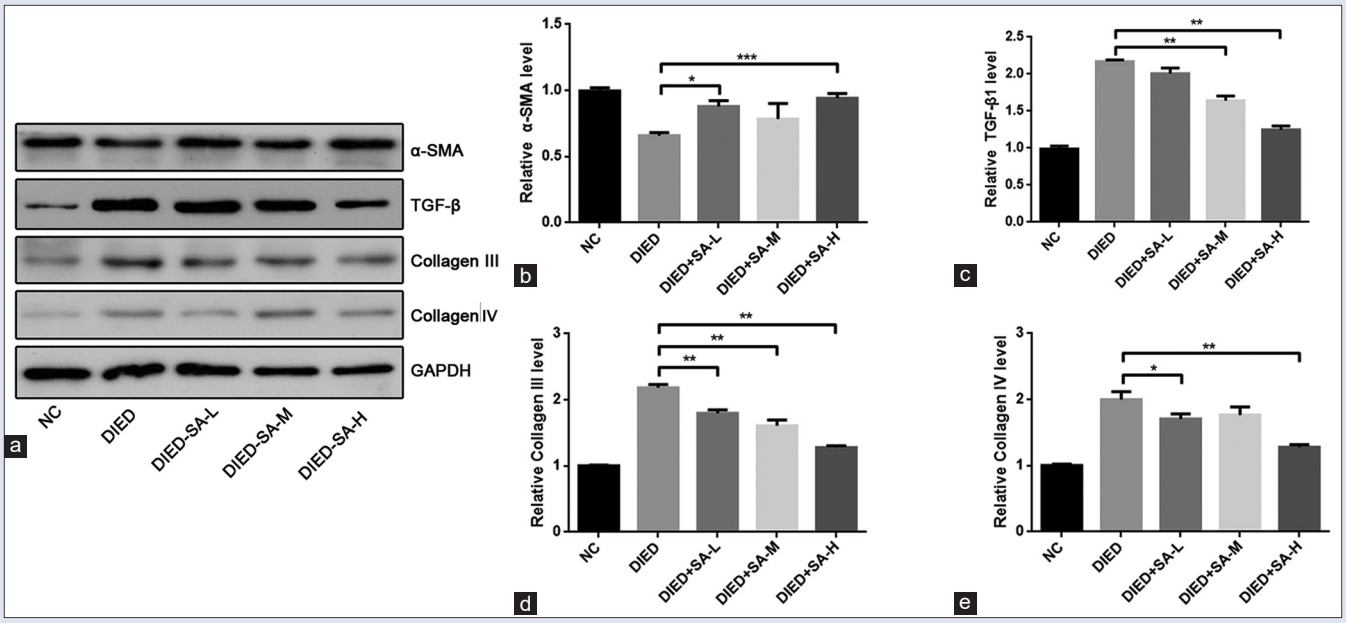

Figure 3: Fibrosis markers of the cavernous corpus. Western blot was used to detect the expression of a-SMA (ab), TGF-β1 (ac), Collagen III (ad), and Collagen IV (ae) in penile tissues of the three groups

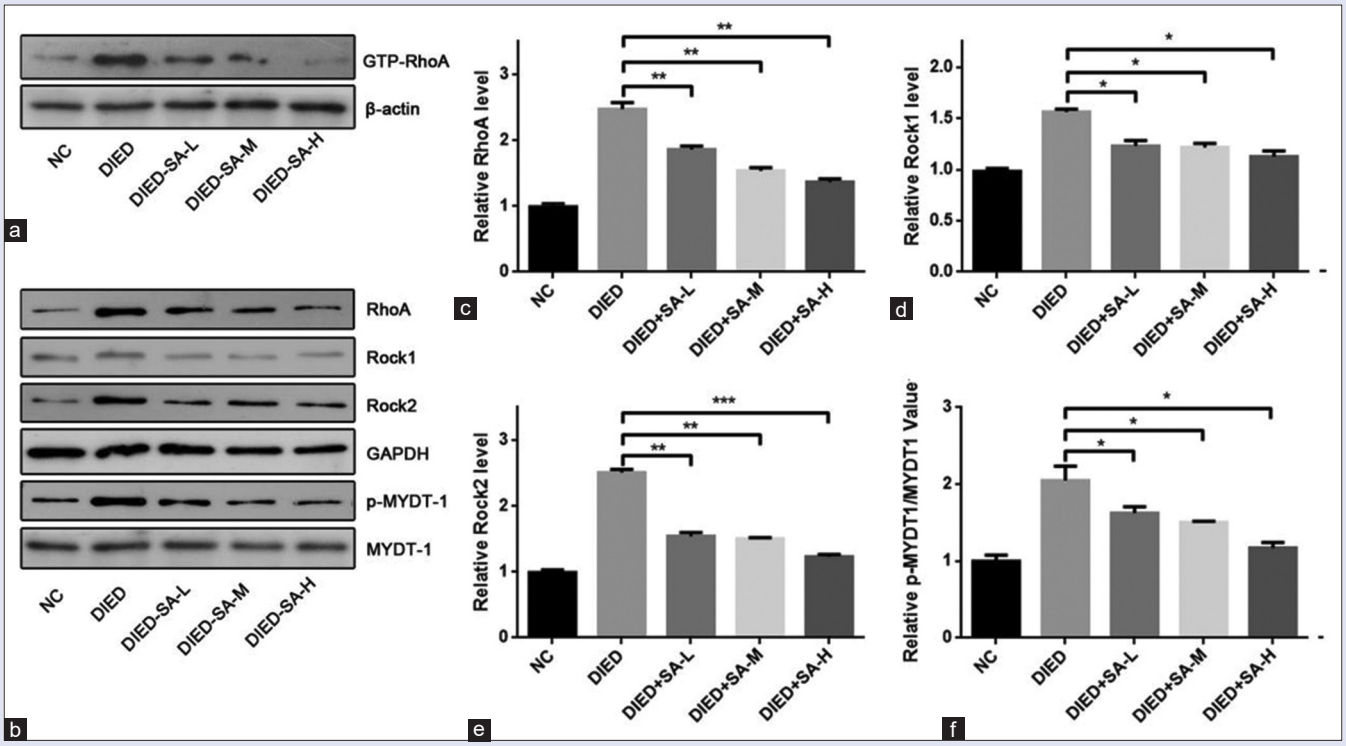

Figure 4: Expression of proteins in the RhoA/ROCK/p-MYPT1 pathway. (a) A Pulldown assay was used to detect the level of GTP-RhoA. (b-e) Western blot was used to detect the expression of RhoA (bc), ROCK1 (bd), and ROCK2 (be). (f) Western blot was used to detect the phosphorylation of MYPT1

Table 3: ICP and ICP/MAP by electrical stimulation of a cavernous nerve in different groups (±s)

Groups	ICP (mmHg)	MAP (mmHg)	ICP/MAP
NC	92.72±5.07	114.00±4.94	0.81±0.03
DIED	28.83±3.96*	109.27±9.28	0.26±0.03*
DIED + SA-L	35.28±3.76*#	111.90±8.81	0.31±0.03*
DIED + SA-M	45.01±5.47*#	110.14±8.61	0.40±0.02*#
DIED + SA-H	57.8±7.39*#¥	112.25±7.30	0.51±0.07*#¥

ICP is intracavernosal pressure; MAP is mean arterial pressure; NC is the normal control group, DIED is the group of diabetes-induced erection dysfunction, DIED + SA-L is DIED with schisandrin (5 mg/kg/d) group, DIED + SA-M is DIED with schisandrin (10 mg/kg/d) group, DIED + SA-H is DIED with schisandrin (20 mg/kg/d) group; There were no significant differences in the levels of MAP among the different groups (P>0.05) Note: Versus group NC, *P<0.001; Versus group DIED, #P<0.05; Versus group L-S, ¥P<0.001.

Table 4: The ALT, AST, ALP, and SR levels of the rats in different groups (±s)

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	SR (μmol/l)
NC	51.33±6.80	150.67±20.55	115.33±24.58	50.00±7.21
DIED	51.67±6.65	162.00±23.25	116.7±13.05	51.33±3.78
DIED + SA-L	52.67±5.03	154.00±25.51	109.67±15.17	48.33±7.76
DIED + SA-M	52.33±4.50	152.67±22.36	111.67±19.14	53.33±4.04
DIED + SA-H	54.00±5.29	153.33±17.03	114.33±17.03	50.67±5.68

NC is normal control group, DIED is the group of diabetes-induced erection dysfunction, DIED + SA-L is DIED with schisandrin (5 mg/kg/d) group, DIED + SA-M is DIED with schisandrin (10 mg/kg/d) group, DIED + SA-H is DIED with schisandrin (20 mg/kg/d) group. There were no significant differences in the levels of ALT, AST, ALP, and SR among the different groups (P>0.05)

DIED group [Figure 4a]. Western blot showed that the expression of RhoA, ROCK1, and ROCK2 decreased in schisandrin A treated groups compared to DIED group and the high dose of schisandrin A displayed is most effective [Figure 4b-e]. Nevertheless, we observed that schisandrin A inhibited phosphorylation of MYPT1 [Figure 4f], which suggested that schisandrin A repaired the ED of diabetic rats by activating the RhoA/ROCK signal pathway.

DISCUSSION

Recent studies have revealed that dysregulation of eNOS and fibrosis of the corpus cavernosum are the major causes of DIED. The first-line drug treatment for ED, such as PDE5i exhibited little benefit in patients with DIED. Thus, exploring novel drugs for DIED is of great significance in ameliorating ED in diabetes patients. The main findings related to the present study are as follows: i) Schisandrin A in a high dose (20 mg/kg/d) could ameliorate ED in rats with DIED. ii) Schisandrin A promoted eNOS and NO levels, and inhibited fibrosis in penile tissues of DIED rats. iii) Schisandrin A inhibited the RhoA/ROCK signal pathway.

Schisandrin A is one of many components present in *Schisandra chinensis*. It has multifold functions and acts as an anti-inflammatory and anti-tumor agent, possesses antioxidant activity, and protects the liver.[9] Moreover, it serves as a sedative and hypnotic and also regulates the immune system. Previous studies have found that schisandrin A has a significant protective effect in endothelial cells damaged by hypoxia/reoxygenation, by increasing intracellular NO levels, reducing MDA levels, and preventing the generation of ROS.[7] Other studies have found that schisandrin A enhances the relaxation of the corpus cavernosum pretreated with sildenafil, suggesting that schisandrin A is beneficial for the recovery of ED.[9] Thus, we constructed a DIED model and administered low, middle, or high doses of schisandrin A to rats. It was carried out to observe the roles of schisandrin A on DIED, and further explore the related mechanism. We found that the erectile rate of schisandrin A treated rats with DIED was partly rescued, which is consistent with the ICP, MAP, and ICP/MAP values.

Table 5: The Na, K, and Ca levels of the rats in different groups (±s)

Groups	Na (mmol/L)	K (mmol/L)	Ca (mmol/L)
NC	88.91±5.47	5.82±0.28	1.05±0.06
DIED	86.01±9.44	5.97±0.52	1.03±0.04
DIED + SA-L	85.51±10.38	5.66±0.41	1.02±0.04
DIED + SA-M	81.11±3.67	5.62±0.05	1.11±0.04
DIED + SA-H	91.86±4.52	5.63±0.23	1.07±0.07

NC is normal control group, DIED is the group of diabetes-induced erection dysfunction, DIED +SA-L is DIED with schisandrin (5 mg/kg/d) group, DIED + SA-M is DIED with schisandrin (10 mg/kg/d) group, DIED + SA-H is DIED with schisandrin (20 mg/kg/d) group. There were no significant differences in the levels of Na, K, and Ca among the different groups (P>0.05)

The mechanisms of ED include increased contractility of the SMCs in the corpus cavernosum,[12] endothelial cells dysfunction,[13] down-regulation of NOS[14] in penile tissues, and increased collagen deposition.[15] nNOS and eNOS play a major role in the initiation and maintenance of a penile erection. Released NO, an important neurotransmitter for penile erection diffuses freely into the CCMSCs. This activates the guanylate cyclase in the CCMSCs and increases cGMP production to induce a relaxed cavernous body which ultimately leads to the erection of the penile.[16] Considering that schisandrin A enhances the relaxation of the corpus cavernosum in rabbits, we explored whether schisandrin A recovered DIED by releasing NO. Our results identified that schisandrin A could rescue the inhibition of eNOS and NO levels in DIED group, which further confirmed the function of schisandrin A on DIED. Schisandrin A with 20 mg/kg/d was the most effective.

Fibrosis is an important pathological manifestation of ED. It leads to the reduction of SMCs and excessive production of extracellular matrix and collagen fibers.[17] Fibrosis exists in many diabetic complications, such as cardiomyopathy and nephropathy.[18] Generally, a-SMA is used to evaluate the ratio of cavernosal smooth muscle cells, as it is considered to be a myofibroblast marker.[19] Whereas, TGF-β1 is an important fibrosis factor to evaluate the fibrosis in the corpus cavernosum.[20] Similar to the previous study, we found increased expression of TGF-β1, while, a-SMA decreased in penile tissues of rats with DIED. Compared with the DIED group, schisandrin A with the concentration of 20 mg/kg/d decreased the TGF-β1 and increased a-SMA effectively. Moreover, we also found that schisandrin A decreased Collagen III and Collagen IV expression. This indicated that schisandrin A rescues ED by reducing corporal fibrosis.

RhoA/ROCK pathway activation is supposed to be an important pathway to maintain penile flaccidity. RhoA is a small G protein with GTPase activity.[21] When GDP-RhoA is converted to GTP-RhoA, GTP-RhoA activates ROCK. Then, the ROCK pathway maintains the contractivity of CCSMCs.[22] In the present study, our results suggested that schisandrin A ameliorates DIED by inhibiting the RhoA/ROCK pathway.

In our previous study, we showed schisandrin A promoted eNOS expression and inhibited the fibrosis of CCSMCs to ameliorate DM-induced ED by RhoA/ROCK signal pathway. However, the detailed mechanisms of schisandrin A ameliorate DM-induced ED need to be further explored. This study was the first study to explore the roles and mechanisms of schisandrin A in the treatment of DIED. However, the mechanism is limited and further studies should be performed to clarify the mechanism of schisandrin A in the treatment of DIED.

CONCLUSION

Schisandrin A ameliorated ED in rats with DIED by promoting eNOS production, inhibiting fibrosis, and inhibiting the RhoA/ROCK1 pathway. Nevertheless, our results provided a novel therapeutic option for the treatment of DIED.

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Conflicts of interest

There are no conflicts of interest.

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