1. Introduction

The incidence of diabetes worldwide is increasing year by year. Erectile dysfunction (ED) in men is a familiar diabetic complication characterized by the inability to achieve or maintain a prolonged erection, resulting in men not getting satisfactory results from sexual intercourse [1]. ED typically becomes more severe with elevated duration of diabetes onset, with the prevalence of ED being approximately 15 percent among diabetic patients under age of 30 and 55 percent after age of 60 [2]. The pathogenesis of diabetic ED involves multiple factors, such as weakened central nervous system stimulation, changes in penile endothelial function, etc. [3]. Among them, endothelial function changes are considered a crucial feature of diabetic ED. Hyperglycemia stimulates the formation of advanced glycation end products (AGEs) and oxidative stress, mediates smooth muscle cell death and disrupts vascular homeostasis in penile functional responses [4, 5]. This induces dysfunction of smooth muscle contraction and reduces corpus cavernosum relaxation, hypopemia, and erectile function [6]. The present cure for diabetic ED is mainly controlled by oral phosphodiesterase type 5 (PDE5) inhibitors [7]. However, it has many drawbacks with safety implications [8]. Therefore, it is imperative to seek a safe and efficient treatment for diabetic ED.

MicroRNAs (miRNAs) with their unique expression patterns can be applied as disease biomarkers [9, 10]. It turns out miRNAs take on a key role in diabetes or ED [11, 12]. MiR-503-5p, a tumor suppressor gene, is reduced in colorectal and cervical cancer [13, 14]. A recent study reports miR-503-5p is clearly associated with diabetes and arterial stenosis, and could affect vascular smooth muscle cell proliferation [15]. Therefore, it was speculated that miR-503-5p might control ED by affecting the viability of penile smooth muscle cells.

This research studied miR-503-5p and its target SYDE2 in diabetic ED and identified a positive role of this axis in disease treatment.
2.2. Establishment of animal models

The ED model was established with reference to the former method [16], and eight-week-old male Sprague-Dawley rats of specific pathogen-free grade (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai) were chosen and maintained at 24 ± 2°C with humidity of 60±5% and 12-h light per day. All rats had free access to clean drinking water and maintenance feed (H10010, Huafukang Biotechnology Co., Ltd., Beijing). 10 mg/mL streptozotocin (STZ), prepared with sodium citrate, was injected into the left lower abdominal cavity at 60 mg/kg once a week (w). After 4 w, the injection was reduced to once every 2 w for 12 w. After each injection, blood was collected from the caudal vein to measure blood glucose (BG). Erection (congestion, growth, and exposure) was observed when 100 μg/kg apomorphine (saline + 0.5% vitamin C) was injected into the skin of the cervix of rats. Rats with BG >16.7 mol/L and no erectile function were considered diabetic ED rats. Conversely, rats with BG values >16.7 mol/L but no change in erectile function were considered normal diabetic rats.

2.3. Grouping of rats

Eighty rats were equally assigned into 8 groups, 10 in each group: the Control, the ED, the ED+miR-503-5p mimic, the ED+NC mimic, the ED + pPLK-shRNA-NC, the ED + pPLK-shRNA-SYDE2, the ED+miR-503-5p mimic + pLenti-SYDE, the ED+miR-503-5p+oe-NC. To elevate or reduce miR-503-5p and SYDE2, ED rats were injected with miR-503-5p/NC mimic (0.5 mg/mL, 2 mL) or shRNA lentiviral plasmid vector targeting SYDE2 and elevated lentiviral plasmid vector and corresponding negative control (pPLK-shRNA-SYDE2, pPLK-sh-NC, pLenti-SYDE2, pLenti-NC, 2 × 10⁶ TU/ml) into the tail vein for 1 week. After 4 w, the rats were euthanized and samples were collected for subsequent experiments. The rats in the control and the ED were injected with the same dose of normal saline. MiR-503-5p mimic and its NC were bought from RiboBio (Guangzhou, China). SYDE2-targeting siRNA and elevation plasmids and NCs were gained from GenePharma (Shanghai, China).

2.4. Intracavernous pressure (ICP)/mean arterial pressure (MAP)

ICP and MAP were evaluated as set forth [17]. The skin of the rat cervix was cut open and the carotid artery was inserted. Then, the pelvic ganglia and cavernous nerves of the penis were exposed and stimulated with a bipolar hook electrode (5.0 mV, 20 Hz, 5 ms, for 1 min). 23 G syringe needle containing 250 U/mL heparin was inserted into the calf of the penis. Pressure sensors were used to measure MAP and ICP values, and ICP/MAP values were calculated to assess erectile function.

2.5. Hematoxylin-eosin (HE) staining

Fixation with 4% paraformaldehyde and dehydration of penile tissue with graded concentrations of ethanol were implemented. Washing of tissues with xylene, waxing, embedding in paraffin, and slicing (approximately 4 μm thickness) of tissues were put into effect. Dewaxing of the sections with ethanol and preparation into tissue sections for HE staining were conducted. Staining of sections with hematoxylin and eosin separately, dehydration with ethanol, clearing with xylene, and seal with neutral glue were implemented. The tissue morphology of the penis was observed under an optical microscope, and 20 fields of view were customarily chosen to detect the cavernous sinus capillaries covering red blood cells. Calculation of the capillary density (capillaries/mm²) was implemented.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cyclic guanosine monophosphate (cGMP) and AGEs in penile tissue were measured with ELISA kits (Assay Designs, Ann Arbor, MI, USA). The optical density value was read at 450 nm on a microplate reader (Thermo Fisher Scientific, USA).

2.7. Isolation of corpus cavernosum smooth muscle cells (CCSMC)

Isolation of CCSMCs was from rat penis as set forth [PMID: 29603682]. The isolated CCSMCs were cultivated in DMEM supplemented with 10% FBS. Differential adhesion was used to eliminate other cells and only CCSMCs were present. The positive rate of α-SMA was tested by flow cytometry to identify CCSMCs.

2.8. Flow cytometry detection of apoptosis

Rat CCSMCs (10⁴ cells/mL) were mixed with 500 μL of binding buffer, treated with 5 μL Annexin-V-FITC and 5 μL PI (Sigma-Aldrich, MO, USA), and examined within 1 h by a FACSCalibur flow cytometer (BD Biosciences, NJ, USA).

2.9. RT-qPCR

After isolation of total RNA using TRizol reagent, synthesis of cDNA was implemented by applying a reverse transcription kit (Sangon, China) or microRNA reverse transcription kit (RiboBio). Quantitative PCR was performed by applying qPCR Master Mix (Promega, USA). Relative mRNA and miRNA were calculated in the light of the 2⁻ΔΔCt method. The primer sequence was clarified in Table 1.

2.10. Western blot

Briefly, lysis of total protein was with lysis buffer covering protease inhibitors. Separation of tissue protein samples on 10% SDS-PAGE and complete transfer to PVDF membranes (Millipore, USA) were conducted. After incubation with primary antibodies, incubation of membranes was with goat anti-rabbit Immunoglobulin G and maintained at 24 ± 2℃ with humidity of 60±5%. The tissue morphology of the penis was observed under an optical microscope, and 20 fields of view were customarily chosen to detect the cavernous sinus capillaries covering red blood cells. Calculation of the capillary density (capillaries/mm²) was implemented.

Table 1. RT-qPCR primer sequence.

<table>
<thead>
<tr>
<th>Primer sequence (5' - 3')</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5’-ATGGGGAAGGTTAGAGTCG-3'</td>
<td>R: 5’-TTACTCCTTTGAGGATG-3'</td>
</tr>
<tr>
<td>U6</td>
<td>F: 5’-CTTCGTCGTCGCCATAGATAT-3'</td>
<td>R: 5’-ACGGCCTAAGTGGCCCT-3'</td>
</tr>
<tr>
<td>MiR-503-5p</td>
<td>F: 5’-TGTTACACCGTTCG-3'</td>
<td>R: 5’-TGCTGTTGCCATAGAT-3'</td>
</tr>
<tr>
<td>SYDE2</td>
<td>F: 5’-ACACGTACAGTGGCCCT-3'</td>
<td>R: 5’-CAGCCTCAGATGCCCT-3'</td>
</tr>
</tbody>
</table>

Note: F, forward; R, reverse.
MicroRNA-503-5p protects ED by SYDE2.


teinSimple, USA). Primary antibodies applied in this study included GAPDH (sc-32233, 1: 1000, Santa Cruz Biotechnology), SYDE2 (NBP1-94015, 1: 1000, Novus Biologicals).

2.11. Immunofluorescence

Replenishment of 3% H₂O₂ to block endogenous enzymes was conducted. Boiling penile tissue sections in antigen retrieval buffer, cooling, and repeating the heating-cooling operation twice were implemented. Blocking sections were with 5% BSA to block nonspecific antibody binding. After removing excess liquid, incubation of sections was with antibody SYDE2 (NBP1-94015, 1: 1000, Novus Biologicals), and Alexa Fluor 594-conjugated secondary antibody. Incubation of cells was with 300 nM DAPI (Sigma-Aldrich, St. Louis, MO, USA) for nuclear counterstaining. Fluorescence images were acquired under a Nikon confocal microscope (Eclipse TE2000U).

2.12. Luciferase activity assay

Cloning wild-type and mutant SYDE2 (WT/MUT-SYDE2) was into pmirGLO dual-luciferase vector (GenePharma) to construct dual-luciferase reporter plasmids. Applying Lipofectamine 2000, co-transfection of HEK293T cells was with wild-type/mutant pmirGLO-SYDE2 and miR-503-5p mimic (or NC). Luciferase activity was analyzed after transfection by applying a dual luciferase reporting kit (Promega, USA).

2.13. Data statistics

Manifestation of the data was as mean ± standard deviation (SD). Student's t-test was applied to determine differences between two groups. Prism Software 8.0 (GraphPad Software, US) was employed to analyze data. * P < 0.05 emphasized obvious statistical meaning. The significance among multiple groups was calculated by applying one-way analysis of variance, and the variance was corrected using the Tukey test. * P < 0.05.

3. Results

3.1. MiR-503-5p is reduced in diabetic ED

To figure out miR-503-5p's role in diabetic ED, it induced diabetic ED by injecting streptozotocin. As clarified in Fig. 1A, the erection rate was reduced in the ED vs. the Control. Meanwhile, the ICP/MAP ratio of the ED declined vs. the Control (Fig. 1B). It came out the penile capillary density of the rats in the ED was distinctly declined vs. the Control (Fig. 1C). The cGMP and AGEs have been shown to be associated with the pathogenesis of ED [18, 19]. It turned out in the comparison with the control, cGMP in the ED was decreased but AGEs were elevated (Fig. 1D). To determine CCSMC apoptosis, CCSMCs were separated from the corpus cavernosum and the isolated cells were identified as positive for α-SMC (Fig. 1E). Moreover, it came out that apoptosis rate of CCSMCs in the ED was elevated vs. the Control (Fig. 1F). This indicated it was successfully established a diabetic ED model by injecting streptozotocin. Subsequently, it was detected that miR-503-5p, as clarified in Fig. 1G, miR-503-5p in the ED rats was declined vs. rats in the Control. These results suggest miR-503-5p is reduced in streptozotocin-induced diabetic ED.

3.2. Elevating miR-503-5p mitigates ED in rats

To figure out miR-503-5p's role in sexual dysfunction in diabetic rats, it was first examined miR-503-5p. miR-503-5p was apparently elevated after injection of miR-503-5p mimic (Fig. 2A). Subsequently, it was examined the effect of elevating miR-503-5p on ED in rats. After treatment with miR-503-5p mimic, the erection rate of ED rats was clearly increased and the ICP/MAP ratio was effectively restored (Fig. 2B-C). The results clarified (Fig. 2D), the penile capillary density of ED rats was distinctly elevated after miR-503-5p mimic treatment. Meanwhile, elevating miR-503-5p memorably enhanced cGMP but declined AGEs in the penis of ED rats (Fig. 2E). Moreover, elevating miR-503-5p also reduced the apoptosis rate of CCSMCs in ED rats (Fig. 2F). Taken together, these results suggest elevating miR-503-5p can effectively treat ED in diabetic rats.

3.3. SYDE2 is elevated in ED and a target gene of miR-503-5p

Bioinformatics website http://starbase.sysu.edu.cn/ discovered SYDE2 and miR-503-5p's latent binding sites (Fig. 3A). For further confirming the direct targeting link between SYDE2 and miR-503-5p, it was conducted the luciferase activity assay. WT SYDE2 clearly reduced the dual luciferase activity in the miR-503-5p mimic, while mutant SYDE2 had no clear effect on the luciferase activity in the miR-503-5p mimic (Fig. 3B). Moreover, it was found that SYDE2 was clearly increased in the ED vs. the Control, while treatment with miR-503-5p mimic significantly decreased SYDE2 (Fig. 3C-D). These results sug-

![Fig. 1. MiR-503-5p is reduced in diabetic ED. A: Erection rate; B: ICP/MAP ratio; C: Representative images of penile HE staining and capillary density; D: ELISA detection of cGMP and AGEs in the penile tissues; E: The positive rate of α-SMA in the isolated cells identified by flow cytometry; F: CCSMC apoptosis rate tested by flow cytometry; G: RT-qPCR detection of miR-503-5p; Apart from E, A-G, in the rats of the Control and the ED, n = 10.](image1)

![Fig. 2. E elevating miR-503-5p mitigates ED in rats. A: RT-qPCR detection of miR-503-5p; B: The erection rate; C: ICP/MAP ratios; D: Representative images and capillaries’ density of penile HE staining; E: ELISA detection of cGMP and AGEs in penile tissue; F: Flow cytometry detection of apoptosis rate of CCSMCs; A-F, in the rats of the ED+NC mimic and the ED+miR-503-5p mimic, n = 10.](image2)
gest SYDE2 is elevated in diabetic ED and is targeted by miR-503-5p.

3.4. Silencing SYDE2 helps to improve ED in diabetic rats

To further demonstrate the role of SYDE2 in diabetic ED, it was silenced SYDE2 in ED rats (Fig. 4A-B). In Fig. 4C, after silencing SYDE2, the erection rate in ED rats was clearly elevated. Furthermore, silencing SYDE2 clearly increased the ICP/MAP ratio in ED rats (Fig. 4D). Moreover, after silencing SYDE2, the capillary density in the penis of ED rats was significantly increased (Fig. 4E). And silencing of SYDE2 resulted in a significant elevation in cGMP and an apparent reduction in AGEs in penile tissue (Fig. 4F). Moreover, silencing SYDE2 also reduced the apoptosis rate of CCSMCs in ED rats (Fig. 4G). This suggests silencing SYDE2 can effectively improve ED in diabetic rats.

3.5. MiR-503-5p/SYDE2 axis plays a key role in diabetic ED

Subsequently, it was explored whether SYDE2 participates in the regulation of miR-503-5p in diabetic ED. It was elevated SYDE2 with strengthening miR-503-5p in ED rats. As clarified in Fig. 5A-B, SYDE2 was memorably elevated in ED rats after injection of miR-503-5p mimic and oe-SYDE2 mixture. Moreover, vs. the ED+miR-503-5p mimic + pLenti-NC, the erection rate, ratio of ICP/ MAP and capillary density of the rats in the ED+miR-503-5p mimic + pLenti-SYDE were significantly reduced, and cGMP in the penis tissue was clearly declined and AGEs was apparently elevated, in addition, the apoptosis rate of CCSMCs was significantly increased (Fig. 5C-G). These results suggest miR-503-5p influences the development of ED in diabetes by regulating SYDE2.

4. Discussion

ED often takes place as a complication of atherosclerosis, diabetes and other diseases [20, 21]. Therefore, in this study, streptozotocin was applied to induce and establish a diabetic ED rat model in order to explore potential molecular targets for the treatment of ED. The results clarified that miR-503-5p was reduced in diabetic ED, and elevating miR-503-5p could improve diabetic ED by depressing SYDE2.

The mechanism of action of miRNAs in ED in diabetes is not fully understood. Previous studies have exhibited that miR-205 is associated with the pathogenesis of diabetic ED by downregulating androgen receptor expression [22]. Furthermore, Li et al. report that miR-328 could ameliorate diabetic ED by regulating cGMP and AGEs in penile tissue [11]. This is consistent with the results. The cGMP can induce relaxation of cavernous smooth muscle
MicroRNA-503-5p protects ED by SYDE2.


Ji-Kan R, Hai-Rong J, Guo N Y, et al., Erectile dysfunction pre-


