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ORIGINAL ARTICLE

Erectile Function

Calpain inhibition improves erectile function in diabetic mice via upregulating endothelial nitric oxide synthase expression and reducing apoptosis

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Calpain activation contributes to hyperglycemia-induced endothelial dysfunction and apoptosis. This study was designed to investigate the role of calpain inhibition in improving diabetic erectile dysfunction (ED) in mice. Thirty-eight-week-old male C57BL/6J mice were divided into three groups: (1) nondiabetic control group, (2) diabetic mice + vehicle group, and (3) diabetic mice + MDL28170 (an inhibitor of calpain) group. Type 1 diabetes was induced by intraperitoneal injection of streptozotocin at 60 mg kg⁻¹ body weight for 5 consecutive days. Thirteen weeks later, diabetic mice were treated with MDL28170 or vehicle for 4 weeks. The erectile function was assessed by electrical stimulation of the cavernous nerve. Penile tissues were collected for measurement of calpain activity and the endothelial nitric oxide synthase (eNOS)-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway. Terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) staining was used to evaluate apoptosis. Caspase-3 expression and activity were also measured to determine apoptosis. Our results showed that erectile function was enhanced by MDL28170 treatment in diabetic mice compared with the vehicle diabetic group. No differences in calpain-1 and calpain-2 expressions were observed among the three groups. However, calpain activity was increased in the diabetic group and reduced by MDL28170. The eNOS-NO-cGMP pathway was upregulated by MDL28170 treatment in diabetic mice. Additionally, MDL28170 could attenuate apoptosis and increase the endothelium and smooth muscle levels in corpus cavernosum. Inhibition of calpain could improve erectile function, probably by upregulating the eNOS-NO-cGMP pathway and reducing apoptosis.

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INTRODUCTION

Erectile dysfunction (ED) is a common complication of diabetes mellitus (DM) and can notably reduce patients' quality of life.¹ The prevalence of ED in diabetic patients is reported to be three times higher than that in nondiabetic patients.² Moreover, diabetic ED is more severe and refractory compared with normal ED.³ Phosphodiesterase type 5 inhibitors are the first-line treatment for ED and are effective for most patients with ED. However, the efficacy of this treatment is much lower for diabetic ED, which was probably associated with the decreased production of nitric oxide (NO) resulting from endothelial dysfunction.^{4,5}

NO promotes the production of cyclic guanosine monophosphate (cGMP) in cavernosal smooth muscle cells, resulting in the relaxation of smooth muscle and subsequent penile erection.⁶ Endothelial nitric oxide synthase (eNOS) is a major source of NO in the corpus cavernosum.^{7,8} Endothelial dysfunction was reported to occur in both diabetic patients and diabetic animal models.^{9,10} The development of diabetic endothelial dysfunction is multifactorial, and calpain may play a critical role in this disorder.¹¹

Calpain is calcium-dependent cysteine protease that participate in various biological processes through the proteolysis of substrate

proteins. Fifteen members of the calpain protein family have been reported in mammals. Calpain-1 (or μ -calpain) and calpain-2 (or m-calpain) are two major isoforms and are ubiquitously expressed, whereas the other isoforms show tissue-specific expression.¹² It was reported that calpain-1 was activated in diabetic animals and inhibition of calpain-1 could rescue diabetes-induced endothelial dysfunction. Calpain can reduce NO production in endothelium via proteolysis of eNOS and other proteins related to eNOS activation, such as heat shock protein 90 (Hsp90) and Akt.^{13–17} Additionally, calpain activation resulted in an increase in the production of reactive oxygen species (ROS) in aortas of diabetic mice.¹⁸ Excessive ROS production could induce eNOS uncoupling, reducing the formation of NO.¹⁹ Hence, we speculated that calpain activation might play a role in the development of diabetic ED.

A previous study showed that calpain inhibition by the calpain inhibitor MDL28170 could preserve the erectile response and neuronal nitric oxide synthase expression in rats suffering from cavernous nerve injury.²⁰ However, whether calpain inhibition can ameliorate diabetic ED has not yet been studied. In the present study, we used a diabetic model of mice induced with streptozotocin to investigate the effect of

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calpain inhibition on diabetic ED and explore the possible underlying mechanism.

MATERIALS AND METHODS

Treatment of animals

The study was approved by the Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Thirty-eight-week-old male specific-pathogen-free C57BL/6J mice obtained from the Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology, were randomly divided into three groups: nondiabetic control ($n = 10$), diabetic mice treated with vehicle (a 1:1 solution of DMSO [dimethyl sulfoxide]: PBS [phosphate buffer saline]; $n = 10$), and diabetic mice treated with MDL28170 (Sigma-Aldrich, M6690, St. Louis, MO, USA; dissolved in a 1:1 solution of DMSO: PBS at a concentration of 2 g l^{-1} ; $n = 10$). All mice were kept under 12-h light/dark conditions. Type 1 diabetes was induced by intraperitoneal injection of streptozotocin (Sigma-Aldrich, S0130) at 60 mg kg^{-1} body weight for 5 consecutive days. Blood glucose levels were measured 1 week after the last injection and mice with a fasting glucose concentration $\geq 16.7 \text{ mmol l}^{-1}$ were considered as diabetic mice. The fasting blood glucose and body weight of all mice were then measured once every 4 weeks. Twelve weeks later, diabetic mice were injected intraperitoneally with 20 mg kg^{-1} body weight MDL28170 or vehicle daily for 4 weeks according to the grouping.²¹

Evaluation of erectile function

After MDL28170 or vehicle treatment, the maximal intracavernosal pressure (MIP) and mean arterial pressure (MAP) were measured in all groups. Mice were anesthetized with pentobarbital sodium (Sigma-Aldrich, P3761; 60 mg kg^{-1} body weight) and fixed on the table in supine position. The left carotid artery was catheterized with a PE-10 tube filled with heparinized saline (100 IU ml^{-1}) and connected to a pressure transducer to measure the arterial pressure. A 29-G needle was inserted into the middle of the right penile shaft to monitor the MIP. The cavernous nerves were exposed and stimulated with a bipolar electrode at 5 V, 15 Hz, and 1.2 ms to induce erection.²² Pressure data were recorded by the BL-420F biological signal acquisition and processing system (Techman Soft, Chengdu, China). The ratio of MIP to MAP (MIP/MAP) was calculated to assess the erectile function. The mice were then sacrificed and penes were harvested for subsequent studies.

Western blot

The cryopreserved penile tissue was minced and incubated in lysis buffer on ice to extract proteins. Protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, P0012, Shanghai, China). $40 \mu\text{g}$ protein lysate of each sample was loaded on 10% sodium dodecyl sulfate/polyacrylamide (SDS-PAGE) gel for electrophoresis at 60 V for 30 min and 110 V for 60 min. Then, the proteins on the gel were transferred to polyvinylidene difluoride membrane at 200 mA for 2 h. After being blocked with 5% bovine serum albumin (BSA) for 2 h, the membranes were incubated with the following antibodies at 4°C overnight: spectrin α -II (1:100, sc-48382, Santa Cruz Biotechnology, Dallas, TX, USA); calpain-1 (1:500, BA0679, Boster, Wuhan, China); calpain-2 (1:500, BA1575, Boster); eNOS (1:500, 610296, BD Biosciences, San Jose, CA, USA); phospho-eNOS (Ser1177; 1:1000, 9570, CST, Danvers, MA, USA); caspase-3 (1:1000, 9665, CST); cleaved caspase-3 (1:1000, 9664, CST); or β -actin (1:500, BM0627, Boster). Membranes were washed and incubated with horseradish peroxidase-conjugated

secondary antibodies (1:1000, CST, 7074, 7076) at room temperature for 1 h. Protein bands were detected with Bio-Rad Clarity Western ECL Substrate (1705061, Bio-Rad Laboratories, Hercules, CA, USA). Densitometry values were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA).

Calpain activity detection

Calpain activity was measured using the fluorescence substrate N-succinyl-LLVY-AMC (13453, AAT Bioquest, Sunnyvale, CA, USA) as described previously.¹⁸ Briefly, penile protein was extracted and protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). N-succinyl-LLVY-AMC was added to the protein extracts at the final concentration of $30 \mu\text{mol l}^{-1}$. After incubation for 1 h, the fluorescence was detected using a fluorescent microplate reader at excitation $\lambda 351 \text{ nm}$ and emission $\lambda 430 \text{ nm}$. Calpain activity of each sample was normalized by the protein concentration.

Western blot was also performed to detect the 145 kDa stable breakdown products (SBDP) of spectrin α -II using a spectrin α -II antibody (Santa Cruz Biotechnology) as described above. SBDP is a calpain-specific cleavage product and can be used to quantify calpain activity.²¹

NO content analysis

NO content in the corpus cavernosum was indirectly detected using a total NO assay kit (Beyotime Institute of Biotechnology, S0023). NO in tissues is metabolized into nitrate and nitrite, and thus the concentration of nitrate and nitrite can reflect the level of NO. Briefly, the penis lysates were incubated with nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and nitrate reductase at 30°C for 30 min, followed by the addition of lactate dehydrogenase (LDH). After 30 min incubation, Griess Reagents were added and the absorbance at 540 nm of each sample was measured with a microplate reader (Thermo, Waltham, MA, USA). NO level was calculated according to the standard curve and normalized by protein concentration.

cGMP concentration measurement

The cGMP concentration in penes was measured with an enzyme-linked immunosorbent assay kit (KGE003, R and D Systems, Minneapolis, MN, USA) following the manufacturer's instruction. The cGMP concentration of each sample was normalized by protein concentration.

Caspase-3 activity analysis

Caspase-3 activity in the penile tissue was detected by caspase-3 Activity Assay Kit (Beyotime, C1116) according to the manufacturer's instruction. Briefly, tissues were grinded and then incubated in cold lysis buffer on the ice for 15 min. The lysed tissues were subsequently centrifuged at $16\,000 \text{ g}$ for 10 min at 4°C and the supernate was collected. Protein concentration was measured by the Bradford Protein Assay Kit (Beyotime, P0006C). The supernatant was transferred to a 96-well plate containing detection buffer, and then Ac-DEVD-pNA was added. After incubation at 37°C for 2 h, the absorbance was measured at 405 nm with a microplate reader (Thermo). Caspase-3 activity of each sample was calculated according to the standard curve and normalized by the protein concentration.²³

Evaluation of apoptosis

Terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) staining was used to measure apoptosis. Penile tissues were fixed in 4% paraformaldehyde for 24 h and paraffin-embedded. The tissues were then sectioned and permeabilized with proteinase K (Beyotime, ST533). Apoptotic cells in the sections

were stained with an *in situ* Cell Death Detection kit (06432344001, Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instruction. Apoptosis index (the ratio of apoptotic cells to all cells) was calculated.²⁴

Immunofluorescence and immunohistochemistry

Immunofluorescence was used to determine the endothelium and smooth muscle content in the corpus cavernosum. Briefly, penile tissues were fixed in 4% paraformaldehyde for 24 h and paraffin embedded. The tissues were then sectioned. The penile slices were dewaxed with dimethylbenzene and dehydrated with gradient ethanol. The endogenous peroxidase was inactivated with 3% hydrogen peroxide. For antigen recovery, the slices were immersed in 0.01 mol l⁻¹ sodium citrate buffer (pH 6.0) at 95°C. Then, the slices were washed with PBS for three times and incubated with normal goat serum at room temperature for 1 h. Slices were incubated with antibodies against CD31 (1:200, AF6191, Affinity, Zhenjiang, China) or α -smooth muscle actin (α -SMA; 1:100, A03744, Boster) at 4°C overnight. Sections were then incubated with DyLight-conjugated secondary antibody (1:200, A23210, Abbkine, Redlands, CA, USA) and nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI; C1006, Beyotime). Images were obtained by fluorescence microscopy (Olympus, Tokyo, Japan) and the percentage of staining-positive area in the field was calculated by Image-Pro plus (Media Cybernetics, Silver Spring, MD, USA).

To perform immunohistochemistry, penile slices were incubated with eNOS antibody (1:200, BD Biosciences) at 4°C overnight. After washing, the sections were incubated with biotinylated secondary antibody, followed by incubation with peroxidase-conjugated streptavidin and staining with diaminobenzidine. After counterstaining with Harris's hematoxylin, images were observed under a microscope (Olympus) and analyzed using Image-Pro plus (Media Cybernetics).

Statistical analysis

All data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and results were presented as mean \pm standard deviation. Statistical analyses were conducted using *t*-test for comparisons of two groups or one-way analysis of variance followed by the Tukey test for comparisons among multiple groups. Intergroup differences were considered statistically significant with $P < 0.05$.

RESULTS

Establishment of diabetes model mice

Thirty C57BL/6J mice were divided into diabetic mice treated with vehicle, diabetic mice treated with MDL28170, and the nondiabetic

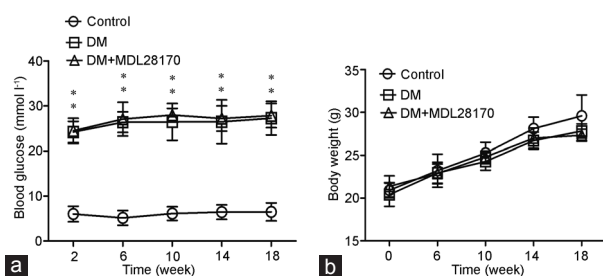


Figure 1: Blood glucose and body weight of mice. (a) One week after the last injection of streptozotocin, the blood glucose of mice was measured once every 4 weeks. (b) The body weight of mice was measured at the beginning of the study, the 6th week, and once every 4 weeks after that. Data were shown as mean \pm standard deviation. $n = 10$ for each group. Statistical analyses were conducted using one-way analysis of variance followed by the Tukey test. * $P < 0.05$, compared with control group. DM: diabetes mellitus.

control groups. Type 1 diabetes was induced in the two diabetes groups by streptozotocin, and the changes of fasting blood glucose levels and body weight were evaluated (Figure 1). After intervention with streptozotocin, the fasting blood glucose concentration in the two diabetes groups was >16.7 mmol l⁻¹ (24.23 ± 2.35 mmol l⁻¹ and 24.47 ± 2.80 mmol l⁻¹ at week 2), which was higher than that in the control group (6.02 ± 1.74 mmol l⁻¹ at week 2, $P < 0.01$ for each). There was no significant difference in blood glucose levels between the two diabetic mice groups ($P = 0.39$). The initial body weight showed no significant differences among the three groups (20.93 ± 0.89 g, 20.40 ± 1.36 g, and 21.38 ± 1.24 g, respectively). The body weight of all mice increased gradually during the experiment. The final body weight among three groups showed no statistically significant differences (29.59 ± 2.45 g, 28.63 ± 1.44 g, and 29.02 ± 1.60 g, respectively).

Erectile function

We next evaluated the effects of the MDL28170 calpain inhibitor on erectile function in diabetic mice. The MIP/MAP in diabetic mice treated with vehicle (0.31 ± 0.06) was lower than that in the nondiabetic control group (0.80 ± 0.05 , $P < 0.01$; Figure 2). Notably, the MIP/MAP of the MDL28170-treated diabetic group (0.48 ± 0.06) was higher than that of the diabetic mice treated with vehicle ($P < 0.01$), indicating that MDL28170 treatment improved erectile function in diabetic mice. However, the MIP/MAP in the MDL28170-treated group was lower than that in the nondiabetic control group ($P < 0.01$). This meant that calpain inhibition could partly restore the erectile function in diabetic mice.

Calpain expression and activity

We next evaluated calpain-1 and calpain-2 expression levels in penes from all three groups by Western blot. No significant differences in expression of both subtypes were observed among three groups. Evaluation of SBDP, which is used to quantify calpain activity, revealed increased SBDP content in mice with diabetic ED compared with control mice ($P < 0.01$; Figure 3a and 3b). MDL28170 treatment reduced the SBDP content in diabetic mice to a lower level than that in the nondiabetic control group ($P < 0.01$). Similarly, calpain activity analysis with N-succinyl-LLVY-AMC also indicated that compared with control mice, calpain activity was enhanced in diabetic mice and partly inhibited by MDL28170 ($P < 0.01$, for each; Figure 3c). These data indicated that calpain activity was enhanced in diabetic mice and MDL28170 administration effectively inhibited calpain activation.

Changes in the eNOS-NO-cGMP pathway

To investigate the mechanism by which MDL28170 ameliorated diabetic

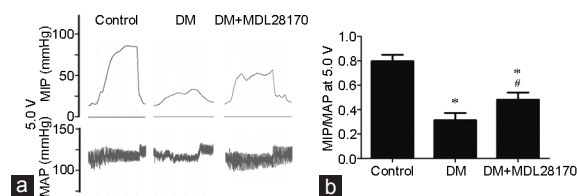


Figure 2: Erectile function of mice. (a) Representative recordings of maximal intracavernosal pressure and arterial pressure during 1-min electrical stimulation at 5.0 V. (b) The ratio of maximal intracavernosal pressure to mean arterial pressure for each group. $n = 10$ for each group. Statistical analyses were conducted using one-way analysis of variance followed by the Tukey test. * $P < 0.05$, compared with control group. # $P < 0.05$, compared with DM group. DM: diabetes mellitus; MIP: maximal intracavernosal pressure; MAP: mean arterial pressure.

ED, we next examined molecular changes in the eNOS-NO-cGMP pathway. Western blot analysis showed that the expression of eNOS in corpus cavernosum was reduced in diabetic mice compared with control mice and restored by MDL28170 treatment ($P < 0.01$ for each; **Figure 4a** and **4c**). Immunohistochemistry with eNOS antibody showed similar results ($P < 0.01$ for each; **Figure 4b** and **4d**). In addition, the phosphorylation of eNOS at Ser1177, an activating phosphorylation, was also diminished in diabetic mice compared with control mice and preserved by MDL28170 intervention ($P < 0.01$ for each; **Figure 4a** and **4c**). Similarly, the production of NO and cGMP in penile tissues was diminished in diabetic mice and partly restored by MDL28170 ($P < 0.01$ for each; **Figure 4e** and **4f**). Together, these data suggested that diabetic ED mice showed decreased expression and decreased activation of eNOS, along with reduced NO and cGMP levels, while treatment of diabetic ED mice with the MDL28170 calpain inhibitor restored eNOS levels and activation, along with increased NO and cGMP levels.

Caspase-3 expression and activity

We next evaluated apoptosis in diabetic ED mice and the effects of MDL28170 treatment. Compared with the control group, the expression of both caspase-3 and cleaved caspase-3 was increased in DM group ($P < 0.01$, for each; **Figure 5a** and **5b**). MDL28170 treatment reduced the production of cleaved caspase-3 in diabetic mice ($P < 0.01$), but did not change the expression of caspase-3 in diabetic mice ($P = 0.11$). Likewise, caspase-3 activity analysis showed that caspase-3 activity in diabetic mice was higher than that in normal mice and MDL28170 could reduce caspase-3 activity in diabetic mice ($P < 0.01$ and $P = 0.03$, respectively; **Figure 5c**). This indicated that MDL28170 intervention could diminish the activation but not the production of caspase-3.

Apoptosis in penile tissues

To further evaluate apoptosis in the corpus cavernosum, we performed the TUNEL staining. The ratio of TUNEL-positive cells to total cells in diabetic mice (0.30 ± 0.04) was increased compared with controls (0.11 ± 0.03 , $P < 0.01$; **Figure 5d** and **5e**). Calpain inhibition by MDL28170 reduced the apoptosis index (0.19 ± 0.01) in penile tissues of diabetic mice ($P = 0.02$). Diabetes led to more cell apoptosis in the penes of mice, and calpain inhibition could reduce the number of apoptotic cells.

Content of endothelium and smooth muscle

Endothelial cells and smooth muscle cells play an important role in penile erection. Immunofluorescence indicated that the

endothelium and smooth muscle content in diabetic mice were both reduced compared with normal mice ($P < 0.01$, for each; **Figure 6**). MDL28170 treatment could increase the content of both endothelium and smooth muscle in diabetic mice ($P = 0.03$ and $P < 0.01$, respectively).

DISCUSSION

Our study showed that calpain activity was elevated in mice with diabetic ED and that the inhibition of calpain by MDL28170 could improve the erectile function of diabetic mice. Calpain inhibition in the corpus cavernosum in diabetic mice led to an increase in eNOS expression and activation by phosphorylation at Ser1177. The production of cGMP and NO was also enhanced by calpain inhibition. In addition, calpain inhibition in diabetic mice resulted in reduced apoptosis and increased endothelium and smooth muscle in the corpus cavernosum compared with nontreated diabetic mice.

Diabetes is a complex disease that can cause damage to various organs and tissues via multiple signal pathways, including the calpain system. Hyperglycemia can activate calpain in a protein kinase C (PKC)-dependent manner or by increasing ROS production.^{25,26} In our study, calpain activity was elevated in penes of diabetic mice compared with control mice, with no changes in the protein levels of calpain-1 and calpain-2. This is consistent with the study from Cheng *et al.*²⁷

The eNOS-NO-cGMP pathway plays a vital role in penile erection.²⁸ eNOS activity is regulated by multiple factors, such as coupling and posttranslational phosphorylation at several serine residues. The phosphorylation of eNOS at Ser1177 could reportedly activate eNOS by reducing the enzyme's requirement of calcium.²² Our results showed that calpain inhibition increased the amount of Ser1177-phosphorylated eNOS, as well as the total protein level of eNOS. NO and cGMP levels showed similar changes. However, whether the changes in NO and cGMP production are direct results of eNOS activation needs further research. In addition, the pathway and mechanism linking calpain inhibition and eNOS activation are still not clear.

A previous study showed that phosphorylation of eNOS at Ser1177 was dependent on Akt activation.²⁹ Akt inhibition diminished the phosphorylation of eNOS and reduced penile erection. A study by Smith and Dodd¹⁴ found that calpain activation could inhibit the Akt signaling pathway. However, whether the reduced phosphorylation of eNOS at Ser1177 caused by calpain activation is mediated by Akt inhibition needs further investigation. Moreover, calpain activation was reportedly able to degrade eNOS,^{30,31} which could explain the reduced eNOS content in mice with diabetic ED in our study. We also found that MDL28170 could not completely restore the levels and phosphorylation of eNOS in diabetic mice, although it decreased calpain activity to a great extent. This indicates that calpain activation is only one of the factors contributing to the degradation and inactivation of eNOS.

Previous studies showed that calpain could enhance apoptosis of cells by directly cleaving caspase-3, a major cysteine protease that participates in apoptosis.^{32,33} Cleaved caspase-3 is the activated form of caspase-3 and an apoptotic executor,³⁴ and thus considered to be the most reliable index of apoptosis.³⁵ Our Western blot showed that calpain activation produced elevated levels of cleaved caspase-3 in diabetic mice, and calpain inhibition decreased the cleavage of caspase-3. Caspase-3 activity assays were in agreement with these results. However, the MDL28170 treatment did not change the amount of inactive precursor caspase-3, indicating that calpain had no effect on the expression of caspase-3.

Normal function and amounts of endothelium and smooth muscle in the corpus cavernosum are essential for penile erection.

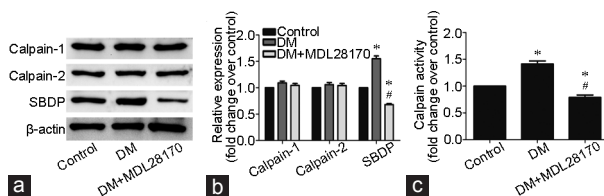


Figure 3: Calpain expression and activity. (a) The expressions of calpain-1, calpain-2, and the 145 kDa stable breakdown products of spectrin α -II were detected with Western blot. (b) Relative density of calpain-1, calpain-2, and stable breakdown products compared with β -actin. Data are shown as the fold changes over the control group. $n = 4$ for each group. (c) Calpain activity measured using a fluorescence substrate N-succinyl-LLVY-AMC. Data are shown as the fold changes over the control group. $n = 3$ for each group. Statistical analyses were conducted using one-way analysis of variance followed by the Tukey test. * $P < 0.05$, compared with control group. # $P < 0.05$, compared with DM group. DM: diabetes mellitus.

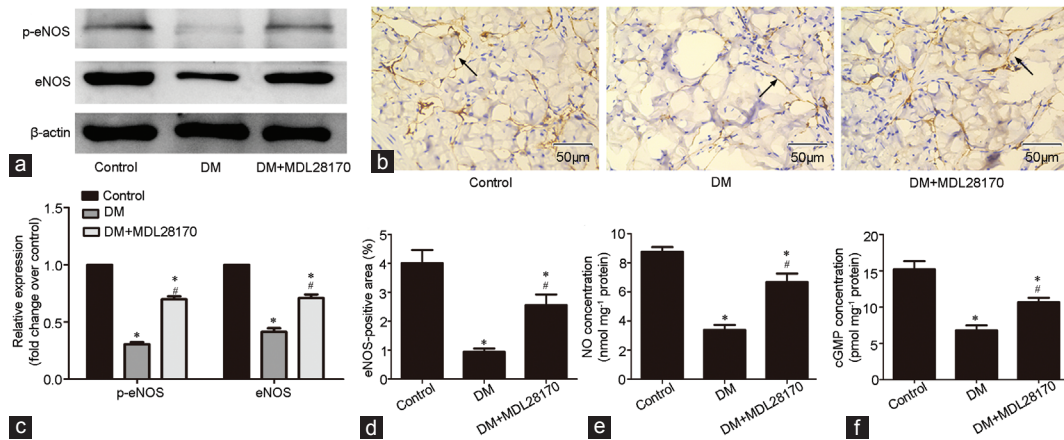


Figure 4: Changes in eNOS-NO-cGMP Pathway. (a) The expression of eNOS and phospho-eNOS (Ser1177) detected with Western blot. (b) Relative density of eNOS and phospho-eNOS (Ser1177) compared with β -actin. Data are shown as the fold changes over the control group. $n = 4$ for each group. (c) eNOS expression detected with immunohistochemistry ($\times 400$). Location of eNOS was indicated with black arrow. (d) eNOS-positive area in the corpus cavernosum detected with immunohistochemistry. $n = 3$ for each group. (e) NO concentration detected using a total NO assay kit and normalized by the protein concentration. $n = 3$ for each group. (f) cGMP concentration measured with an enzyme-linked immunosorbent assay kit and normalized by the protein concentration. $n = 3$ for each group. Statistical analyses were conducted using one-way analysis of variance followed by the Tukey test. * $P < 0.05$, compared with control group. # $P < 0.05$, compared with DM group. DM: diabetes mellitus; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; cGMP: cyclic guanosine monophosphate.

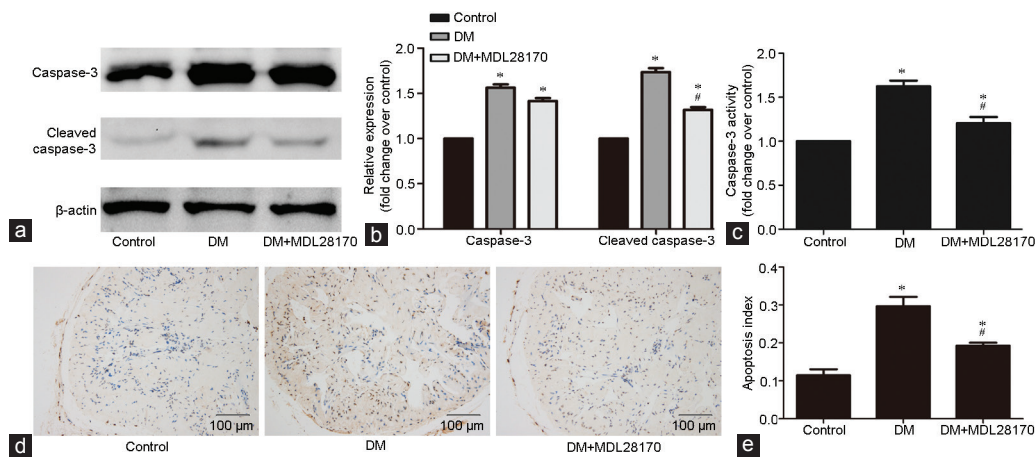


Figure 5: Caspase-3 expression and apoptosis in penile tissues. (a) The expression of caspase-3 and cleaved caspase-3 detected with Western blot. (b) Relative density of caspase-3 and cleaved caspase-3 compared with β -actin. Data are shown as the fold changes over the control group. $n = 4$ for each group. (c) Caspase-3 activity detected with a caspase-3 activity assay kit and normalized by the protein concentration. $n = 3$ for each group. (d) Apoptosis detected with terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling staining ($\times 200$). Apoptotic cells were stained in brown. (e) Apoptosis index in the corpus cavernosum. $n = 3$ for each group. Statistical analyses were conducted using one-way analysis of variance followed by the Tukey test. * $P < 0.05$, compared with control group. # $P < 0.05$, compared with DM group. DM: diabetes mellitus.

Our results suggested that the content of endothelium and smooth muscle was reduced in mice with diabetic ED. However, calpain inhibition could preserve the endothelium and smooth muscle content in diabetic mice. The loss of endothelium might contribute to the reduced content of eNOS, while apoptosis of smooth muscle may damage the relaxation function of corpus cavernosum, diminishing penile erection.

Our study has a few limitations. First, we only evaluated the amount of calpain-1 and calpain-2 in penes. The expression of other calpain family members such as calpain-10, a diabetes-related calpain subtype, should also be examined in future studies. In addition, the mechanism of how calpain influences the content and phosphorylation of eNOS needs further research. Although MDL28170 is widely used as calpain inhibitor, it might also inhibit other proteases. We will use transgenic mice or another calpain

inhibitor that works in a different way from MDL28170 to further investigate its effect on erectile function.

CONCLUSIONS

Calpain was activated in the corpus cavernosum of mice with diabetic ED. The inhibition of calpain activity by MDL28170 could improve the erectile function in diabetic mice, and the mechanism might involve upregulation of the eNOS-NO-cGMP pathway and reduction in apoptosis of endothelial cells and smooth muscle cells in penile tissues. Together, our results suggest that inhibition of calpain might be a novel alternative therapy for diabetic ED.

AUTHOR CONTRIBUTIONS

HL and LPC carried out the studies and drafted the manuscript; TW and SGW performed the statistical analysis; JHL conceived of the study

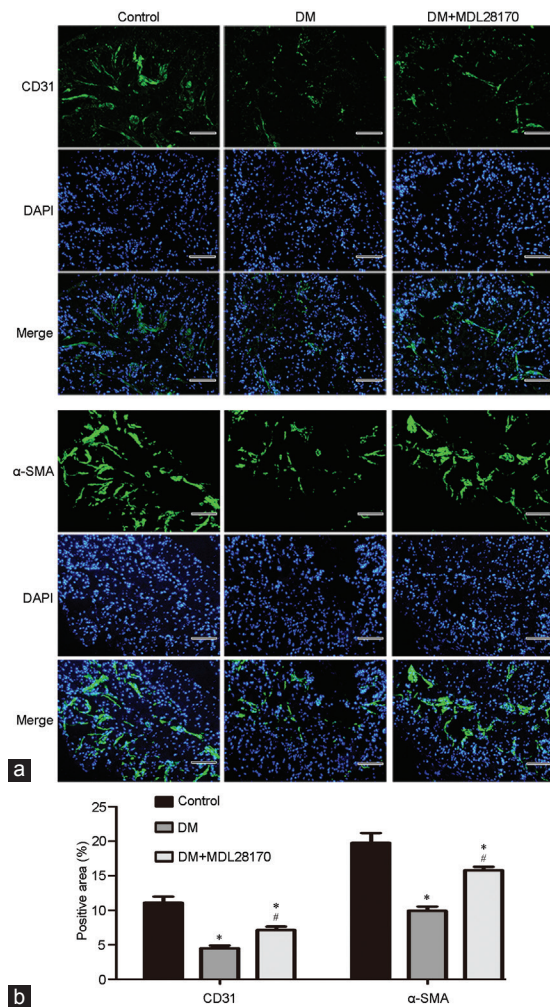


Figure 6: Endothelium and smooth muscle content in corpus cavernosum. (a) Endothelium and smooth muscle in corpus cavernosum was detected with immunofluorescence using antibodies against CD31 or α -SMA (green, $\times 200$). Nuclei were stained by DAPI (blue). Scale bars=100 μ m (b) Percentage of endothelium and smooth muscle content. $n = 3$ for each group. Statistical analyses were conducted using one-way analysis of variance followed by the Tukey test. * $P < 0.05$ compared with control group. # $P < 0.05$, compared with DM group. DM: diabetes mellitus; DAPI: 4',6-diamidino-2-phenylindole; α -SMA: α -smooth muscle actin.

and participated in its design and coordination. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declared no competing interests.

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