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ORIGINAL ARTICLE

# Diabetes associated with hypertension exacerbated oxidative stress–mediated inflammation, apoptosis and autophagy leading to erectile dysfunction in rats

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## Abstract

**Background:** Diabetes or hypertension contributes to erectile dysfunction (ED). We hypothesized that excess reactive oxygen species (ROS) production evoked by diabetes combined with hypertension may further suppress endothelial nitric oxide (NO) expression/activity and promote oxidative stress in the ED penis.

**Methods:** Twenty-four adult male Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were divided into four groups: normal WKY, diabetic WKY, normal SHR and diabetic SHR. Intraperitoneal streptozotocin (65 mg/kg) was applied to induce type I diabetes. After 4-week diabetes and/or hypertension induction, we determined the intra-cavernous pressure (ICP) using electrical stimulation of cavernous nerves, intra-cavernosum NO amount using an electrochemical NO probe, and blood ROS using an ultrasensitive chemiluminescence-amplified analyzer. Western blot analysis and immunohistochemistry were used to explore the pathophysiologic mechanisms of inflammation, apoptosis and autophagy in the penis. A novel NO donor, CysaCysd Lu-5 (CCL5, $(RCH_2CH_2S)(R'R''CHCH_2S)Fe(NO)_2$, 1-4 μg), was intravenously administered to these ED rats for evaluating their ICP responses.

**Results:** In the baseline status, the lucigenin- and luminol-amplified blood ROS were significantly enhanced in the diabetic SHR rats vs normal WKY rats. Significantly decreased ICP, eNOS expression and NO amount were found in the normal SHR, diabetic WKY, and diabetic SHR vs normal WKY rats. Intravenous NO donor L-Arginine markedly increased ICP and NO amount, whereas eNOS inhibitor, $N_\omega$-Nitro-L-Arginine methyl ester hydrochloride depressed ICP in all four groups. Diabetes and/or hypertension alone increased fibrosis, proinflammatory NF-kB/ICAM-1 expression, mast cell numbers, CD68 expression and infiltration, Caspase 3-mediated apoptosis, Beclin-1/LC3-II–mediated autophagy and mild Nrf-2/HO-1 expression and depressed eNOS expression in the ED penis. The novel NO donor, CCL5, was more efficient than L-arginine to improve diabetes and/or hypertension–induced ED by the significant increase of ICP.

**Conclusion:** Diabetes combined with hypertension synergistically exacerbated ED through enhanced oxidative stress, inflammation, apoptosis and autophagy and depressed eNOS activity and NO production.

**Keywords:** Apoptosis; Autophagy; Erectile dysfunction; Hypertension; Nitric oxide; Reactive oxygen species; Type I diabetes

## 1. INTRODUCTION

Sexual dysfunction affects millions of people with an increasing prevalence, worldwide. The disease pathophysiology shares several similarities with cardiovascular disease, including atherosclerosis, endothelial dysfunction, structural vascular damage, and subclinical inflammation. Erectile dysfunction (ED) and female sexual dysfunction are common among patients with cardiovascular diseases with risk factors such as hypertension, diabetes, obesity, and metabolic syndrome.[1] ED could be induced by testosterone insufficiency,[2] and impairment in the vascular endothelial system,[3] corpus cavernosum smooth muscle,[4] and autonomic system.[5] ED of 35%-75% occurred in diabetic patients.[6] ED of 35.2% occurs in hypertensive patients compared to 14.1% ED in normal hypertension subjects.[7] Hypertension

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and cardiovascular diseases contribute to ED in men,[8] and the severity of ED was positively correlated with the time course and degree of hypertension.[9] Erectile function was normally regulated through the intact veno-occlusive mechanism.[10] However, long-term hypertension and/or diabetes would induce structural and functional alterations in the ED penis including the appearance of fibrosis in the corpus cavernosum and vessels, the decrease in compliance, diameter and blood flow and the impairment of veno-occlusive system.[4,11] We hypothesized whether diabetes combined with hypertension could exacerbate ED through oxidative stress and inflammation pathophysiologic mechanisms mediating endothelial dysfunction.

Oxidative stress evoked neural and vascular injury contributed to diabetic ED.[12] Exacerbated reactive oxygen species (ROS) production leads to tissue damage through ROS-evoked abnormal signal transduction, inflammatory monocyte/macrophage (ED-1) infiltration, cellular dysfunction and programmed cell death in several kinds of cells.[13,14] Among the ROS, superoxide ($O_2^-$) reacts with NO to form peroxynitrite resulting in a decrease in the NO amount and impairment of NO-mediated smooth muscle relaxation.[15] Diabetes and/or hypertension may increase excess ROS to induce apoptosis and autophagy. ROS formation may induce apoptosis or autophagy via execution by caspases, lysosomal proteases, or endonucleases.[16,17] The increased ROS can trigger apoptosis by activating Bax expression/caspase 3 activity/poly-(ADP-ribose)-polymerase fragments pathway or enhance autophagy by activating Beclin-1/Atg5-Atg12/LC3-II pathway.[14] We suggest that diabetes and/or hypertension may evoke excess ROS to induce apoptosis and autophagy in the corpus cavernosum.

Nitric oxide synthase (NOS) exists in the penis and is regulated by the release of L-arginine via endothelial NOS (eNOS) to produce NO contributing to erectile function.[18] Exogenous NO or NOS inhibitor can affect the intra-cavernous pressure (ICP).[19] A previous report indicated that oral or intravenous L-arginine increased the ICP level by the electric cavernous nerve.[20] In this study we explore the diabetes and/or hypertension effects on eNOS activity and NO amount in the corpus cavernosum. Nuclear Factor E2-related Factor 2 (Nrf-2) is a protective transcription factor and Nrf-2-Kelch–like ECH-associated protein-1 (Keap1) pathway is a self-defense mechanism in response to external stress or toxicity. Nrf-2 through downstream antioxidant response element and heme oxygenase 1 (HO-1) exerts antioxidant and anti-inflammatory reactions to play a protective role in reducing diseases and programmed cell death.[21,22] We will examine the role of Nrf-2/Keap1 signaling in diabetes and/or hypertension–induced ED.

We first established a model for simultaneous measurement of intra-cavernosum pressure and NO amount. We next explored the hypertension and/or diabetes effects on NO donor and inhibitor on erectile function. We also evaluated the inflammation, apoptosis and autophagy responses in diabetes and/or hypertension–induced ED. The Nrf-1/HO-1 pathway was also examined in diabetes and/or hypertension–induced ED.

## 2. METHODS

### 2.1. Animals

We used spontaneously hypertensive rats (SHR) as a hypertension induction model because preliminary data indicated ED was found in SHR.[23] Wistar-Kyoto rats (WKY) were selected as a control. All animals were purchased from BioLASCO Co. Ltd (I-Lan, Taiwan) and housed at the Experimental Animal Center, National Taiwan Normal University, at a constant temperature and with a consistent light cycle (light from 07:00 to 18:00). The rats were fed standard chow and tap water ad libitum. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan Normal University with Approval Number 108001. The guidelines of the National Science Council of Republic of China (1997) were followed. The animals were divided into four groups: WKY-CON (n = 6), WKY-STZ (n = 6), SHR-CON (n = 6), and SHR-STZ (n = 6).

### 2.2. Type I diabetes induction

The protocol for diabetes induction was previously demonstrated.[24] We used intraperitoneal streptozotocin (STZ at 65 mg/kg; Sigma, MO) to destroy pancreatic β cells. Rats were fasted 8 hours before and under 2% isoflurane and treated STZ in 10 mg/mL of 0.1 M sodium citrate buffer (pH 4.5; Sigma). We determined the body weight and fasted blood glucose before and after STZ treatment every week. After 4 weeks of STZ treatment, the fasted (8 hours) blood glucose level was examined using the Contour plus blood glucose monitoring system (Bayer, Leverkusen, Germany). The fasted blood glucose >250 mg/dL was identified as successful diabetes induction.

### 2.3. Simultaneous recording of mean arterial pressure, intra-cavernosal pressure, and NO amount

Under anesthesia (Urethane, 1.5 g/kg, i.p.; Sigma), the rat trachea was intubated for spontaneous ventilation. Mean arterial blood pressure (MAP) was determined by a PE50 tubing containing heparinized saline with a pressure transducer (DPT-100, Blood Pressure Transducer; Utahmed, UT) introduced into the left carotid artery and was recorded on a polygraph (Power Lab 8/30; AD Instruments, Dunedin, New Zealand). The anesthetized animals were placed in a supine position on a heated surgical table. A lower midline abdominal incision was made after the abdomen was shaved and sterilized. The prostate gland was exposed and the posterolateral tracking cavernous nerves were identified and isolated.

### 2.4. ICP recording

A needle (24G*3/4″ Scalp Vein Set; Nipro, Osaka, Japan) filled with heparin (250 U/mL) was attached to PE-50 tubing, inserted into the right crura, and connected to a pressure transducer (DPT-100, Blood Pressure Transducer; Utahmed) to permit continuous ICP measurement. The isolated cavernous nerve was stimulated with an electrical stimulator (S9 Stimulator; Grass, Warwick, RI) with a stainless-steel bipolar hook was placed around the cavernous nerve, proximal to the injury site. After ICP stabilization, the cavernous nerve was stimulated with 60 seconds of electric stimulation (6 V/60 Hz/duration = 10 ms). ICP was measured with the help of a pressure transducer and amplifier (Quad Bridge Amp; AD instruments) and recorded on a polygraph (Power Lab 8/30; AD Instruments).

### 2.5. Real-time intra-cavernous NO determination

The NO probe (INC-020, NO Electrode; Inter Medical co., Ltd, Aichi, Japan) was first calibrated using NO donor S-nitroso-N-acetylpenicillamine (SNAP; Molecular Probes, Eugene, OR) before use as described previously.[25] The anesthetized rats were fixed on a heating table. We inserted a needle (19G*11/2″, Terumo Needle; Terumo, Tokyo, Japan) into the penis as a guide tube and then the precalibrated NO-selective microelectrode was inserted through the guide tube to measure the NO amount in the corpus cavernosum. The recorded signals were amplified by an amplifier (IMEC-601/601A, Electrochemical Amplifier; Inter Medical co., Ltd) and recorded on a polygraph recorder (Power Lab 8/30; AD Instruments).

### 2.6. Effect of L-arginine, L-NAME or CCL5 on MAP, ICP and intra-cavernous NO determination

The intravenous administration of 0.2 mL of saline was set as control. The intravenous treatment of 0.2 mL of L-arginine (25 mg/kg body weight; Sigma),[20] intravenous administration of 0.2 ml eNOS inhibitor ($N_{\omega}$-Nitro-L-Arginine methyl ester hydrochloride, L-NAME; Sigma) at dose of 3 mg/kg body weight) or CysaCysd Lu-5 (CCL5, $(RCH_2CH_2S)(R'R''CHCH_2S)Fe(NO)_2$, 1-4 μg iv) provided by Professor Wen-Feng Liaw of National Tsin Hua University was performed.

### 2.7. Western blot

The method for determining β-actin (Sigma), Nrf-2 (Cell Signaling, Inc.), cleaved-Caspase 3 (Cell Signaling, Inc.), CD68 (AbD Serotec), and Beclin-1 (Protein Tech Group, Chicago, IL) expression from the penis of these four groups of rats were evaluated using the Western blot technique as described before.[16]

### 2.8. Chemiluminescence measurement for ROS production

We determined blood ROS by luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma) amplified chemiluminescence (CL) and lucigenin (10-Methyl-9-(10-methylacridin-10-ium-9-yl) acridin-10-ium dinitrate; Sigma) amplified CL as described previously.[26] The CL was continuously monitored for an additional 600 seconds; integrating the area under the curve and subtracting it from the background level was used to calculate the total CL amount. The assay was performed in duplicate for each sample and was expressed as CL counts per 10 seconds for whole-blood CL.

### 2.9. In situ demonstration of fibrosis, inflammation, apoptosis and autophagy formation

We compared the degree of fibrosis using Masson's trichrome stain for detection of collagen deposits, inflammation by toluidine blue staining for specific mast cell labeling, by CD68 immunostaining to evaluate monocyte/macrophage distribution, apoptosis by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay (DNA Fragmentation Kit; BioVision, Milpitas, California),[26] and autophagy by Beclin-1 staining. Twenty high-power (×400) fields were randomly selected for each section. The level of each oxidative stress was analysed with a Sonix Image Setup (Sonix Technology Co., Ltd., Hinschu, Taiwan, ROC).

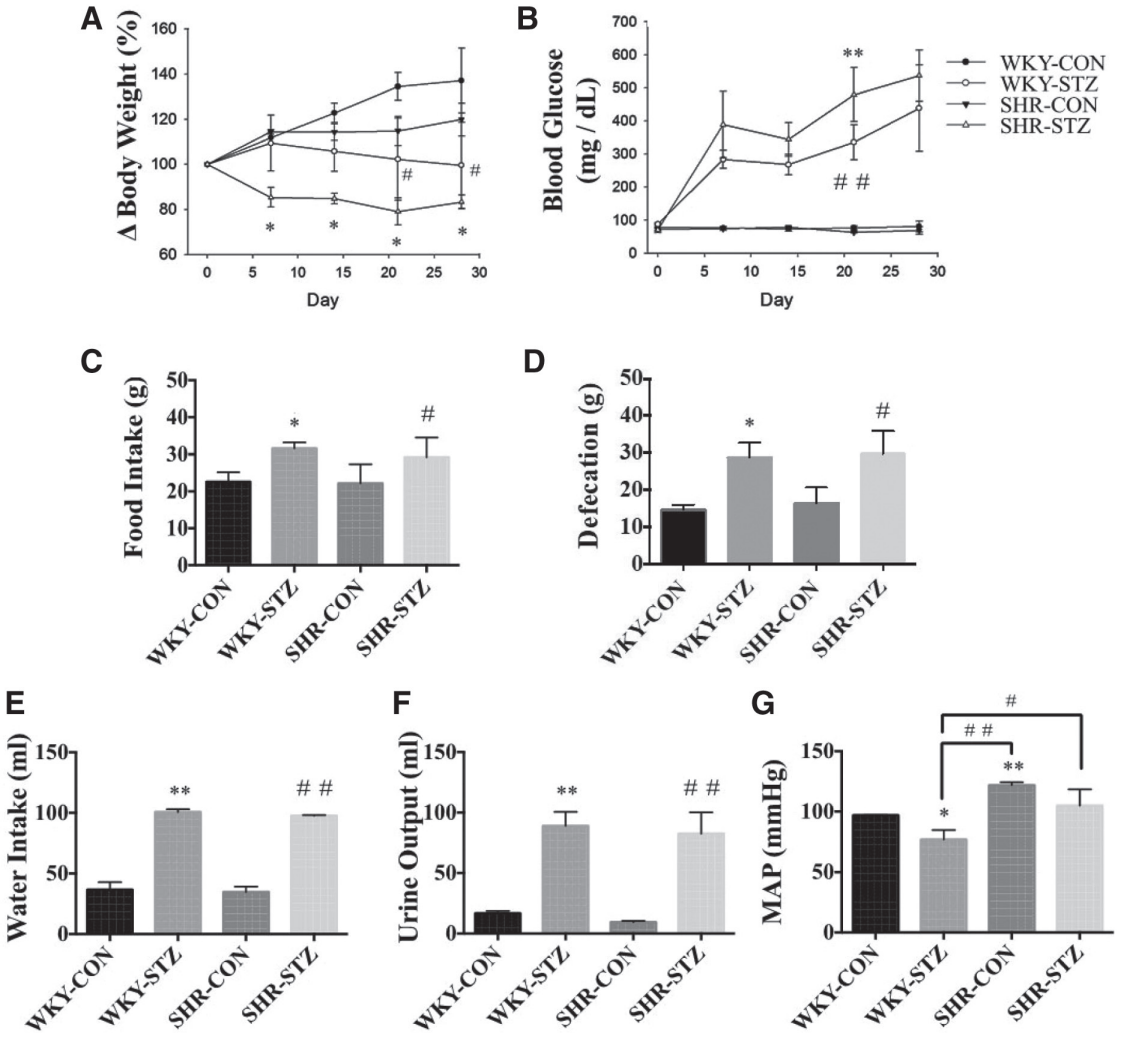


**Fig. 1** The basal physiologic parameter of percentage change of body weight (A), fasted blood glucose (B), food intake (C), feces amount (defecation) (D), water intake (E), urine output (F), and mean arterial blood pressure (MAP) (G) in the WKY-CON, WKY-STZ, SHR-CON, and SHR-STZ rats. After 4 weeks of STZ induction, these physiological parameters are significantly increased in WKY-CON and SHR-CON rats as compared to WKY-CON and SHR-CON rats. Data are expressed as mean ± SEM (n = 6) using the single values. *$p < 0.05$ vs WKY-CON, **$p < 0.01$ vs WKY-CON. #$p < 0.05$ vs SHR-CON, ##$p < 0.01$ vs SHR-CON.

### 2.10. Statistical analysis

We used GraphPad Prism 6 (GraphPad Software, CA) to analyze all data. All values were expressed as mean ± standard error mean. Data were subjected to one-way analysis of variance, followed by Duncan's multiple-range test for assessment of the difference among groups. Differences within groups were evaluated by a paired t test. Differences were regarded as significant if $p < 0.05$ was attained.

## 3. RESULTS

### 3.1. The basal levels of several physiologic parameters

We determined the basal level of several physiological parameters in four groups of rats and ascertained the induction of diabetes and hypertension in our animal models. We confirmed STZ significantly ($p < 0.05$) decreased body weight (Fig. 1A) and elevated fasted blood glucose ($p < 0.05$, Fig. 1B) after 1 week of STZ induction vs CON rats. We evaluated the effect of 4 weeks of STZ on several physiological parameters in the 24 h metabolic cage analysis. The food intake value in WKY-STZ (31.6 ± 1.6 g) and SHR-STZ (29.2 ± 5.4 g) and feces amount in WKY-STZ (28.7 ± 7.6 g) and SHR-STZ (29.7 ± 14.8 g) were significantly higher than the food intake value in WKY-CON (22.6 ± 2.5 g) and SHR-CON (22.2 ± 5.1 g) (Fig. 1C) and feces amount in WKY-CON (14.6 ± 1.4 g) and SHR-CON (16.5 ± 4.2 g) (Fig. 1D). The water intake in WKY-STZ (100.7 ± 2.3 mL) and SHR-STZ (97.5 ± 0.7 mL) and urine output in WKY-STZ (89.0 ± 11.5 mL) and SHR-STZ (82.5 ± 17.7 mL) were significantly higher than that in WKY-CON (36.5 ± 6.4 mL) and SHR-CON (34.7 ± 4.5 mL) (Fig. 1E) and that in WKY-CON (16.5 ± 2.12 mL) and SHR-CON (9.3 ± 1.2 mL) (Fig. 1F). The MAP was significantly higher in SHR-CON rats, whereas diabetes decreased the MAP in WKY-STZ and SHR-STZ vs WKY-CON and SHR-CON rats, respectively.

### 3.2. Diabetes and hypertension increased blood ROS

Our data found that diabetes combined with hypertension significantly enhanced the level of lucigenin amplified blood ROS (5820 ± 259 counts/sec) in SHR-STZ rats vs SHR-CON (784 ± 452 counts/sec), WKY-STZ (643 ± 192 counts/sec) and WKY-CON (445 ± 160 counts/sec) (Fig. 2A, B). The level of luminol-amplified blood ROS was higher in SHR-STZ rats (4550 ± 1537 counts/sec) than in SHR-CON (741 ± 132 counts/sec), WKY-STZ (393 ± 158 counts/sec), and WKY-CON (117 ± 74 counts/sec) (Fig. 2C, D).

### 3.3. Diabetes associated with hypertension inhibited ICP

As shown in Fig. 3A, the simultaneous MAP, ICP, and ICNO recordings were displayed in four groups of rats in response to L-arginine or L-NAME treatment. Our data (Fig. 3B) demonstrated that the ICP level in WKY-STZ (29.78 ± 2.24 mmHg, n = 6) and SHR-STZ (36.40 ± 1.18 mmHg, n = 6) was lower than that in WKY-CON (43.47 ± 1.09 mmHg, n = 6) ($p < 0.01$). The ICP/MAP ratio in WKY-STZ (0.43 ± 0.01, n = 6) and

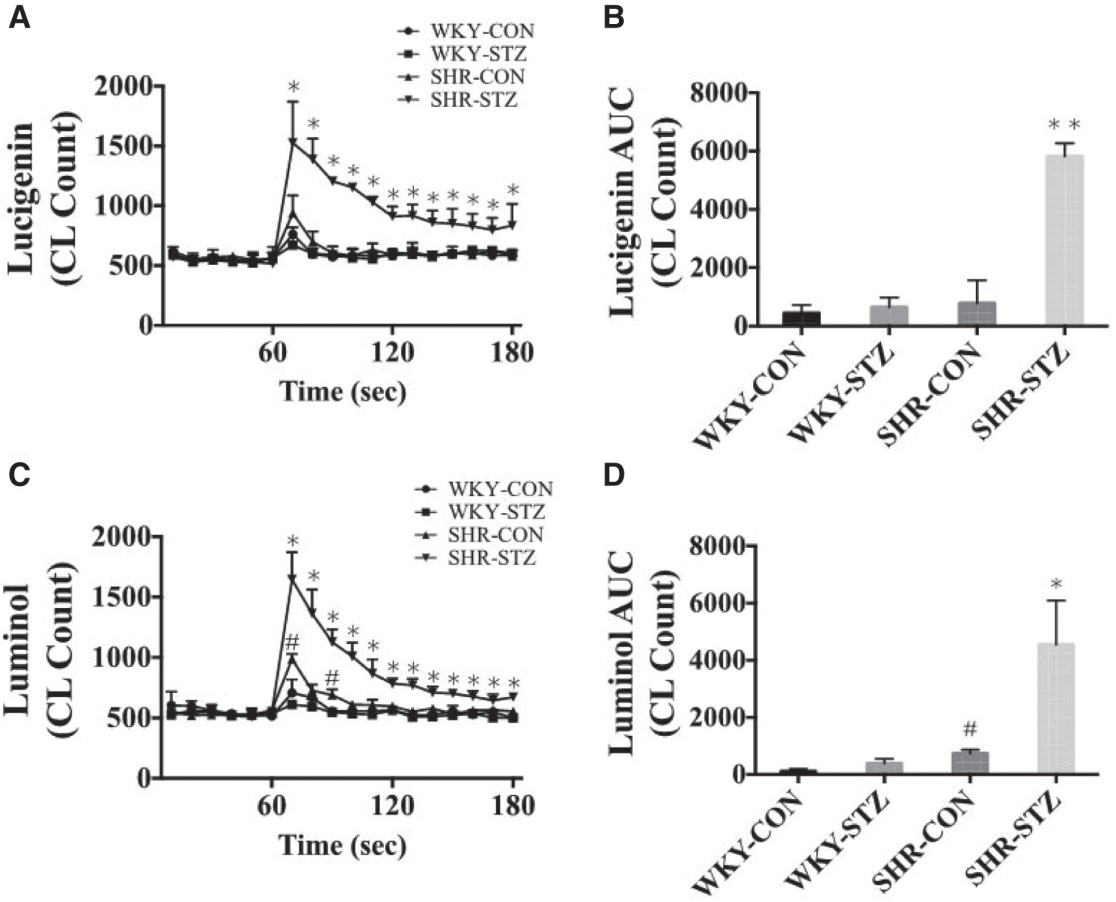


**Fig. 2** Diabetes and hypertension increased the lucigenin ROS (A and B) and luminol ROS (C and D) in the blood. The highest level of lucigenin or luminol blood ROS was found in the SHR-STZ group vs other three groups. Data are expressed in mean ± SEM, n = 6 in each group. *$p < 0.05$ SHR-STZ vs SHR-CON; #$p < 0.05$ SHR-CON vs WKY-CON. ROS = reactive oxygen species.

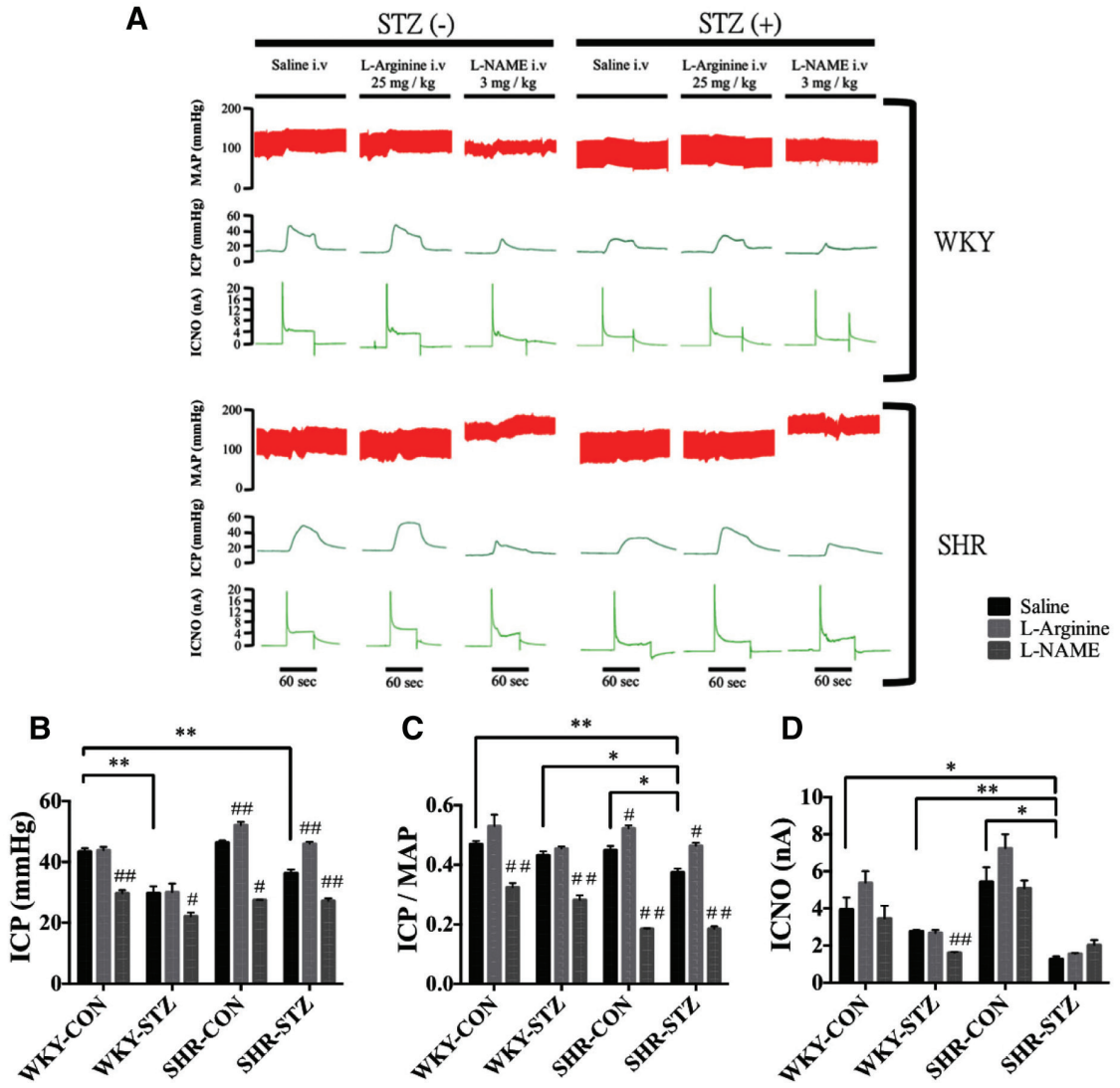


**Fig. 3** A, The original response of MAP, ICP, and ICNO to L-arginine or L-NAME in the WKY-CON, WKY-STZ, SHR-CON, and SHR-STZ rats. Statistic data (B and C) indicated that diabetes associated with hypertension significantly depressed electrical stimulation of cavernosal nerve enhanced ICP level in SHR-STZ as compared to other three groups. Data are expressed as mean ± SEM (n = 6) using the single values. *$p < 0.05$ vs WKY-CON, **$p < 0.01$ vs WKY-CON; #$p < 0.05$ vs SHR-CON, ## $p < 0.01$ vs SHR-CON.

SHR-CON (0.45 ± 0.01, n = 6) was not significant from that of WKY-CON (0.46 ± 0.01, n = 6). The ICP/MAP ratio in SHR-STZ (0.37 ± 0.01, n = 6) was significantly lower than that in WKY-CON ($p < 0.01$), WKY-STZ ($p < 0.05$) and SHR-CON ($p < 0.05$) (Fig. 3C). Diabetes combined with hypertension markedly inhibited ICP leading to ED.

### 3.4. Effect of L-arginine or L-NAME on ICP and ICNO

As shown in Fig. 3C, L-Arginine treatment (25 mg/kg body weight) significantly ($p < 0.05$) increased ICP/MAP ratio in SHR-CON (0.52 ± 0.01, n = 6) and SHR-STZ (0.46 ± 001, n = 6) but did not significantly enhance the ICP/MAP ratio in WKY-CON (0.53 ± 0.04, n = 6) and WKY-STZ (0.45 ± 0.01, n = 6). eNOS inhibitor, $N_{\omega}$-Nitro-L-Arginine methyl ester hydrochloride (L-NAME) (3 mg/kg body weight) treatments significantly decreased the ICP/MAP ratio in the four groups of rats (WKY-CON: 0.32 ± 0.01, n = 6; WKY-STZ: 0.28 ± 0.02, n = 6; SHR-CON: 0.19 ± 0.00, n = 6; SHR-STZ: 0.19 ± 0.01, n = 6) ($p < 0.01$).

We determined the ICNO in these four groups of rats. Our data (Fig. 3D) showed that during cavernous nerve electric stimulation for inducing erectile function, the ICNO level was significantly ($p < 0.05$) decreased in SHR-STZ (1.28 ± 0.14 nA, n = 6) vs SHR-CON (5.44 ± 0.79 nA, n = 6), WKY-STZ (2.79 ± 0.05 nA, n = 6), or WKY-CON (3.95 ± 0.64 nA, n = 6). L-arginine treatment significantly increased ICNO in WKY-CON and SHR-CON rats, but not in WKY-STZ and SHR-STZ. L-NAME treatment significantly decreased ICNO in WKY-CON, SHR-CON, and WKY-STZ, but not in SHR-STZ. We suggest that dysregulated NO in corpus cavernosum was found in the SHR-STZ rats.

### 3.5. Diabetes and hypertension promoted fibrosis and inflammation in corpus cavernosum

The blue fibrosis appeared in the areas around the cavernous nerve and corpus cavernosum of WKY-STZ, SHR-CON, and SHR-STZ rats (Fig. 4A). The fibrosis markedly appeared in the cavernous nerve, corpus cavernosum, and dorsal artery areas of SHR-STZ rats (Fig. 4E). The increased mast cells were found in the cavernous nerve, corpus cavernosum and dorsal artery areas in WKY-STZ, SHR-CON, and SHR-STZ rats (Fig. 4C), and the increased mast cell number was displayed in the order

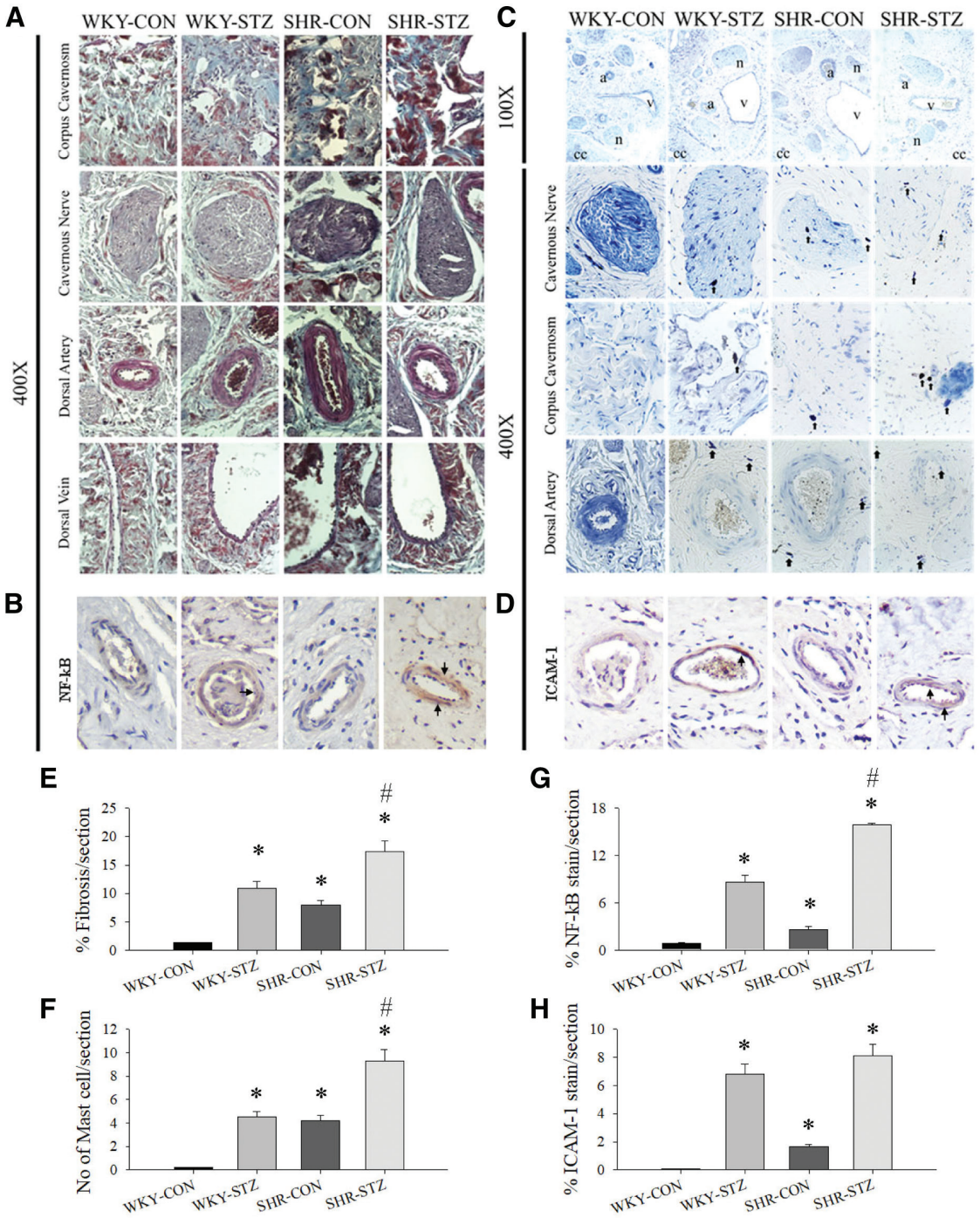


**Fig. 4** Diabetes and/or hypertension evoked fibrosis (blue stain, A), inflammatory NF-κB expression (brown color, B), mast cell accumulation (arrowheads, C), and ICAM-1 expression (arrow heads, D) in WKY-CON, WKY-STZ, SHR-CON, and SHR-STZ rats. Statistic data of these parameters are indicated in E (% fibrosis), F (number of mast cells), G (% NF-κB expression/section), and H (% ICAM-1 expression/section) in four groups of rats. *$p < 0.05$ vs WKY-CON. #$p < 0.05$ vs WKY-STZ.

SHR-STZ > WKY-STZ = SHR-CON > WKY-CON (Fig. 4F). The proinflammatory NF- κB (Fig. 4B) and its downstream ICAM-1 expression (Fig. 4D) were increased in the vessels of WKY-STZ and SHR-STZ. Statistic data showed that the vessels in SHR-STZ displayed the highest brown color of NF- κB (Fig. 4G) and ICAM-1 expression (Fig. 4H) compared to other three groups.

### 3.6. Diabetes and/or hypertension increased inflammatory CD68 cells in penis

The western blot data indicated a significant ($p < 0.01$) increase of CD68 cells in the penis was found in WKY-STZ (1.230 ± 0.050 fold, n = 6), SHR-CON (1.742 ± 0.043 fold, n = 6), and SHR-STZ (1.937 ± 0.095 fold, n = 6) as compared to that in WKY-CON rats (1-fold, n = 6) (Fig. 5A, B). Furthermore,

the CD68 value in SHR-STZ was higher ($p < 0.05$) than WKY-STZ (Fig. 5B). Immunohistochemistry data displayed a consistent finding that the CD68 stain was markedly increased in the WKY-STZ, SHR-CON, and SHR-STZ as compared to WKY-CON rats (Fig. 5C).

### 3.7. Diabetes and/or hypertension increased apoptotic cleaved-Caspase 3 expression in penis

The western blot demonstrated that the cleaved-Caspase 3 expression was significantly higher ($p < 0.01$) in WKY-STZ (1.083 ± 0.036 fold, n = 6), SHR-CON (1.097 ± 0.047 fold, n = 6), and SHR-STZ (1.298 ± 0.093 fold, n = 6) when compared to that in WKY-CON (1 fold, n = 6) (Fig. 6A, B). Furthermore, the cleaved-Caspase-3 level in SHR-STZ was higher ($p < 0.05$) than WKY-STZ (Fig. 6B). Immunohistochemistry data displayed a consistent finding that the cleaved-Caspase 3 stain was significantly increased in the WKY-STZ, SHR-CON, and SHR-STZ as compared to WKY-CON rats (Fig. 6C).

### 3.8. Diabetes and/or hypertension increased autophagic Beclin-1 and LC3-II expression in penis

The western blot demonstrated that the autophagic Beclin-1 expression was significantly higher ($p < 0.01$) in WKY-STZ (1.069 ± 0.019 fold, n = 6), SHR-CON (1.123 ± 0.023 fold, n = 6), and SHR-STZ (1.158 ± 0.043 fold, n = 6) when compared to that in WKY-CON (1 fold, n = 6) (Fig. 7A, B). Furthermore, the Beclin-1 level in SHR-STZ was higher ($p < 0.05$) than WKY-STZ (Fig. 7B). Another autophagic marker LC3-II expression was also significantly higher ($p < 0.01$) in WKY-STZ, SHR-CON, and SHR-STZ when compared to that in WKY-CON (Fig. 7C, D). The LC3-II expression in SHR-STZ was much higher ($p < 0.05$) than WKY-STZ (Fig. 7D). Immunohistochemistry data displayed the Beclin-1 stain was markedly increased in the WKY-STZ, SHR-CON, and SHR-STZ as compared to WKY-CON rats (Fig. 7E).

### 3.9. Diabetes and hypertension mildly enhanced Nrf-2/HO-1 in the penis

The western blot data demonstrated that upstream Nrf-2 protein expression was significantly higher ($p < 0.01$) in WKY-STZ (1.100 ± 0.040 fold, n = 6), SHR-CON (1.153 ± 0.036 fold, n = 6), and SHR-STZ (1.330 ± 0.065 fold, n = 6) as compared to that in WKY-CON (1-fold, n = 6) (Fig. 8A, B). The Nrf-2 level in SHR-STZ was higher ($p < 0.05$) than WKY-STZ (Fig. 8B).

As shown in Fig. 8C, D, the western blot showed that the downstream HO-1 protein expression was significantly higher ($p < 0.01$) in, SHR-CON (1.123 ± 0.015 fold, n = 6) and SHR-STZ (1.390 ± 0.220 fold, n = 6) as compared to that in WKY-CON (1 fold, n = 6) or WKY-STZ (1.052 ± 0.060 fold, n = 6). The HO-1 expression level in SHR-STZ was higher ($p < 0.05$) than WKY-STZ or SHR-CON (Fig. 8D). Immunohistochemistry data displayed a consistent finding that the HO-1 stain was markedly increased in the WKY-STZ, SHR-CON, and SHR-STZ as compared to WKY-CON rats (Fig. 8E).

### 3.10. Artificial NO donor, CCL5, improved ED

CCL5 is an artificial NO donor (Fig. 9A), which can confers NO directly. In combination with electrical stimulation of carvenous nerve, CCL5 dose-dependently and significantly increased ICP in the severe ED of SHR-STZ rats (Fig. 9B, C). Immunohistochemical data indicated the eNOS expression was clearly displayed in the dorsal artery of WKY-CON and SHR-CON rats. However, the eNOS expression was markedly decreased in WKY-STZ and SHR-STZ rats (Fig. 9D, E). As compared to L-arginine treatment, CCL5 significantly improved ED by the increase of ICP in WKY-STZ, SHR-CON, and SHR-STZ rats (Fig. 9F).

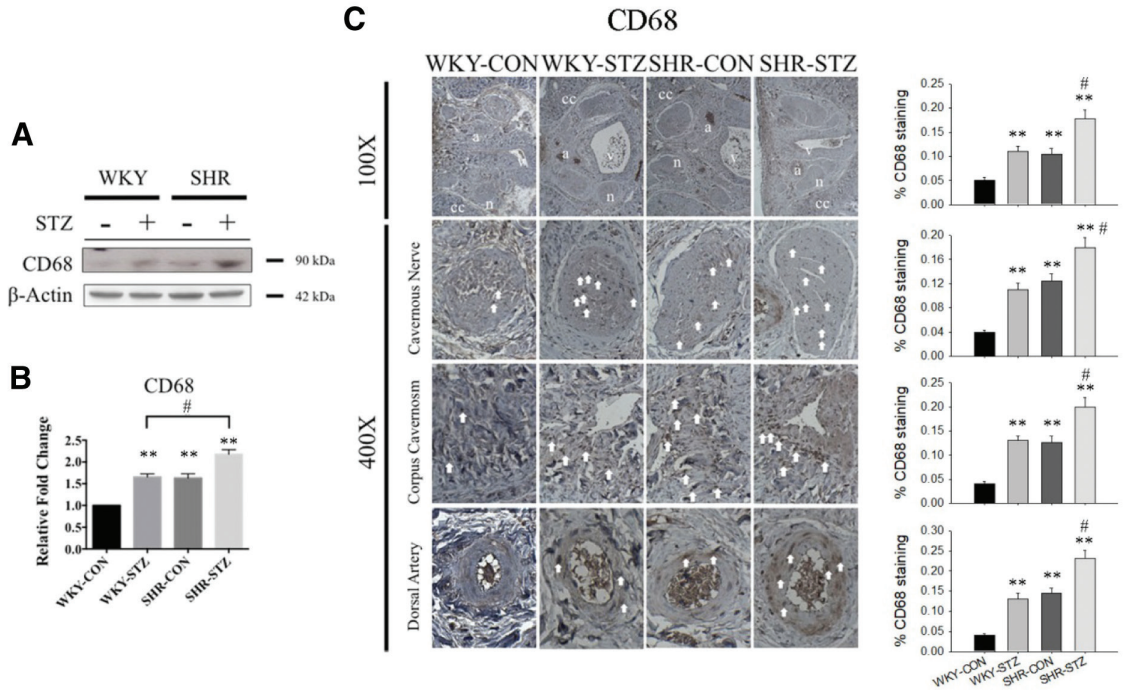


**Fig. 5** Western blot (A), statistic data (B), and immunohistochemistry (C) of CD68 expression in four groups of rats. Data are expressed as mean ± SEM (n = 6) using the single values. $^{**}p < 0.01$ vs WKY-CON; #$p < 0.05$ vs WKY-STZ.

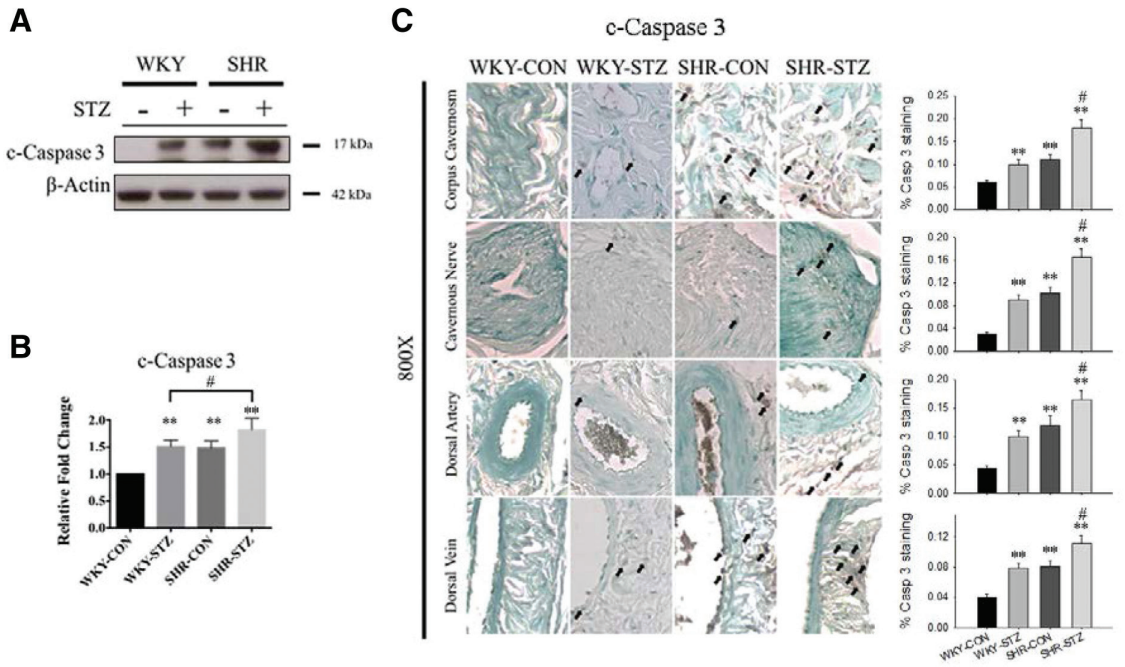


**Fig. 6** Western blot (A) and statistic data of cleaved-Caspase 3 expression (B) and TUNEL immunohistochemistry (C, brown color indicated with arrows) of four groups of rats. Data are expressed as mean ± SEM (n = 6) using the single values. $^{**}p < 0.01$ vs WKY-CON; $\#p < 0.05$ vs WKY-STZ. TUNEL = transferase-mediated nick-end labeling.

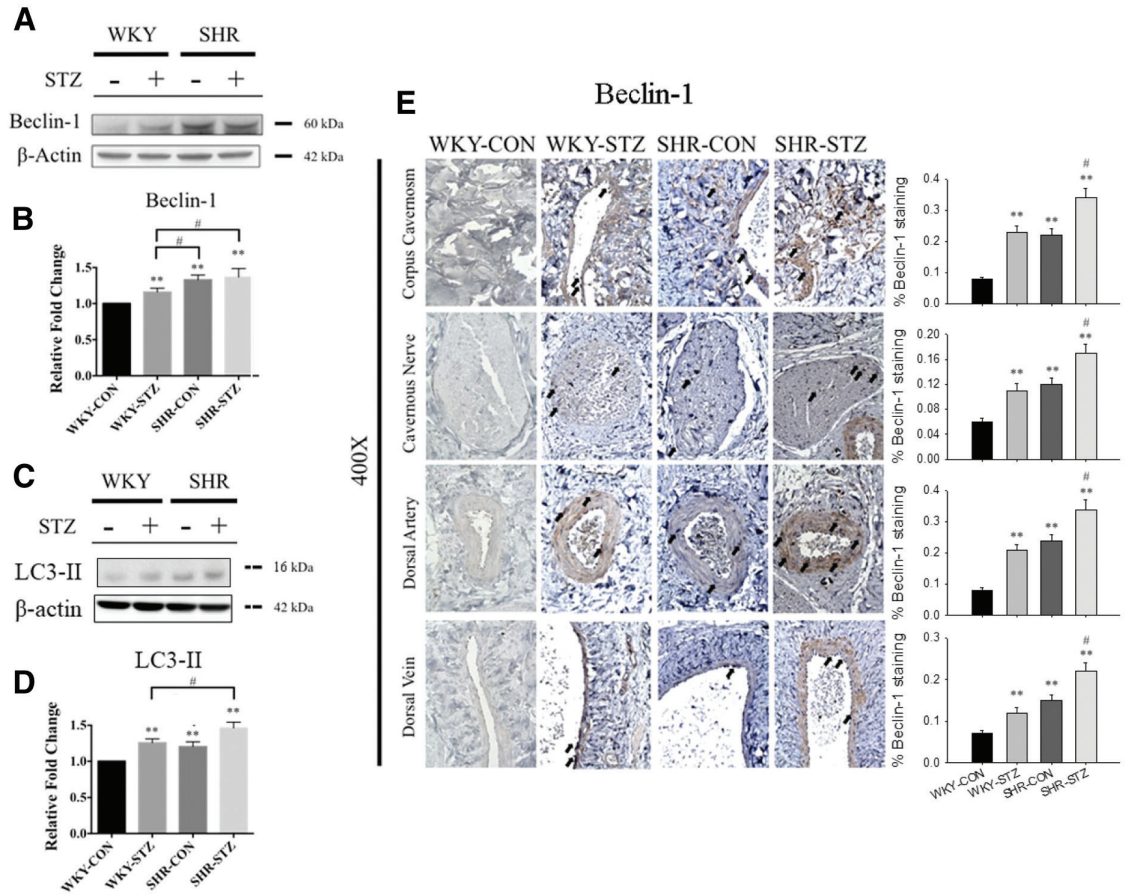


**Fig. 7** Western blot (A), statistic data (B), and immunohistochemistry (E) of Beclin-1 expression and western blot (C) and statistic data (D) of LC3-II in four groups of rats. Data are expressed as mean ± SEM (n = 6) using the single values. $^{**}p < 0.01$ vs WKY-CON. $\#p < 0.05$ vs WKY-STZ.

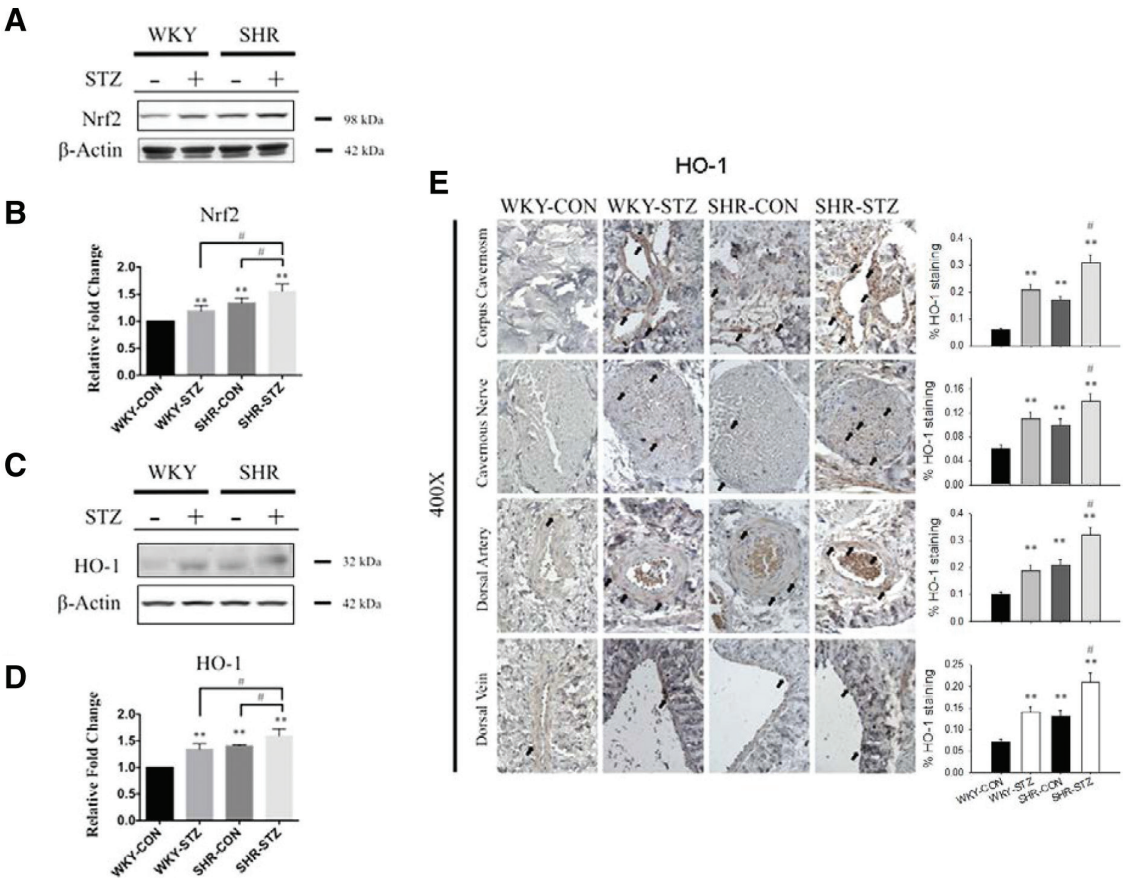


**Fig. 8** The western blot of Nrf-2 (A and B) and HO-1 expression (C and D) and HO-1 immunohistochemistry (E) in the four groups of rat. Data are expressed as mean ± SEM (n = 6) using the single values. *$p < 0.05$ vs WKY-CON, **$p < 0.01$ vs WKY-CON. # $p < 0.05$ vs WKY-STZ.

## 4. DISCUSSION

We established one animal model in this study for simultaneous recording of MAP, ICP, and the intra-cavernous NO amount in rats. This model has not been reported before, possibly due to the difficulty in surgical technique and available equipment for NO amount determination. Using this model we aimed to directly determine the role and response of NO and eNOS and hemodynamics in erectile function during hypertension and/or diabetes. This model is quite important and useful in screening novel drugs or treatments for their future therapeutic potential on ED.

Increased oxidative stress was displayed by the increased blood ROS and the increased CD68 and mast cells infiltration in the penis, possibly inducing penile apoptosis and autophagy and subsequently leading to ED. In diabetes increased mast cells may release histamine–mediated oxidative stress and/or inflammation to desensitize renal chemoreceptors and mechanoreceptors with the decreased basal discharge and responses to several stimulations, leading to sensory neuropathy.[27] However, antihistamine treatment or chronic insulin treatment recovered the sensory neuropathy in diabetic rats.[27] Furthermore, increased mast cell number and degranulation could trigger ICAM-1 expression and leukocyte adhesion to the venous endothelium to enhance ROS level in the inflamed tissue.[28] In the inflamed tissue, increased NF-κB/ICAM-1 expression, ROS amount, neutrophils adhesion to venous endothelium, CD68 (monocyte/macrophage) and mast cell infiltration were observed.[29] We also found these inflammatory markers in the penis of diabetes and/or SHR rats in the present study.

Several epidemiological studies reported that ED is a marker of cardiovascular diseases.[1] After sexual stimuli, the NO concentration was significantly increased because of its release from the cholinergic and non-noradrenergic, non-cholinergic fibers and the endothelium.[1] Enhanced $O_2$–. production and reduced basal NO activity augmented vasoconstriction in macrovascular penile arteries and coronary arteries and the severity of the structural and functional abnormalities in penile arteries might anticipate the vascular dysfunction of the more preserved coronary vascular bed.[30] Our data implicated that hypertension and/or diabetes–induced ED mainly through the decreased eNOS expression and NO production. Our immunocytochemical results found eNOS expression in the vascular beds of corporal cavernosum and dorsal artery was significantly decreased after diabetes and/or hypertension. Our data further demonstrated that exacerbated oxidative stress, inflammation, apoptosis, and autophagy were found in the cavernous nerve, corpus cavernosum, and dorsal artery of the SHR-STZ penis resulting in functional and structural impairment in the penis. In SHR rats, acetylcholine–mediated relaxation of corporal cavernous strips was significantly impaired for a defect in endothelium-dependent reactivity and a reduction in NO.[31] Diabetes downregulates eNOS expression by mediate activation of the RhoA/Rho-kinase pathway.[32] Our data from direct recording also showed the lowest NO amount in the corpus cavernosum of the SHR-STZ penis. Diabetes combined with hypertension synergistically exacerbated the impairment of endothelial NO activity.

The increased $O_2^-$ may react with NO to produce peroxynitrite ($ONOO^-$), a powerful oxidant that further decomposes to hydroxyl ($HO^·$) and impairs cavernous nerves, vessels, and corpus cavernosum of the penis.[33] According to our data, diabetes and/or hypertension evoked the increased oxidative stress,

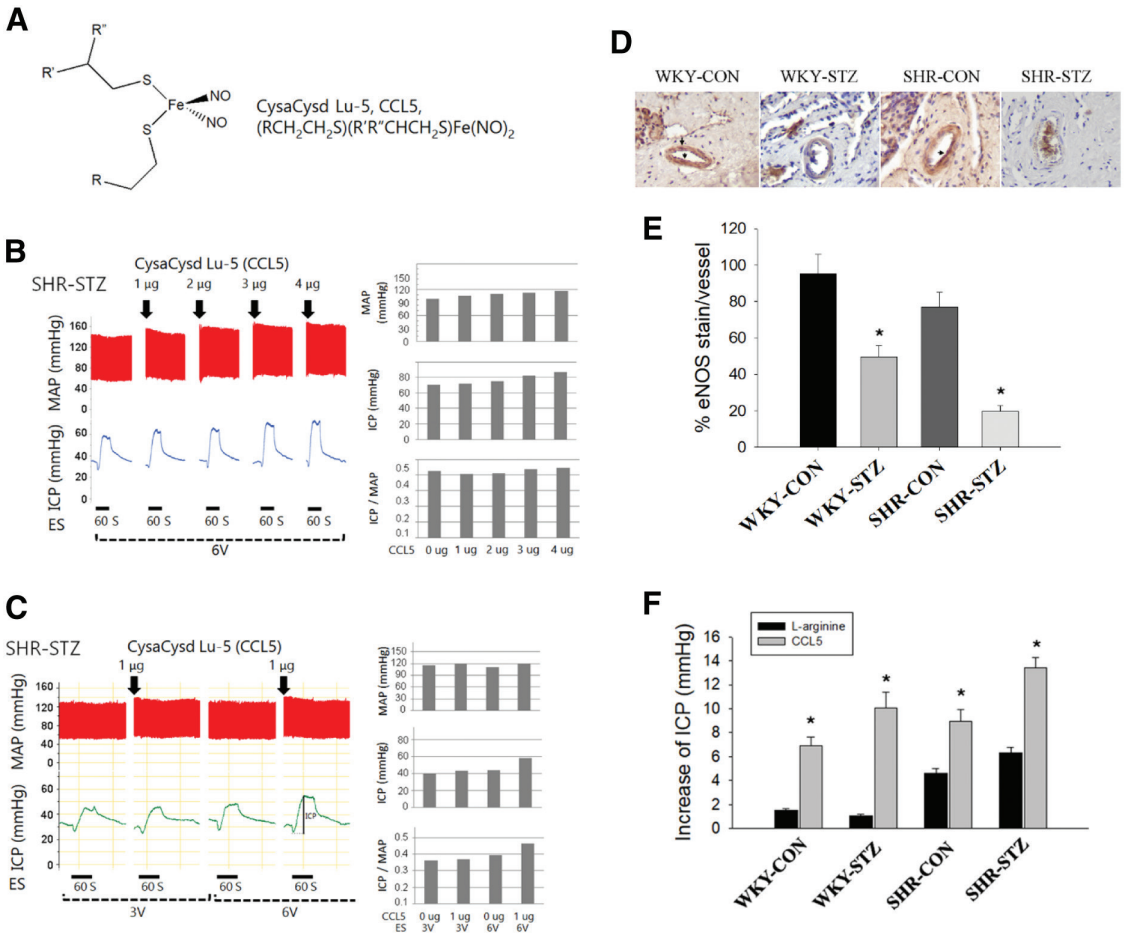


**Fig. 9** The CysaCysd Lu-5 (CCL5) (A) chemical structure. CCL5 dosage effect combined with cavernous nerve electric stimulation on MAP, ICP, and ICP/MAP response (B and C) in one SHR-STZ rat. Effect of diabetes and/or hypertension on the eNOS expression (brown color indicated by the arrows) in the arteries of WKY-CON, WKY-STZ, SHR-CON, and SHR-STZ (D). The eNOS expression in these arteries of four groups is indicated in E. Statistical data for increased ICP using L-arginine or CCL5 in these four groups of rats (F). *$p < 0.05$ vs L-arginine treated WKY-CON.

inflammation, apoptosis, and autophagy in the cavernous nerve, corpus cavernosum, and dorsal artery of the SHR-STZ penis. All structural and functional alterations by these oxidative parameters could decrease the eNOS expression and NO amount, subsequently leading to lower ICP level and ED. Our results displayed that intravenous administration of L-arginine, a NO donor, markedly increased the ICP and NO amount in hypertensive or diabetic rats. In addition, using a novel NO donor CCL5 further increased the ICP level compared to L-arginine. Using eNOS inhibitor (L-NAME) significantly depressed ICP and NO in all rats consistently demonstrating the therapeutic role of NO.[20] We suggest that NO donor may improve ED directly through the increased NO amount. Further mechanistic studies are required to clarify the role of NO donor on the interaction between inflammation, apoptosis and autophagy in the rat ED model.

Elevated MAP–like SHR induced hypertension could induce ED with the decreased the level of maximum penile intracavernous pressure/MAP (ICPmax/MAP) by the decreased eNOS and HSP90 interaction,[34] and a lower eNOS expression in sinusoidal endothelium.[35] These messages informed that elevated MAP could reduce ICP by the functional and structural alteration in vessels as well as in the cavernous spaces of the erectile tissue. Our data indicated that the decreased level of ICP was displayed in the order of SHR-STZ > WKY-STZ = SHR-CON > WKY-CON indicating the combination of hypertension and diabetes further impaired the erectile function. In comparison with other studies on chronic diabetes mellitus induction for 24 weeks[8] or at least 10 weeks,[36] the ICP in our animal model with 4-week STZ induction was not severely decreased. Long-term hypertension and diabetes action would further damage the penis and decrease the erectile function.

On the other hand, by recording ICP, erectile responses to nerve stimulation at peripheral sites can be evaluated objectively in our anesthetized animals. It is thereby possible to isolate and study the effects of autonomic nervous system regulatory mechanisms on erection, and to quantify the pharmacological modulation effect on nerve–induced responses as changes in ICP. However, in the conscious rodent model, erectile responses include activities from supraspinal and spinal regulatory units, which may result in enhancement of both autonomic and somatic pathways to the corpus cavernosum or the penile striated muscles.[37] These components can be evaluated by recording ICP in conscious animals, allowing the investigator to avoid possible deleterious effects of anesthesia on supraspinal and spinal transmitter pathways that may be involved in the regulation of erection.[37]

On the other hand, excess oxidative stress would increase collagen accumulation and smooth muscle cell proliferation and thus reduce blood flow to the penis as well as lower compliance

of the cavernous sinuses.[4,10,38] Our data consistently found that fibrosis occurred in the rat penis with diabetes and/or hypertension. Long-term diabetes associated with hypertension would further increase fibrosis in the penis leading to lower compliance and severe ED. Decreased fibrosis in the penis would be another therapeutic target for ameliorating ED.

The apoptosis and autophagy mechanisms in the penile tissue of STZ-induced diabetic rats or SHR rats may be related to oxidative stress and inflammation regulation. In the corpus cavernosum of the diabetic or hypertensive penis, our data evidenced that the increased CD68, mast cells and proinflammatory NF-κB and ICAM-1 expression levels result in increased ROS production. The increased ROS may act as upstream modulators of autophagy and apoptosis induction. Both autophagy and apoptosis affect cell death and these two important physiological roles can be coordinated. In some cases, autophagy inhibits apoptosis for cell survival, whereas in some cases autophagy induces cell death or interacts with apoptosis to induce cell death. In the present study, we found that DM or SHR enhanced Beclin-1/LC3-II–mediated autophagy and cleavage Caspase 3-mediated apoptosis appearance in the type 1 DM and SHR penis. This finding was consistent with previous reports that in the diabetic rat ED model, autophagy was over-activated and icarisid II improved erectile function partially by reducing autophagy levels.[39,40] However, rapamycin, an autophagy inducer, has been reported to improve erectile function in rats with DM–induced ED, by promoting autophagy, inhibiting apoptosis and fibrotic activity, and ameliorating endothelial function.[41] This discrepancy in the downregulation or upregulation in autophagy signaling in ED penis could be due to the tissue sampling, the duration of disease induction, and the pathologic types of ED models.

Excess oxidative stress may induce the Nrf-2/HO-1 protective mechanism to inhibit apoptosis and autophagy.[13] Although Nrf-2/HO-1 signaling is mildly enhanced after hypertension and/or diabetes, the upregulation seems to be inadequate to confer protection in our data. Our recent results from heart implicated that 8 weeks of diabetes exacerbated ischemia/reperfusion-induced myocardial dysfunction through down-regulated Bcl-2/Nrf-2/HO-1 expression, increased caspase 3 mediated apoptosis-mediated oxidative injury and impaired microvascular reactivity.[24] The mild increase of Nrf-2/HO-1 expression in the corpus cavernosum may be ascribed to an early stage in 4 weeks of diabetics and/or hypertension in our experimental model. Using anti-hypertensive drugs can decrease oxidative stress markers, restore plasma NO abnormality and the protein expressions of eNOS, Nrf-2, and HO-1 in L-NAME treated rats.[42] The data–informed that activating Nrf-2/HO-1 signaling pathway may preserve or upregulate eNOS signaling. In the present study, we found that diabetes associated with hypertension evoked oxidative stress, inflammation, fibrosis, apoptosis, and autophagy in the penis leading to ED. Our present data with CCL5, an artificial NO donor, significantly improved diabetes and/or hypertension–induced ED. We found that CCL5 is more efficient than L-arginine to increase ICP during cavernous nerve stimulation. In the future, we will study the potential therapeutic ability of Nrf-2 activator in hypertension and diabetes–induced ED. Our results are summarized in Fig. 10.

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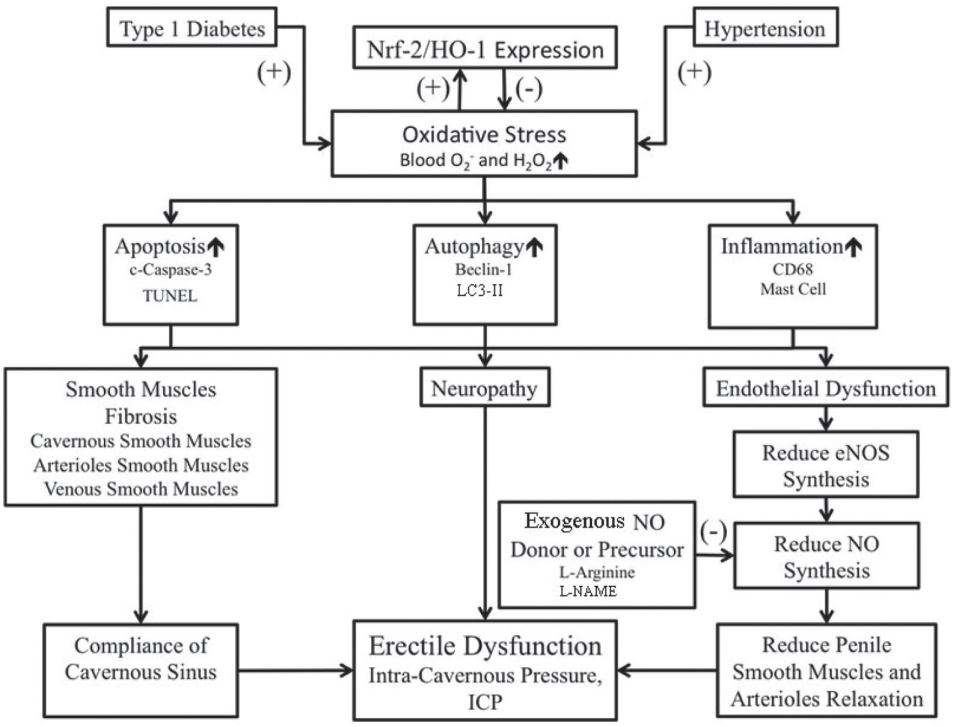


**Fig. 10** The summary diagram is demonstrated. Diabetes and hypertension exacerbate oxidative stress to induce apoptosis, autophagy, and inflammation leading to erectile dysfunction via the smooth muscle fibrosis, neuropathy, and endothelial dysfunction.

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