

## Neural and vascular alterations in the penis of diabetic rats with erectile dysfunction

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**Abstract:** The present study was designed to determine the pathological alterations in the nerves and blood vessels of the penis in diabetic rats with erectile dysfunction (ED). Localization and content of neural nitric oxide synthase (nNOS) and endothelial NOS (eNOS) in the penis were determined by immunohistochemistry. Ultrastructural alterations in the nerves were detected by electron microscopy. Neuropeptide Y (NPY)-expressing nerve fibers in the wall of the internal pudendal arteries were detected by immunohistochemistry. Plasma NPY was determined by radioimmunoassay. Endothelium-dependent vascular function was examined in isolated corpus cavernosum. Compared with control and insulin-treated rats, there was a significant decrease in nNOS and eNOS expression in the dorsal nerve of the penis and the endothelial lining and the cavernous sinusoids in the penis of diabetic rats. The total number of myelinated fibers and G ratio in the pudendal nerve of diabetic rats significantly decreased when compared to that of the control and insulin-treated group. Besides, there was a significant increase in NPY content in the plasma and expression in the nerves located in the wall of the internal pudendal artery. The relaxation response of corpus cavernosum strips to acetylcholine, but not sodium nitroprusside, was impaired in diabetic rats and was restored in insulin-treated animals. The decreased level of NO might lead to degenerative alterations of the pudendal nerves that innervate the penis. These changes, together with the elevated NPY expression and decreased eNOS expression in the blood vessels might comprise the pathological neural and vascular basis of impaired vascular relaxation of the cavernous penis, which results in ED.

[Zhong MF, Wang WJ, Hu JJ, Li F, Ding WL. **Neural and vascular alterations in the penis of diabetic rats with erectile dysfunction.** *Life Sci J* 2013;10(2):2792-2797] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 387

**Key words:** corpus cavernosum function, diabetes mellitus, erectile dysfunction, neuropeptide Y, nitric oxide synthase

### Introduction

Diabetic patients have an increased risk of vascular and neural dysfunction. Vasculopathy results in endothelial-cell abnormalities that involve reduced production or action of vasodilators, such as nitric oxide (NO), and altered responses to vasoconstrictors, such as neuropeptide Y (NPY) (Cai and Harrison, 2000; Cameron and Eaton, 2001). Hyperglycemia, oxidative stress, and altered lipid profiles contribute to vascular complications, including peripheral nerve perfusion deficits, which play an important role in the etiology of diabetic neuropathy (Cameron and Cotter, 1997; Cameron and Eaton, 2001; De Young and Yu, 2004).

Endothelial and nonadrenergic and noncholinergic (NANC) nerve-derived, NO-mediated, smooth-muscle relaxation is diminished by diabetes in the corpus cavernosum of animal models and humans (Andersson and Wagner, 1995; Azadzo and Saenz de Tejada, 1992; Bivalacqua and Champion, 2000; Gocmen and Secilmis, 2000; Keegan and Cotter, 1999; Saenz de Tejada and Goldstein, 1989). Normal erectile function involves nerve-mediated increases in arterial inflow to the corpus cavernosum, relaxation of smooth muscle, and restriction of venous outflow. NANC nerves provide the majority of NO during erection; however,

neuropeptides and vasodilators released from the endothelium (including NO) also play a role physiologically (Andersson and Wagner, 1995; Bivalacqua and Champion, 2000).

Penile erection results from a complex interaction between nervous and local factors that regulate the tone of trabecular smooth muscle and penile vasculature (Andersson and Wagner, 1995; Simonsen and Garcia-sacristan, 2002). Sympathetic nerves cause detumescence of the erect penis and also maintain the penis in a flaccid state (Andersson and Wagner, 1995; Giuliano and Bernabe, 1993). Noradrenaline (NA) released from nerves contracts trabecular smooth muscle through  $\alpha$ 1-adrenoceptors, and penile cavernous and helicine arteries through a heterogeneous population of  $\alpha$ 1-adrenoceptors (Andersson and Wagner, 1995; Bivalacqua and Champion, 2000; Simonsen and Prieto, 1997). NPY is usually co-localized with NA in sympathetic perivascular nerves and contributes to the vasoconstriction elicited by activation of sympathetic nerves (Edvinsson and Ekblad, 1984).

The main arterial supply to the penis is via the internal pudendal artery, which branches off the hypogastric artery and splits into the bulbourethral,

dorsal and cavernosal arteries (Andersson and Wagner, 1995; Simonsen and Garcia-sacristan, 2002). Immunohistochemical studies have demonstrated the presence of numerous NPY-immunoreactive nerves in the erectile tissues of the penis, with a high density around the helicine arteries (Kirkeb and Jorgensen, 1991; Wespes and Schiffmann, 1988; Schmalbruch and Wagner, 1989).

Initially, NPY was suggested to play a role in detumescence (Giuliano and Bernabe, 1993). Despite the evidence for a rich NPY-peptidergic innervation in penile resistance or helicine arteries, no information is available concerning the role of NPY in the regulation of the tone of these arteries. Penile resistance arteries play a major role in the physiology of erection since they act as sphincters by regulating the blood flow between the systemic circulation and the cavernous sinusoids (Andersson and Wagner, 1995; Simonsen and Garcia-sacristan, 2002). NO of neural and endothelial origin is one of the main vasodilators in these arteries, whereas NA released from sympathetic nerves is a powerful vasoconstrictor (Simonsen and Prieto, 1997).

The aims of the present study were to investigate: NPY expression in nerves located in the wall of the internal pudendal artery and NPY volume in plasma in rats with experimental diabetes; changes in nNOS and eNOS expression in nerve fibers and the penis, respectively; and the relaxation response of the corpus cavernosum to vasoconstrictors and vasodilators.

## Methods

### Animals

All experimental procedures were performed under protocols approved by the Animal Care Committee of the Animals Center at the Chinese Academy of Science in Shanghai, and by the Committee on the Care and Use of Animals in Research at Shanghai Jiao Tong University School of Medicine.

Male Sprague–Dawley rats at 2 months of age, obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Science, were housed individually in a temperature-controlled room with a 12:12-h light–dark cycle. Water and rat chow were provided *ad libitum*. The animals were divided into three groups: diabetic, age-matched controls, and insulin-treated rats. The control group (n=6) received 0.1 mol/L citrate buffer, the solvent of streptozotocin (STZ), intraperitoneally (i.p.). In the second group (n=6), diabetes mellitus (DM) was induced with STZ (65 mg/kg body wt i.p.; Sigma, St. Louis, MO, USA). In the third group (n=6), diabetic rats received daily evening injections of ultralente insulin (Iletin II, 10–20 U/kg/day; Eli Lilly, Indianapolis, IN, USA) in doses individually adjusted to maintain blood glucose levels between 5 and 7 mM. Forty-eight hours after STZ treatment, rats with blood glucose level >16.8 mmol/L

were considered diabetic.

### Immunohistochemistry

Penis segments not used in the in vitro study were immersed in 4% paraformaldehyde and embedded in paraffin for detection of nNOS and eNOS. Immunohistochemical detection of NPY was performed on segments of the internal pudendal artery. Whole-mount preparations were prepared after removal of the surrounding adipose and connective tissue. Segments of artery were opened longitudinally and pinned to the base of a dish filled with Sylgard elastomer, with the adventitial side facing upward. Tissues were then immersed in 4% paraformaldehyde and embedded in paraffin within 48 h. Histological sections, 5- $\mu$ m thick, prepared from paraffin-embedded tissue samples were used for immunohistochemical analysis. nNOS, eNOS and NPY expression was determined using antibodies against nNOS, eNOS and NPY. Sections were incubated in primary antibodies: monoclonal anti-eNOS antibody (1:1000 dilution; Sigma); polyclonal anti-nNOS antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); or polyclonal anti-NPY antibody (1:200 dilution; Boster, China). After incubation with the primary antibodies overnight at 4°C, sections were rinsed three times, followed by incubation with horseradish-peroxidase-complex-ligated secondary antibody. Sections were incubated with 3,3'-diaminobenzidine substrate and finally counterstained with Gill's hematoxylin before sealing and microscopic examination.

### Transmission electron microscopy (TEM)

The pudendal nerve was cut into 1-mm<sup>3</sup> blocks and fixed in 2% glutaraldehyde over 48 h at 4°C. The blocks were post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon 618 (Ladd Research Industries, Burlington, VT, USA). Random areas with transverse and longitudinal parts identified on semithin sections were selected for TEM. Ultrathin sections were cut at 50–80 nm and stained with lead citrate and uranium acetate, and observed under a Philips CM-120 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV. Three blocks of pudendal nerves from each group were sectioned and used for TEM.

### Plasma NPY concentrations

Blood samples were taken in an anticoagulant tube following overnight fasting. Antipeptide enzyme (500 U) was added to the tube and mixed evenly. The blood samples were centrifuged at 3500–4000 rpm for 15 min. The supernatants were transferred to another tube and stored at –70°C until use. Plasma NPY concentration was measured by radioimmunoassay methods using commercial kits.

### Functional study

The functional studies were performed as

previously described. Each tissue strip was suspended in a 10-mL bath filled with Krebs–Henseleit solution: 118.0 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L MgSO<sub>4</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 25.0 mmol/L NaHCO<sub>3</sub>, 11.0 mmol/L glucose and 0.5 mmol/L Na<sub>2</sub>-EDTA at 37°C, which was continuously gassed with a 95% O<sub>2</sub>/5% CO<sub>2</sub> mixture throughout the course of all functional studies. One end of each strip was connected to a force-displacement transducer and the isometric forces were recorded and analyzed with the MacLab/8c system. Each strip was stretched until optimal resting tension (0.1–0.2 g/mm<sup>2</sup>) developed, and then was allowed to equilibrate for 30 min before starting the experiments. The response to 80 mmol/L KCl was assessed two or three times at 10-min intervals until the response was reproducible.

For evaluation of muscle contractility in the three groups, concentration–response curves for 10  $\mu$ mol/L phenylephrine (Sigma) were constructed. For evaluation of endothelium-dependent relaxation in the three groups, the concentration–response curves to 2  $\mu$ mol/L acetylcholine (Sigma) in the strips pre-contracted with 10  $\mu$ mol/L phenylephrine were constructed. Concentration–response curves to 1  $\mu$ mol/L sodium nitroprusside (Sigma) in the strips pre-contracted with 10  $\mu$ mol/L phenylephrine were constructed. Relaxation was expressed as the percentage of the pre-contraction tension induced by phenylephrine.

#### Statistical analysis

All results are expressed as the mean  $\pm$  SEM. Statistical analysis was performed by ANOVA.  $P < 0.05$  was considered statistically significant.

### Results

#### Body weight and blood glucose

Blood glucose ( $25.38 \pm 5.36$  and  $25.60 \pm 1.19$  for 7-week and 12-week groups, respectively) in STZ-treated rats was significantly higher than that in the control and insulin-treated groups ( $5.25 \pm 0.49$  and  $5.30 \pm 0.98$ , respectively). Control and insulin-treated rats exhibited a consistent weight gain throughout the 12 weeks of the study. In contrast, there was a remarkable weight loss in diabetic rats compared to the control and insulin-treated rats by 12 weeks ( $P < 0.05$ , Table 1).

Table 1. Blood glucose and body weight of 12 weeks rats (Mean $\pm$ SD)

Group	Blood glucose (mM)	Weight (g)
C	$5.33 \pm 0.68$	$656.67 \pm 6.06$
DM	$25.60 \pm 1.19^{* \#}$	$431.67 \pm 26.13^{* \#}$
IT	$5.56 \pm 0.31^{*}$	$650.00 \pm 7.07^{*}$

\* vs C,  $P < 0.01$ ; # vs IT,  $P < 0.05$ ;

#### nNOS expression in the pudendal nerve

Strong dark-brown staining of nNOS was seen in the nerve fibers of the pudendal nerve of control rats. There was a significant decrease in nNOS expression in the pudendal nerve of diabetic rats, as suggested by fewer positively stained fibers and lower optical density (Figure. 1, Table 2). The quantity and density of positively stained fibers increased in the insulin-treated group when compared to those in the diabetic rats ( $P < 0.01$ ), but was lower than those in the controls ( $P < 0.01$ ).

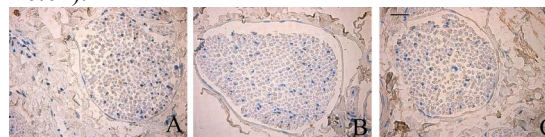


Figure 1. Penile erection induced by Apomorphine injection (Mean $\pm$ SD). The total times of penile erection during the 30-minute recording significantly reduced in diabetic groups compared to that of control. \* \*  $P < 0.01$ , vs control; #  $P < 0.05$ , vs 8 weeks DM group.

Table 2. nNOS expression in the dorsal nerve of the penis (Mean $\pm$ SD)

Group	nNOS-positive nerve fibers	Density
C	$80.67 \pm 2.16$	$184.41 \pm 11.47$
DM	$25.83 \pm 3.66^{* \#}$	$81.75 \pm 10.26^{* \#}$
IT	$62.50 \pm 1.87^{*}$	$162.59 \pm 6.13^{*}$

\* vs C,  $P < 0.01$ ; # vs IT,  $P < 0.05$ ;

#### Alterations in the ultrastructure of the pudendal nerve

By 7 weeks after DM induction, the compact myelin sheath was disrupted. This alteration was even more severe in 12-week DM rats. G ratio and the number of myelinated fibers were also reduced significantly in diabetic rats, and were even lower in the nerves of 12-week DM rats. Insulin treatment reduced significantly the number of disrupted myelin sheaths, and also elevated the G ratio (Figure. 2).

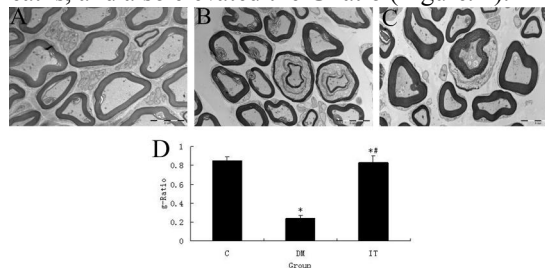


Figure 2. iNOS expression in blood vessels of penis. Positively stained cells were indicated by arrows. There was a significant increase in iNOS expression in the endothelium of the blood vessels of diabetic groups. Scale bar=25 $\mu$ m. A: 8 weeks control; B: 8 weeks DM; C: 12 weeks control; D: 12 weeks DM.

### NPY content in the plasma and nerves in the wall of the internal pudendal artery

NPY nerve fibers were found in the wall of the internal pudendal artery of normal rats as thin brown fibers with few nerve bulks. There was a significant increase in the number of NPY-positive nerve fibers, as suggested by wider arborization and more nerve bulks. Insulin treatment, however, reduced significantly the number of NPY-positive nerve fibers (Figure. 3). There was a significant increase in NPY content in the plasma of diabetic rats, which was reduced after insulin treatment (Figure. 3).

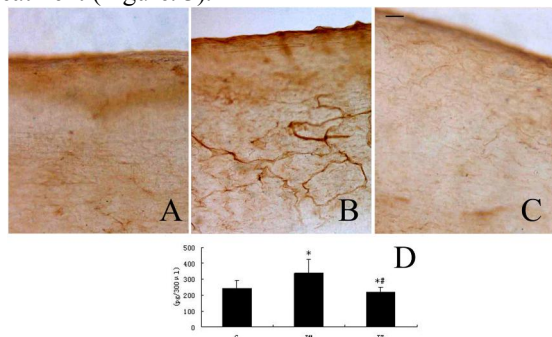


Figure 3. iNOS expression in dorsal nerve of penis. Positively stained cells were indicated by arrows and were found surrounding the axons in the nerve bundles. The number of positively stained cell was significantly increased in diabetic groups. Scale bar=25 $\mu$ m. A: 8 weeks control; B: 8 weeks DM; C: 12 weeks control; D: 12 weeks DM group.

### eNOS expression in the blood vessels of the penis

eNOS expression was detected in the endothelium of penile blood vessels, as suggested by the strong dark-brown staining with anti-eNOS antibody. This expression was decreased significantly in the blood vessels of diabetic rats when compared to that of the controls ( $P<0.01$ ). eNOS expression in 12-week diabetic rats was even lower than that in the 7-week diabetic group. Insulin treatment enhanced eNOS expression in diabetic rats. However, the expression of eNOS in insulin-treated rats was still lower than that in the controls ( $P<0.01$ , Figure. 4, Table 3).

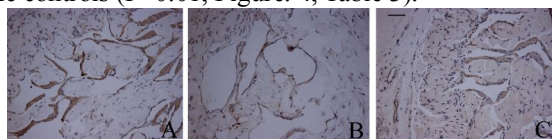


Figure 4. iNOS expression in endothelial of corpus cavernosum. Positively stained cells were indicated by arrows. iNOS expression was significantly increased in the smooth muscles of the corpus cavernosum of diabetic groups. Scale bar=25 $\mu$ m. A: 8 weeks control; B: 8 weeks DM; C: 12 weeks control; D: 12 weeks DM group.

Table 3. eNOS expression in the sinoid of the corpus cavernous penis (Mean $\pm$ SD)

Group	C	DM	IT
OD	20866.14 $\pm$ 679.13	9893.16 $\pm$ 207.90 <sup>#</sup>	16015.18 $\pm$ 436.89 <sup>*</sup>

\* vs C,  $P<0.01$ ; <sup>#</sup> vs IT,  $P<0.05$ ;

### Functional test

The vasodilative function of the corpus cavernosum penis was detected by isometric tension studies. There was a significant reduction in the maximum relaxation induced by acetylcholine in diabetic rats compared with that in the control and insulin-treated groups. In contrast, there were no differences between the three groups in the maximum relaxation induced by the NO donor sodium nitroprusside (Figure. 5).

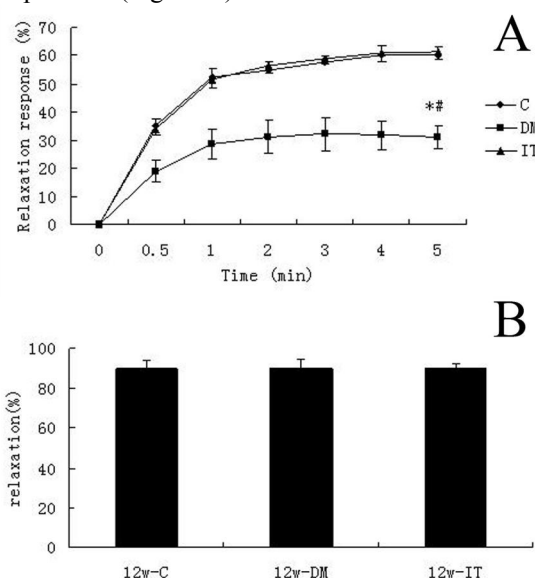


Figure 5. iNOS expression in the penile tissues of control and DM groups (A). Columns in the histogram represent ratio of 3-NT versus  $\beta$ -actin (B). iNOS expression was significantly increased in the penile tissues of diabetic groups when compared to that of control, and was even higher in 12 weeks diabetic animals compared to that of 8 weeks diabetic animals. \*  $P<0.01$ , vs control; #  $P<0.05$ , vs 8 weeks DM group.

### Discussion

The mechanism of DM erectile dysfunction (ED) is very complex and involves a series of neural and vascular alterations, to which a series of small molecules, such as NO and NPY, might contribute.

NO has long been regarded as an important antioxidant that protects cell from oxidative injury. In the present study, expression of nNOS in the pudendal nerve fibers was reduced significantly, accompanied by



disruption in the lamina structure of the myelin sheath. This may have resulted from oxidative injury caused by reduced expression of nNOS, which leads to decreased NO content. Myelin sheath formation by Schwann cells in the peripheral nerve is pivotal to the normal physiological functions of the peripheral nerves. It insulates the axonal surface, facilitates leap conduction, and ensures prompt conduction velocity. Disruption of the myelin sheath in myelinated nerve fibers directly impairs the physiological properties of these nerves, which results in decreased conduction velocity and impaired perception of peripheral sensations. In penile tissue from STZ-diabetic rats, nNOS expression within nitrergic nerves is lost selectively and gradually until apoptosis of the cell bodies within the pelvic ganglia nullifies the effects of restoration of glycemic control by insulin treatment (Cellek and Foxwell, 2003). In our study, the decrease in nNOS expression in the nerves and impaired structure of myelin lamina suggest that neuropathy is a possible cause of diabetic ED.

Vasculopathy caused by DM often results from endothelial dysfunction, which plays an essential role in the physiology of penile erection (Montorsi and Montorsi, 2003; Sullivan and Thompson, 1999). Endothelial cells, located at the interphase between the blood and the vascular wall, play a key role in the regulation of the vascular tone and homeostasis, and have the ability to synthesize short-life vasoactive mediators such as NO (Busse and Fleming, 2003). eNOS is present in the endothelium that lines the cavernous sinusoids and penile blood vessels (Baranowska and Bik, 2006; Kirkeby and Jorgensen, 1991; Prieto and Rivera, 2004; Wespes and Schiffmann, 1988). In our study, decreased expression of eNOS in the endothelial cells that line the cavernous sinusoids in diabetic rats was evident. This indicates that vasculopathy caused by DM another possible cause of diabetic ED.

NO has recently been shown to be the most important transmitter for relaxation of smooth muscle in the penile corpus cavernosum of mammals, and also an important antioxidant (Andersson, 2001). To determine the function of endothelium-derived NO in the mediation of penile erection, relaxation of rat corpus cavernosum was induced by acetylcholine stimulation in an isometric tension study. Our results demonstrated significantly decreased relaxation responses of the penile corpus cavernosum in diabetic rats, which suggests a role for endothelium-derived NO in mediating penile erectile function. However, impairment of smooth muscle function of the penile corpus cavernosum may also lead to a decreased relaxation response. To exclude the possibility of impaired muscle response caused by diabetic lesions, we also induced relaxation of the penile corpus cavernosum with exogenous sodium nitroprusside. It is

known that sodium nitroprusside is the raw material for the synthesis of NO, which activates soluble guanylate cyclase, elevates tissue cGMP level, and relaxes smooth muscles. The present study showed that the penile corpus cavernosum of diabetic rats displayed the same relaxation response to that of control and insulin-treated rats. This suggests normal smooth muscle function and that induction of diabetic ED does not involve the NO-cGMP pathway.

Endothelial dysfunction may be more susceptible to diabetic ED and may even precede hyperglycemia and oxidative damage of nitrergic nerves. Indeed, endothelial dysfunction is thought to be a causative factor for many nerve dysfunctions in DM. In our study, decreased eNOS endothelial cells and nNOS nerve fibers may have been associated with diminished acetylcholine-induced smooth-muscle relaxation.

NPY plays a pivotal role in the control of metabolic homeostasis (Baranowska and Bik, 2006). Besides, it is also an important vasoconstrictor. NPY usually co-localizes with NA in sympathetic perivascular nerves. It is distributed widely in penile erectile tissues, with a particularly high density around the helicine arteries (Kirkeby and Jorgensen, 1991; Prieto and Rivera, 2004; Wespes and Schiffmann, 1988). NPY is also a potent angiogenic factor, as well as a powerful stimulator of vascular-smooth-muscle proliferation and atherogenesis in vitro and in vivo (Abe and Tilan, 2007). It has also been shown that NPY has a direct vasoconstrictive effect on vascular smooth muscle and enhances the NA-induced vasoconstrictive response through the Y1 receptor in vitro (Tseng and Robertson, 1988). In vivo, blood pressure increases after NPY infusion, as a result of constriction of resistance vessels (Corder and Lowry, 1986).

DM is associated with an increased incidence of macro- and microvascular diseases (Colwell and Halushka, 1979). Apart from noradrenergic innervation, the vasculature also receives peptidergic innervation by neuropeptide-containing nerve fibers. The present study demonstrated higher blood plasma NPY levels in diabetic rats than in control and insulin-treated rats. Besides, our results also demonstrated increased NPY nerve fibers in the wall of internal pudendal arteries of diabetic rats. The elevated content of NPY in the plasma and the increased number of NPY-positive nerve fibers might lead to reduced blood perfusion during penile erection, by increasing the resistance of penile arteries.

In summary, the present study indicated severe disruption in the lamina of the myelin sheath of the pudendal nerve. This, together with elevated NPY expression and decreased eNOS expression in the blood vessels might constitute the neural and vascular pathological basis of impaired vascular relaxation of

the cavernous penis, which results in ED.

### Acknowledgement

This work was supported by the national science foundation of china. Project number: 81271380.

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