

·Original Article·

## Effects of icariin on erectile function and expression of nitric oxide synthase isoforms in castrated rats

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### Abstract

**Aim:** To investigate the effect of icariin on erectile function and the expression of nitric oxide synthase (NOS) isoforms in castrated rats. **Methods:** Thirty-two adult male Wistar rats were randomly divided into one sham-operated group (A) and three castrated groups (B, C and D). One week after surgery, rats were treated with normal saline (groups A and B) or oral icariin (1 mg/[kg·day] for group C and 5 mg/[kg·day] for group D) for 4 weeks. One week after treatment, the erectile function of the rats was assessed by measuring intracavernosal pressure (ICP) during electrostimulation of the cavernosal nerve. The serum testosterone (ST) levels, the percent of smooth muscle (PSM) in trabecular tissue, and the expression of mRNA and proteins of neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and phosphodiesterase V (PDE5) in corpus cavernosum (CC) were also evaluated. **Results:** ICP, PSM, ST and the expression of nNOS, iNOS, eNOS and PDE5 were significantly decreased in group B compared with those in group A ( $P < 0.01$ ). However, ICP, PSM and the expression of nNOS and iNOS were increased in groups C and D compared with those in group B ( $P < 0.05$ ). Changes in ST and the expression of eNOS and PDE5 were not significant ( $P > 0.05$ ) in groups C and D compared with those in group B. **Conclusion:** Oral treatment with icariin (> 98.6 % purity) for 4 weeks potentially improves erectile function. This effect is correlated with an increase in PSM and the expression of certain NOS in the CC of castrated rats. These results suggest that icariin may have a therapeutic effect on erectile dysfunction. (*Asian J Androl* 2005 Dec; 7: 381–388)

**Keywords:** icariin; corpus cavernosum; intracavernosal pressure; nitric oxide synthase; erectile dysfunction

### 1 Introduction

Specific phosphodiesterase V (PDE5) inhibitors have been demonstrated to be safe and effective for erectile dysfunction (ED) management. These agents are commonly considered as the first line therapy for this disease.

However, the clinical effect of a PDE5 inhibitor requires at least a minimal nitric oxide (NO) signal triggered by sexual stimulation. This, in turn, causes the induction of cGMP turnover. Hence, without sexual stimulation, the PDE5 inhibitor itself is unable to increase intracellular cGMP synthesis and induce erection [1, 2].

The pathological conditions of NO synthesis involve cavernosal nerve injury during radical prostatectomy or severe diabetic neuropathy [3, 4], which can lead to ED; treatment with PDE5 inhibitors has had limited success even under sexual stimulation. Therefore, direct enhance-

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Received 2004-11-12 Accepted 2005-03-11

ment of NO synthesis may be an effective approach for the treatment of ED [5, 6].

The nitric oxide synthase (NOS) family includes two “constitutive”,  $\text{Ca}^{2+}$ -dependent enzymes. They are originally discovered in neurons (nNOS) and endothelial cells (eNOS) and are also present in the corpus cavernosum (CC) [2]. The third, a  $\text{Ca}^{2+}$ -independent enzyme, an “inducible” isoform (iNOS) was also found in human and rat penis [7]. Previous studies have demonstrated that the expressions and activities of NOS isoforms in the CC are well correlated with erectile function [5, 6].

Epimedium herba has been used as a tonic drug in traditional Chinese medicine (TCM) for centuries. This medicinal herb is believed to be a potent enhancer of erectile function. However, it is difficult to perform a prospective, placebo-controlled study to determine its safety and efficacy as well as to investigate its mechanism on ED because of multiple compounds in this herb. Icariin ( $\text{C}_{33}\text{H}_{40}\text{O}_{15}$ , molecular weight: 676.67) has been determined to be the main active component of Epimedium herba [8].

In our previous study, we observed that icariin induced relaxation of the isolated CC of rabbits in a dose-dependent manner [8]. Such relaxation could be inhibited by NOS specific inhibitor,  $\text{N}^{\omega}$ -Nitro-*L*-arginine (*L*-NNA). We also observed that icariin increased intracavernosal pressure (ICP) significantly, which could be conversely inhibited by *L*-NNA, without any influence on the mean arterial pressure (MAP) in another model [9].

Icariin showed a stronger inhibitory effect on cGMP-specific PDE5 than on cAMP-specific PDE4. The inhibition of icariin on PDE5 (0.43 mol/L) was not significantly different from that of papaverine (0.68 mol/L). However, the selective activity of icariin on PDE5 (PDE4/PDE5 of  $\text{IC}_{50}$ ) was 36.93-fold higher than that of papaverine [10]. These results suggested that icariin could increase the activity of NOS and/or PDE5 and thus could ultimately lead to increased cGMP to enhance erectile function. However, the long-term effects of icariin on ED and its exact mechanism remain unknown. Our previous study with a 4-week oral administration of icariin-enriched fraction (51 % purity) on castrated rats showed that this preparation could improve erectile function and increase nNOS and iNOS expression in CC [11].

In the present study, we further purified icariin and investigated its effect on ED by analyzing the serum testosterone (ST) level, cavernous tissue histology and the

mRNA and protein expression of NOS isoforms in castrated rats. We selected castrated rats as our ED animal model because castration may decrease substantially the erectile responses to various stimulations. This castration model has been widely used for the assessment of a variety of agents in ED treatment [11–13].

## 2 Materials and methods

### 2.1 Animals

Thirty-two adult male Wistar rats weighing 250 g–300 g (Grade A, certificate No. scxk11-00-0006) were obtained from the Animal Breeding Center of Beijing Medical University (Beijing, China). The rats were randomly divided into four groups with eight rats each: one sham operated group (A) and three castrated groups (B, C and D). Under anesthesia, rats from groups B, C and D underwent bilateral orchiectomy through a 2-cm midline scrotal incision and both testes were removed. Rats in group A were sham operated. Seven days after the operation, rats from groups A and B were intragastrically administered with normal saline per day for 4 weeks, whereas rats from groups C and D were similarly administered with icariin 1 mg/(kg·day) and 5 mg/(kg·day), respectively for 4 weeks.

### 2.2 Drugs and reagents

Icariin was purified from the extract of Epimedium herba by high-speed counter-current chromatography with a two-phase solvent system composed of n-hexane-n-butanol-methanol-distilled water (1:4:2:6, v/v) as described previously [7, 9]. The purity was higher than 98.6 %. Trizol (Gibco/BRL, Carlsbad, USA) is used to extract RNA from the tissue. Reagents for the reverse transcriptase polymerase chain reaction (RT-PCR) were obtained from Sigma (St. Louis, MO, USA). The PCR primers were synthesized by SBS (Beijing, China). Specific antibodies against nNOS, iNOS and eNOS were products of Santa Cruz (Santa Cruz, CA, USA) and purchased from Zhongshan Biochemical Company (Beijing, China). The antibody against phosphodiesterase type 5A was a product of Fab Gennix (No. PD5A-101AP, Shreveport, LA, USA). The testosterone ELISA assay kit was purchased from DRG. Inc. (Marburg, Germany).

### 2.3 ICP measurement

After orally administered with icariin for 4 weeks, the rats were kept for 7 days for voiding to avoid the

residual effect of icariin. The rats were then anesthetized with intramuscular sodium pentobarbital (50 mg/kg) and the operation was carried out according to Adachi *et al.* [17]. Briefly, the pelvic nerve and the main penile nerve (cavernous nerve) were surgically exposed and stimulated with a square pulse stimulator connected to a platinum bipolar electrode positioned on the erectile nerve. Two 25-gauge butterfly needles were inserted into the CC and carotid artery and the ICP and MAP were recorded with a pressure transducer integrated into a computerized data acquisition system (BioPac system, Goleta, CA, USA) that was calibrated with a manometer to express the response in millimeters of mercury. The unilateral electrical field stimulation of the cavernous nerve was undertaken for 60 s using a square wave stimulator (5 V at a frequency of 15 Hz with square wave duration of 1 ms and the stimulating intervals were 1 min with at least 15 min between stimulations). The effects of icariin on erectile function were evaluated by changes in the ratio of ICP to MAP and the area under the curve (AUC) of ICP.

#### 2.4 Serum testosterone assay

After the assessment of erectile function was completed, a 2-mL blood sample was obtained from each rat and stored at 4 °C. Analysis was carried out according to the user's manual of testosterone ELISA assay kit.

#### 2.5 Smooth muscle staining

After assessing the erectile function and blood sampling, the entire penis including the penile shaft and caudal were excised. The skeletal muscles and the connective tissue were removed. Vertical sections of the middle part of the penile shaft were harvested for tissue histology study with paraffin sections and with Masson's trichrome staining. CC was isolated from the remaining parts of the penis and stored in a liquid nitrogen tank as samples for RT-PCR and Western blot.

To evaluate percent of smooth muscle (PSM) changes, 12 areas were randomly selected from the same tissue section and observed by computerized photomicrographs. The results were analyzed by the ratio of smooth muscle and collagen using Adobe Photoshop 7.0 software (Adobe, San Jose, CA, USA) and presented as the number of pixel counts of muscle or reddish brown areas divided by the pixel count of collagen or green areas. For statistical analysis, the mean of the calculations was used per animal. The hematoxylin and eosin staining was

also performed to make the selected regions comparable.

#### 2.6 RT-PCR

Total RNA of the CC was isolated by means of TRIZOL. Three micrograms of total RNA were used for the amplification of nNOS, eNOS and iNOS by RT-PCR.  $\beta$ -actin was used as an internal control. The PCR primers were designed with computer software as follows: forward 5'-CAACACAAAGGAATGAATCC-3', reverse 5'-GCTGAAAACCTCATCTGC-3' for nNOS (NM\_052799.1, gi|16258810, corresponding to 4487 bp–4005 bp); forward 5'-GGAAATAGAAACAACAGGAACC-3', reverse 5'-AGCGCATACCACTTCAGC-3' for iNOS (NM\_012611.2, gi|31377499, corresponding to 689 bp–1222 bp); forward 5'-CCTAAGTTGCC TTGCATCC-3', reverse 5'-GCTTTACCACTGAGCT AAATCC-3' for eNOS (XM\_342615.1, gi|34853989, corresponding to 3010 bp–3769 bp); forward 5'-CATG TTTGAGACCTTCAACACC-3', reverse 5'-TAATGTC ACGCACGATTTCC-3' for  $\beta$ -actin (BC063166.1, gi|38648901, corresponding to 447 bp–718 bp). The amplification was initiated with incubation at 95 °C for 10 min, followed by 27 cycles consisting of 94 °C for 45 s, 58 °C for 50 s and 72 °C for 60 s, and ended with 72 °C for 10 min. PCR products were detected by electrophoresis on 1 % agarose/ErBr gel. Specificity of RT-PCR products was confirmed by sequencing. The density was measured at five points for each band. Data were presented as the mean density of the mRNA band in question relative to that of the  $\beta$ -actin band in the same sample.

#### 2.7 Western blot

Tissues of the CC were homogenized in ice-cold lyses buffer (25 mmol/L HEPES, pH 8.0, 5 mmol/L EDTA, 1 % sodium deoxycholate, 1 % Triton X-100, 0.1 % SDS, and protease inhibitors). Homogenated tissues (25 % w/v) were centrifuged at 10 000  $\times g$  for 20 min at 4 °C to remove tissue debris without precipitating plasma membrane fragments that contained eNOS. Protein concentration was determined by the Lowry method [14]. Twenty micrograms of total protein were used in SDS-PAGE. Anti-NOS (nNOS, iNOS and eNOS) serum and anti-PDE5 serum were diluted at 1:1000 in Tris-buffered saline (TBS) and used as primary antibodies. A 1:8000 dilution of horseradish peroxidase-labeled rabbit anti-rat IgG fragment was used as a secondary antibody;  $\alpha$ -actin was used as an internal control. Exposure of luminol-reacted membrane onto X-ray films was performed. The films were scanned

with a BioRad GS-670 densitometer and the band intensity was semi-quantified with the UTHSCSA Image Tool for Windows (2.0) (Triad Scientific Inc., Swarthmore Ave, Lakewood, New Jersey, USA). The density was measured at five points for each band. Data were presented as the mean density of the protein band in question relative to that of the  $\beta$ -actin band in the same sample.

2.8 Statistic analysis

The software SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis including the Student-Neumann-Keul's test and the paired *t*-test. The values were presented as the mean  $\pm$  SEM. *P* < 0.05 was considered to be statistically significant.

3 Results

3.1 Effects of icariin on ICP and MAP

The values of the peak ICP after cavernous nerve stimulation were showed in Figure1. No obvious changes were observed for MAP ([102.00  $\pm$  3.22] mmHg). The increase of ICP in castrated rats (group B) was significantly lower than that in the sham-operation group (group A) (*P* < 0.05). Castrated rats treated with icariin (groups C and D) significantly increased the erectile response (ICP) to cavernous nerve stimulation compared with group B, as measured by ICP/MAP and total ICP values.

3.2 Effects of icariin on serum ST level

The mean serum ST levels in groups A, B, C and D were (2.41  $\pm$  0.91) pmol/mL, (0.38  $\pm$  0.15) pmol/mL, (0.38  $\pm$  0.10) pmol/mL and (0.38  $\pm$  0.11) pmol/mL, respectively. The mean ST level was significantly lower in all castrated rats (groups B, C and D) regardless of icariin treatment (*P* < 0.05). No significant differences were seen in the icariin-treated rats (groups C and D) compared

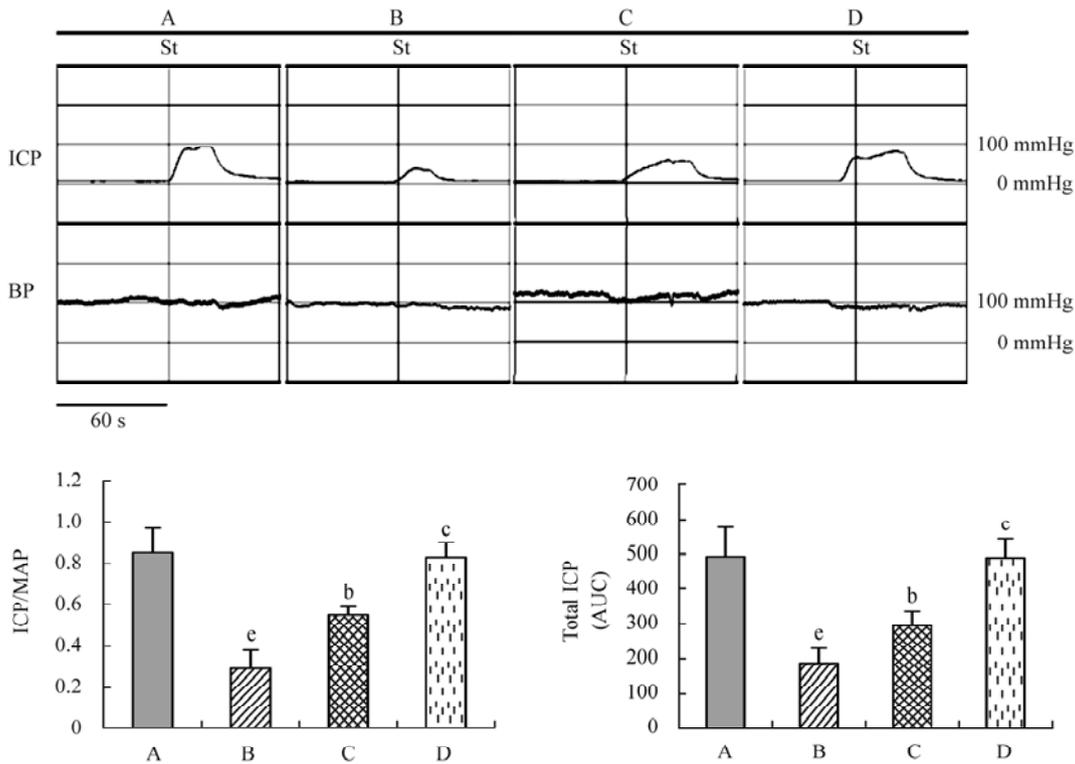


Figure 1. Upper: Representative time course of changes in intracavernosal pressure (ICP) during 5-V cavernous nerve stimulation in the sham-operated rats (A), castrated rats (B) and castrated rats treated with 1 mg/(kg-day) icariin (C) and 5 mg/(kg-day) icariin (D). Lower: Statistic analysis of the effects of icariin on the ICP/mean arterial pressure (MAP) ratio (left) and area under the curve (AUC) (right) of sham-operated rats (A), castrated rats (B) and castrated rats treated with 1 mg/(kg-day) (C) and 5 mg/(kg-day) icariin (D). Mean  $\pm$  SEM (*n* = 8), <sup>b</sup>*P* < 0.05, <sup>c</sup>*P* < 0.01, vs. corresponding castrated rats; <sup>e</sup>*P* < 0.05, vs. corresponding sham-operated rats; St: stimulation.

with untreated rats (group B) ( $P > 0.05$ ).

### 3.3 Effects of icariin on PSM

The mean PSM in the castrated rats (group B) was significantly decreased when compared with the sham-operated rats ( $P < 0.05$ ); however, oral icariin treatment (groups C and D) showed an obvious increase in PSM compared with the untreated rats (group B) ( $P < 0.05$ ) (Figure 2).

### 3.4 Effects of icariin on mRNA expression of NOS isoforms and PDE5

The RT-PCR results (Figure 3) showed that the mRNA of nNOS, iNOS, eNOS and PDE5 were significantly decreased in the castrated rats in group B compared with that in the sham-operated rats (group A) ( $P < 0.05$ ).

However, the nNOS and iNOS mRNA in groups C and D were significantly increased in both icariin-treated rats ( $P < 0.05$ ,  $P < 0.01$ ); however, no obvious changes in the expression of eNOS and PDE5 were observed ( $P > 0.05$ ).

### 3.5 Effects of icariin on protein expression of NOS isoforms and PDE5

The Western blot results (Figure 4). The protein expression of nNOS, iNOS, eNOS and PDE5 was significantly decreased in castrated rats in group B compared with that in the sham-operated rats (group A) ( $P < 0.05$ ). However, the nNOS and iNOS protein expression in groups C and D were significantly increased in both icariin-treated rats ( $P < 0.05$ ,  $P < 0.01$ ); however, no obvious changes in the expression of eNOS or PDE5

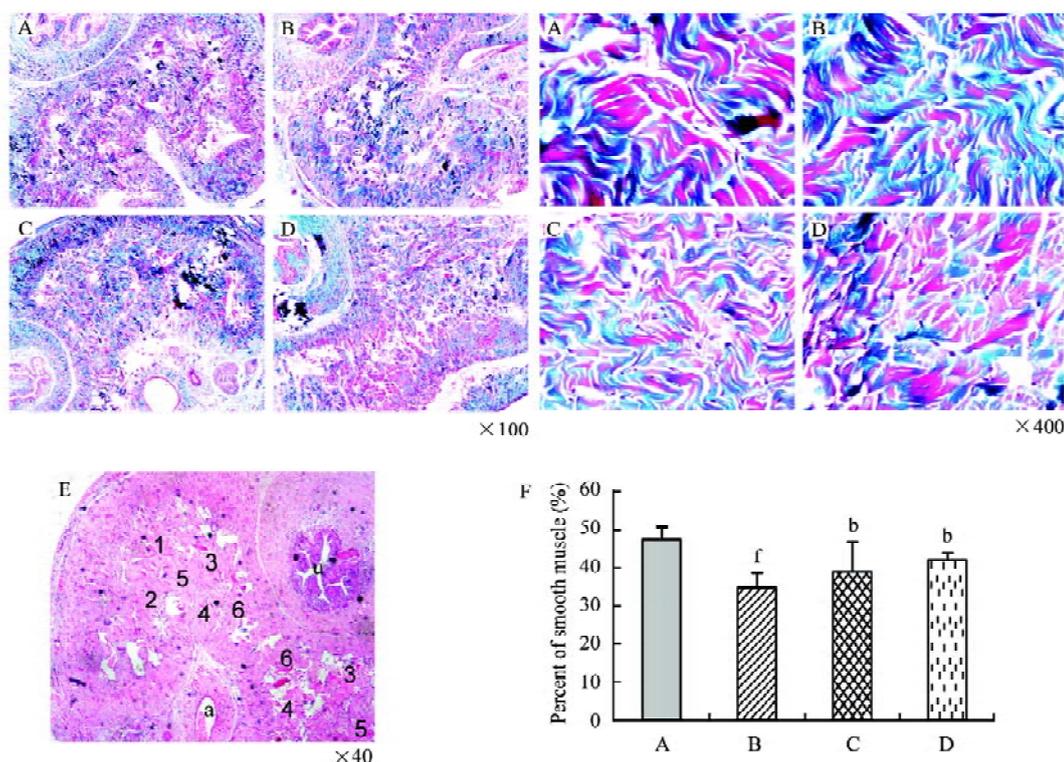


Figure 2. Upper: Masson's trichrome stain showed smooth muscle (red areas), fibrotic tissue (blue areas) and empty space (white areas) of representative rats in group A (A), group B (B), group C (C) and group D (D). (left:  $\times 100$ ; right:  $\times 400$ ). Lower: Hematoxylin and eosin stains showed selected regions for data analysis (E). a: dorsal penis artery; u: urethra. Results of statistical analysis (F) showed percent of smooth muscle was significantly decreased in group B, compared with group A; however, percent of smooth muscle was significantly increased in the icariin-treated groups (C, D) compared with group B. Mean  $\pm$  SEM,  $n = 8$ ;  $^{\dagger}P < 0.05$ , vs. corresponding castrated rats;  $^{\ddagger}P < 0.05$ , vs. corresponding sham-operated rats.

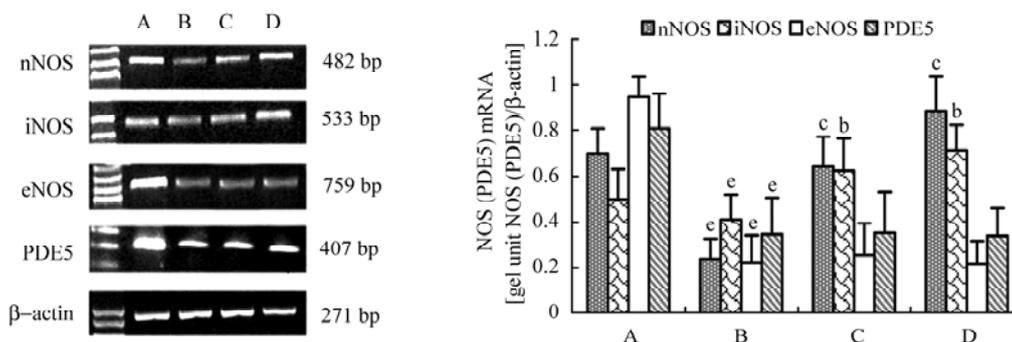


Figure 3. Left: Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis demonstrates expression of nNOS at 482 bp, iNOS at 533 bp, eNOS at 759 bp and PDE5 at 407 bp in the corpus cavernosum of sham-operated rats (lane A), castrated rats (lane B) and castrated rats treated with 1 mg/(kg-day) icariin (lane C) and 5 mg/(kg-day) icariin (lane D). Right: Densitometry and statistic analysis of NOS and PDE5 mRNA (ratio to- $\beta$ -actin) in the corpus cavernosum. Sham-operated rats (A), castrated rats (B) and castrated rats treated with 1 mg/(kg-day) (C) and 5 mg/(kg-day) icariin (D). Mean  $\pm$  SEM,  $n = 8$ ; <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , vs. corresponding castrated rats; <sup>e</sup> $P < 0.05$ , vs. corresponding sham-operated rats.

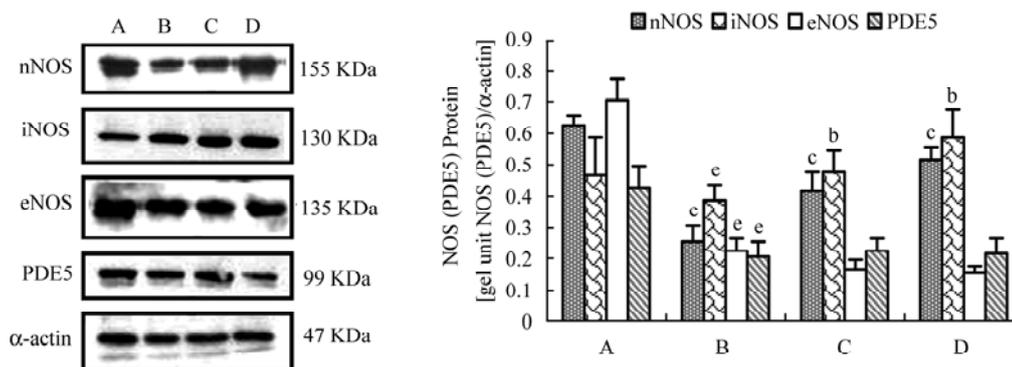


Figure 4. Left: Western blot analysis demonstrates expression of nNOS at 155 kDa, iNOS at 130 kDa, eNOS at 135 kDa and PDE5 at 99 kDa in the corpus cavernosum of sham-operated rats (lane A), castrated rats (lane B) and castrated rats treated with 1 mg/(kg-day) icariin (lane C) and 5 mg/(kg-day) icariin (lane D). Right: Densitometry and statistic analysis (bottom) of NOS and PDE5 protein (ratio to- $\alpha$ -actin) in the corpus cavernosum. Sham-operated rats (A), castrated rats (B) and castrated rats treated with 1 mg/(kg-day) (C) and 5 mg/(kg-day) icariin (D). Mean  $\pm$  SEM,  $n = 8$ ; <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , vs. corresponding castrated rats; <sup>e</sup> $P < 0.05$ , vs. corresponding sham-operated rats.

were observed ( $P > 0.05$ ).

#### 4 Discussion

In the present study, the castrated rats showed significantly reduced erectile function that was paralleled with the decreased peak and total ICP. These observations were consistent with the previous studies [12, 13, 15]. In our previous study, we showed that a 4-week oral administration of icariin-enriched fraction (51%

purity; 1 mg/[kg-day] and 5 mg/[kg-day]) improved erectile function and increased nNOS and iNOS expression in CC of castrated rats [11]. In this study, we further demonstrated that 4-week oral treatment with pure icariin (> 98.6 %); 1 mg/(kg-day) and 5 mg/(kg-day) significantly increased the peak and total ICP (area under the erectile curve) in castrated rats compared with controls but without influence on ST levels. These results suggested that long-term oral treatment with icariin-enriched fraction and/or pure icariin might improve the erectile

function of castrated rats.

It has been known that the trabecular smooth muscle content is the key structure in normal erectile function and the degree of loss of corporeal smooth muscle content correlates with the extent of impairment in corporeal veno-occlusive function [16]. Since icarrin administration increases smooth muscle content in the CC, this medicinal herb active component may have an effect on reversing ED. Whether icariin could affect smooth muscle apoptosis and/or collagen synthesis remains to be studied.

In the present study, icariin was found to increase the mRNA and protein expression of nNOS and iNOS in the CC of castrated rats, which was well correlated with the changes of PSM and ICP. In addition, the ST levels of the castrated rats (group B) were significantly decreased compared with those of the control rats (group A) and were not altered by icariin administration (groups C and D). These results suggested that the mechanisms of icariin on ED and NOS mRNA and protein expressions are not associated with testosterone synthesis. Whether icariin can directly affect the activity of NOS promoter and/or the related nuclear transcription factors in smooth muscle cells is currently under investigation.

The levels of NOS proteins were known to be influenced by several signals via transcriptional or translational mechanisms and the changes in the expression of NOS isoforms after several chemical and physical stimulations have been reported [17]. For example, nNOS and eNOS levels in rat cavernosal tissue were observed to increase after repeated PGE1 treatment [18]. Similarly, changes in nNOS and eNOS protein content were observed following orchietomy and androgen replacement [12, 13]. iNOS and eNOS content in rat cavernosal tissue can also be upregulated by single reagent VEGF [5]. In our previous study, chronic oral treatment with Icariin increases the erectile function (ICP) and restores the eNOS expression in corpus cavernosum of arteriogenic erectile dysfunction rats [9].

Studies noted that icariin could produce two metabolic products of icarinoside II and icaritin after oral administration [19]. Our own studies showed that oral icariin specifically inhibited PDE5 and upregulated NOS expression. These observed effects could be associated with the icariin metabolic products, however, this needs to be further investigated.

In summary, the 4-week oral administration of icariin to castrated rats can promote erectile function, which is correlated with the increased PSM and the mRNA and

protein expression of NOS but with no influence on ST levels. Although its mechanism remains to be explored, this study suggests that icariin may have long-term therapeutic effects on ED.

### Acknowledgment

The authors would like to acknowledge the National Nature Science Foundation of China (No. 30070927) and Beijing National Nature Science Foundation (No. 7012023) for grants for this work.

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