ORIGINAL RESEARCH—BASIC SCIENCE

Icariin Combined with Breviscapine Improves the Erectile Function of Spontaneously Hypertensive Rats

Yongxian Li, MS,* Jun Jiang, MD, PhD,† Yanzheng He, MD, PhD,† Rui Jiang, MD, PhD,‡ Junxiang Liu, BSc,§ Zhongcai Fan, MD, PhD,* and Yong Cheng, BSc‡

*Department of Cardiovascular Diseases, Affiliated Hospital, Luzhou Medical College, Luzhou, China; †Department of Vascular Surgery, Affiliated Hospital, Luzhou Medical College, Luzhou, China; ‡Department of Urology, Affiliated Hospital, Luzhou Medical College, Luzhou, China; §Department of Statistics, Luzhou Medical College, Luzhou, China

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ABSTRACT

Introduction. The impaired erectile response in spontaneously hypertensive rats (SHR) is caused by increased signaling of RhoA/Rho-kinase and decreased signaling of nitric oxide (NO). Icariin improves erectile function via upregulating multitargets in NO/cyclic guanosine monophosphate (NO/cGMP) pathway, which breviscapine accomplishes by downregulating RhoA/Rho-kinase pathway.

Aim. To investigate the effect and mechanism of icariin combined with breviscapine on the erectile function of SHR.

Methods. Five 12-week-old male Wistar-Kyoto (WKY) rats and 20 age-matched male SHR were evenly randomized into WKY rats control group, SHR control group, icariin-treated group, breviscapine-treated group, and combined treatment group treated by vehicle, icariin, breviscapine, and icariin plus breviscapine, respectively, by gavage for four successive weeks. Maximum intracavernosal pressure/mean arterial pressure (ICPmax/MAP) and the expression of endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), phosphodiesterase type 5 inhibitors (PDE5), and Rho-associated, coiled-coil containing protein kinase 1 and 2 (ROCK1 and ROCK2) in the cavernous tissues were determined.

Results. The ICPmax/MAP in the combined treatment group was significantly increased compared with SHR control group, icariin-treated group, and breviscapine-treated group. The expression of eNOS and nNOS was significantly higher in the combined treatment group than in SHR control group, icariin-treated group, and breviscapine-treated group ($P < 0.05$). The expression of PDE5 was significantly lower in the icariin-treated group than in SHR control group ($P < 0.05$). The expression of ROCK1 was significantly lower in the combined treatment group than in other groups ($P < 0.05$). The expression of ROCK2 was significantly higher in SHR control group than in WKY rats control group, icariin-treated group, and combined treatment group ($P < 0.05$). Among these groups, the expression of eNOS and nNOS was the strongest, and ROCK1 was the lowest in WKY rats control group.

Conclusion. Icariin combined with breviscapine has synergistic effects on erectile function of SHR through different signal pathways. **Li Y, Jiang J, He Y, Jiang R, Liu J, Fan Z, and Cheng Y. Icariin combined with Breviscapine improves the erectile function of spontaneously hypertensive rats. J Sex Med 2014;11:2143–2152.**

Key Words. Breviscapine; Erectile Dysfunction; Erectile Function; Hypertension; Icariin; Rat

Introduction

Erectile dysfunction (ED), defined as the inability to attain or maintain a penile erection sufficient for successful vaginal intercourse, is a common disease, and its prevalence mounts with increasing age [1]. The world ED population is predicted to reach 322 million by the year 2025 [2]. Epidemiologic data have confirmed that ED is linked to the presence of morbid medical conditions including diabetes mellitus, obesity, cardio-

Yongxian Li and Jun Jiang equally contributed to this study.

vascular disease, and hypertension [3]. The incidence of ED was 52–68% in 34- to 75-year-old hypertensive population [4,5]. On one hand, hypertension decreased the production of nitro oxide (NO) by downregulating the expression of NOS in cavernous tissue; on the other hand, it increased smooth muscle tension by upregulating RhoA/Rho-kinase signaling in the cavernosal tissue to impair the erectile function [6]. Phosphodiesterase type 5 inhibitors (PDE5) became the first-line therapy for ED caused by hypertension and other factors by its key role in NO signal pathway during erection to promote cyclic guanosine monophosphate (cGMP) accumulation. It was estimated that 11–44% of patients would become therapeutic nonresponders to PDE5 monotherapy [7]. ED nonresponsive to PDE5 inhibitors were referred to as refractory ED [8]. How to improve the therapeutic effect to ED, in particular, to refractory ED is a hot point of study. Therapeutic strategies of choosing different drugs according to different pathogenesis of ED may be a key to resolving the complex issues. Another approach to treat ED patients is aimed at the inhibition of pathways controlling smooth muscle contraction. Abundant evidence demonstrates that RhoA/Rho-kinase pathway is elevated in ED of various etiologies [9]. Different Rho-kinase inhibitors such as Y-27632, HA-1077, and H-1152 improved erectile function in vitro and in vivo, and they also improved erectile function in rat models associated with ED risk factors such as aging, diabetes mellitus, hypertension, and hypogonadism [10]. This suggests that RhoA/Rho-kinase inhibitors could offer an alternative treatment for ED patients with upregulated RhoA/Rho-kinase pathway.

Chinese herbal medicine has a long history of use in the treatment of ED and is still currently used. *Epimedium* is one of the important Chinese herbs widely used to improve sexual function. It possesses many biological effects, such as improvement of cardiovascular function, hormone regulation, immune modulation, anti-tumor, and improved erectile function of aged rats [11]. It has two important active constituents: icariin and icariin-II. Biliary excretion is the major elimination pathway for icariin disposition [12]. The underlying mechanism of icariin involves dose-dependent inhibition of PDE5 activities, increase of the ratio of smooth muscle cells, and the expression of neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase, endothelial nitric oxide synthase (eNOS), and cGMP [13]. Therefore, it regulates the activity of the NO/cGMP signal pathway in corpus cavernosum to enhance erectile function in castrated rats [14]. Icariin may also have neurotrophic effects. It preserves penile hemodynamics, smooth muscle and endothelial integrity, and nNOS expression in penis of diabetic rats [15]. Icariin-II increases cGMP concentration, NOS expression, and maximum intracavernosal pressure/mean arterial pressure (ICPmax/MAP) values in diabetic ED. Meanwhile, icariin-II downregulates the transforming growth factor beta1/Smad2/connective tissue growth factor signaling pathway and decreases apoptosis in penis [16]. There is no significant difference between icariin and icariin-II for sexual function [16]. However, the effect of icariin on ED is weaker than other PDE5 (such as sildenafil, vardenafil and tadalafil) now used in western medicine [17,18].

Breviscapine was extracted from the herb *Erigeron breviscapus*, another Chinese herb. Breviscapine has the effect of dilation of cerebral blood vessels, decreasing cerebral vascular resistance, increasing cerebral blood flow, improving microcirculation, and inhibiting platelet aggregation. It is useful in treatment of ischemic cerebrovascular disease, coronary disease, angina pectoris, and myocardial infarction [19]. The data showed that breviscapine significantly decreased the expression of ROCK1 mRNA, ROCK2 mRNA, and Rho-kinase protein [20]. However, there is no report of breviscapine use for the treatment of ED patients.

In medicine, combined use of multiple drugs with different pharmacological actions are often more effective than a single drug. Combination therapy has gradually been used in the treatment of refractory ED [21,22]. For example, intracavernosal injection of two or more vasoactive drugs has gotten much more effect in the treatment of nonresponder ED. In addition, combination strategies such as the adjunctive testosterone therapy in men with low testosterone level [23] and concomitant use of the vacuum erection device with PDE5 as salvage therapy in the nonresponder have been reported [24]. The crosstalk between the two pathways (NO/cGMP and RhoA/Rho-kinase) on smooth muscle cellular functions in corpus cavernosum may be a key role for erectile function. Upregulated NO/cGMP pathway combined with depressed RhoA/Rho-kinase pathway might have synergistic effect. Thus, considering the potential efficiency of breviscapine in decreasing corpus cavernosum tone and icariin in enhancing NO/cGMP pathway, the concurrent administration of both drugs to spontaneously hypertensive

rats (SHRs), theoretically, may potentiate the beneficial effects of each drug administrated alone, and this protocol may represent a new treatment strategy for refractory ED patients.

To our knowledge, this is the first study to treat ED caused by hypertension with combined icariin and breviscapine extracted from two Chinese herbs with different pharmacological actions.

Materials and Methods

Experimental Animal and Grouping

In this study, all procedures were performed in accordance with the guidelines of the Chinese Council on Animal Care. Five 12-week-old male Wistar-Kyoto (WKY) rats and 20 age-matched male SHR purchased from Vital River Laboratory Animal Technology Co. Ltd. in China (NO. of Certificate: SCXK [Jing] 2012-0001) had free access to tap water and a standard chow throughout the experiment. Icariin and/or breviscapine was dissolved in a 50:50 mix of normal saline and dimethyl sulfoxide [15,25]. The SHRs were randomized into four groups (N = 5): SHR control group received a 50:50 mix of normal saline and dimethyl sulfoxide (10 mL/kg); icariin-treated group received icariin (Xi'an TonKing Biotech Co., Xi'an, China) at a concentration of 10 mg/mL in 2.5 mg/kg; breviscapine-treated group received breviscapine (Yunnan Phytopharmaceutical Co., Ltd., Yunnan, China) at a concentration of 20 mg/mL in 80 mg/kg; and combined treatment group received icariin (10 mg/mL in 2.5 mg/kg) combined with breviscapine (20 mg/mL in 80 mg/kg). WKY rats were the control group which exposed to the same protocol as SHR control group. All treatments were done by gavage per day for 4 weeks successively. It was no more than 2 hours between last treatment and experiments. All rats (N = 5) were analyzed in each experiment.

Measurement of Body Weight, Serum Testosterone, and ICP/MAP in Groups

After 4 weeks of treatment, rats were anesthetized with sodium pentobarbital (30 mg/kg), and the body weight and ICPmax/MAP were examined as previously described [26,27]. The right carotid artery was exposed and inserted with a polyethylene-50 tube filled with 50 IU/mL heparinized saline to monitor the MAP. Electrical stimulations of the cavernous nerve (square-wave pulses of 5 milliseconds, duration of 60 seconds, 3 and 5 V) at 12 Hz frequencies were performed in a randomized manner and repeated twice for each rat. These conditions were obtained from a preliminary study designed to determine the optimal stimulatory conditions. The ICP responses elicited by electrical stimulations were quantified by calculating the ratio ICPmax (mm Hg)/MAP (mm Hg), and then the blood samples were taken from the tail vein and analyzed for serum testosterone with radioimmunoassay (Bayer Inc., Berlin, Germany).

Immunohistochemical Analysis of eNOS, nNOS, ROCK1, ROCK2, and PDE5 Expression

The rats were killed with an anesthetic overdose, and the sacrificed rats were perfused by intracardial injection of sterile saline buffer. The whole penis including the penile shaft and caudal was harvested and micro-dissected so that all extra-tunical tissues including the prepuce and urethra were removed. The midshaft segments were a 2-mm section fixed in 10% formalin and used for embedding in paraffin for immunohistochemical staining by the avidin-biotin-peroxidase complex horseradish peroxidase conjugated technique. The rest was frozen at –80°C for Western blot analysis [28]. In the study, continuous slices dyeing by hematoxylin-eosin staining and immunohistochemistry was performed. Nonspecific binding of Immunoglobulin G was blocked using normal goat serum 1:50 in 0.1% bovine serum albumin in phosphate-buffered saline. Sections were incubated with primary monoclonal antibodies against eNOS (1:50 dilution; Cell Signaling Technology Inc., Beverly, MA, USA), nNOS (1:50 dilution; Leica Biosystems, St. Louis, MO, USA), ROCK1 (1:50 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), ROCK2 (1:50 dilution, Santa Cruz Biotechnology Inc.), PDE5 (1:50 dilution, Santa Cruz Biotechnology Inc.), and then the sections were washed and incubated with secondary antibodies (1:100 dilution; Zhong Shan Goldenbridge Biotechnology Co., Beijing, China). The primary antibodies were replaced with the normal serum of the host species with the secondary antibody as a negative control. Sections were examined under a light microscope. Immunohistochemical analyses in rats were performed using the technique described previously [25,27]. Slides were examined by an observer blinded to treatment group. nNOS positivity was ascertained by counting positive fibers in four random high-power fields of the midline dorsal neurovascular bundle of the penis. eNOS, ROCK1, ROCK2, and PDE5 positivity was quantified by selection of four random fields of the corporal bodies. The computerized densitometric analyses of the expression of eNOS,

nNOS, ROCK1, ROCK2, and PDE5 in cavernous tissue in the images were performed by Image-Pro Plus version 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA). The levels of these proteins expression were quantitated by measurement of integral optical density (IOD). The IOD was calculated for each image using the following equation: positive area × average density. Results are presented as the mean ± standard deviation.

Western Blot Analysis of eNOS, nNOS, ROCK1, ROCK2, and PDE5 Expression

The rest of frozen rat penis was chosen and was immediately purified for protein analysis as previously described [26] with modifications. In short, the cavernous tissues homogenized were lysed in radioimmunoprecipitation assay protein extraction reagent and centrifuged at 12,000 rpm for 3 minutes at 4°C. The supernatant was collected. Protein concentration was determined by the bicinchoinic acid protein assay kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were denatured at 100°C for 5 minutes, and then electrophoresis was done by 12% sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to 0.22 μm polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) for 150 minutes at 25 V. The membranes were reacted with blocking buffer (Beyotime Institute of Biotechnology) for 60 minutes at room temperature, and then incubated with primary antibodies against eNOS (1:500 dilution; Cell Signaling Technology Inc.), nNOS (1:500 dilution; Leica Biosystems), ROCK1 (1:1,000 dilution; Santa Cruz Biotechnology Inc.), ROCK2 (1:200 dilution; Santa Cruz Biotechnology Inc.), and PDE5 (1:500 dilution; Santa Cruz Biotechnology Inc.) overnight at 4°C, respectively. The membrane was washed three times using Tris-buffered saline Tween-20 (TBST) at intervals of 10 minutes. Biotinylated Goat Anti Rabbit IgG (1:10,000, 1:4,000, 1:3,000, 1:5,000, 1:4,000 dilution, respectively; Boster, Wuhan, China) was the secondary antibody and was reacted for 60 minutes at room temperature and the membrane was washed again with TBST three times with an interval of 10 minutes between each washing. Antibody specific bands were detected using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). The computerized densitometric analyses of the expression of proteins at the bands were normalized by β-actin (1:2,000 dilution; Bio-rad, Hercules, CA, USA) expression and performed by Bio1D software (version 97; Vilber Lourmat, Rue Des Coutures, France). The levels of these proteins expression were quantitated by measurement of IOD/β-actin IOD.

Data Processing

Data were expressed as mean ± standard deviation (mean ± SD). Analysis of variance was performed to evaluate the difference between groups followed by Student–Newmann–Keuls test. The results were assessed statistically using the SPSS for Windows 13.0 software (SPSS Inc., Chicago, IL, USA) and Stata 10.0 (Computer Resource Center, Boston, MA, USA). The level of significance for all was set at $P < 0.05$.

Results

General Condition

Animals showed no obvious signs of systemic illness throughout the period of the study.

The level of the body weights, testosterone, and mean artery pressure (MAP) in different groups are shown in Table 1. There were no significant differences of the body weights and testosterone among these groups ($P > 0.05$). The level of MAP in WKY rats control group was significantly lower than others ($P < 0.05$).

Assessment of ICP

After electro-stimulation by 3 and 5 V, the ratio of ICPmax/MAP in each group was shown in

Table 1 General conditions of the studied rats (N = 5, mean ± SD)

Group	Body weight (g)	Testosterone (ng/dL)	MAP (mm Hg)
WKY rats control group	275.3 ± 4.5	475.11 ± 34.33	90.75 ± 6.54*
SHR control group	278.8 ± 8.5	400.7 ± 18.20	173.72 ± 6.23
Icariin-treated group	283.2 ± 2.1	489.66 ± 60.95	180.45 ± 5.62
Breviscapine-treated group	274.0 ± 2.8	428.42 ± 46.60	181.52 ± 6.73
Combined treatment group	277.4 ± 3.5	483.11 ± 42.33	179.43 ± 6.25

*$P < 0.05$ vs. SHR control group, icariin-treated group, breviscapine-treated group, and combined treatment group, respectively.

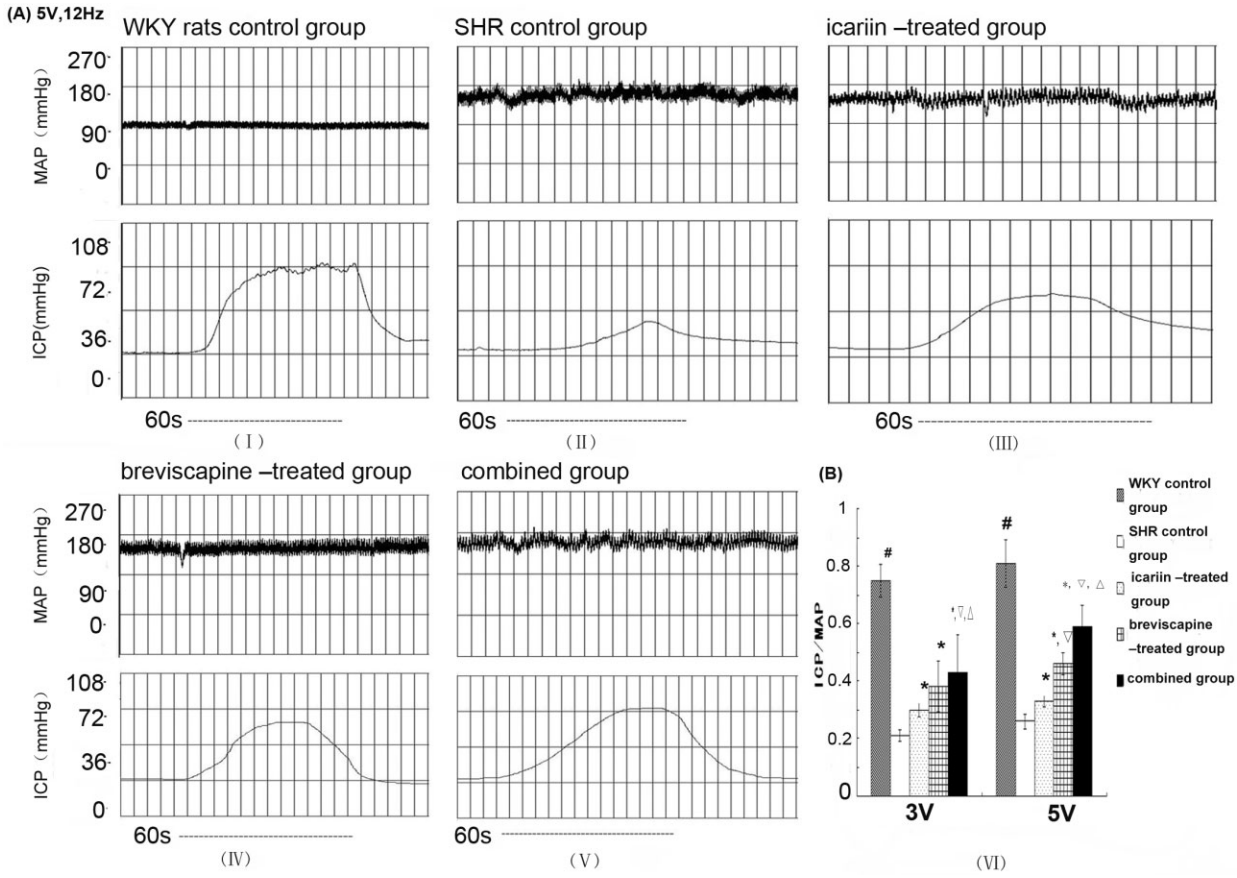


Figure 1 Increasing of maximum intracavernosal pressure/mean arterial pressure (ICPmax/MAP) ratio in spontaneously hypertensive rats (SHR) treated by icariin combined with breviscapine. (A) Representative digitalized tracing of an original recording of MAP and ICP when electric-stimulating the pelvic nerve (5 V, 12 Hz, 1 ms, 60 seconds) in SHR and in age-matched Wistar-Kyoto rats (WKY). (B) Erectile function of the five groups presented as voltage-dependent erectile response (ICPmax/MAP) at 3 V or 5 V. Each bar depicted the mean values ± standard deviation (N = 5). $^*P < 0.05$ vs. SHR control group. $\nabla P < 0.05$ vs. icariin-treated group. $\triangle P < 0.05$ vs. breviscapine-treated group. $\#P < 0.05$ vs. SHR control group, icariin-treated group, breviscapine-treated group, and combined treatment group, respectively.

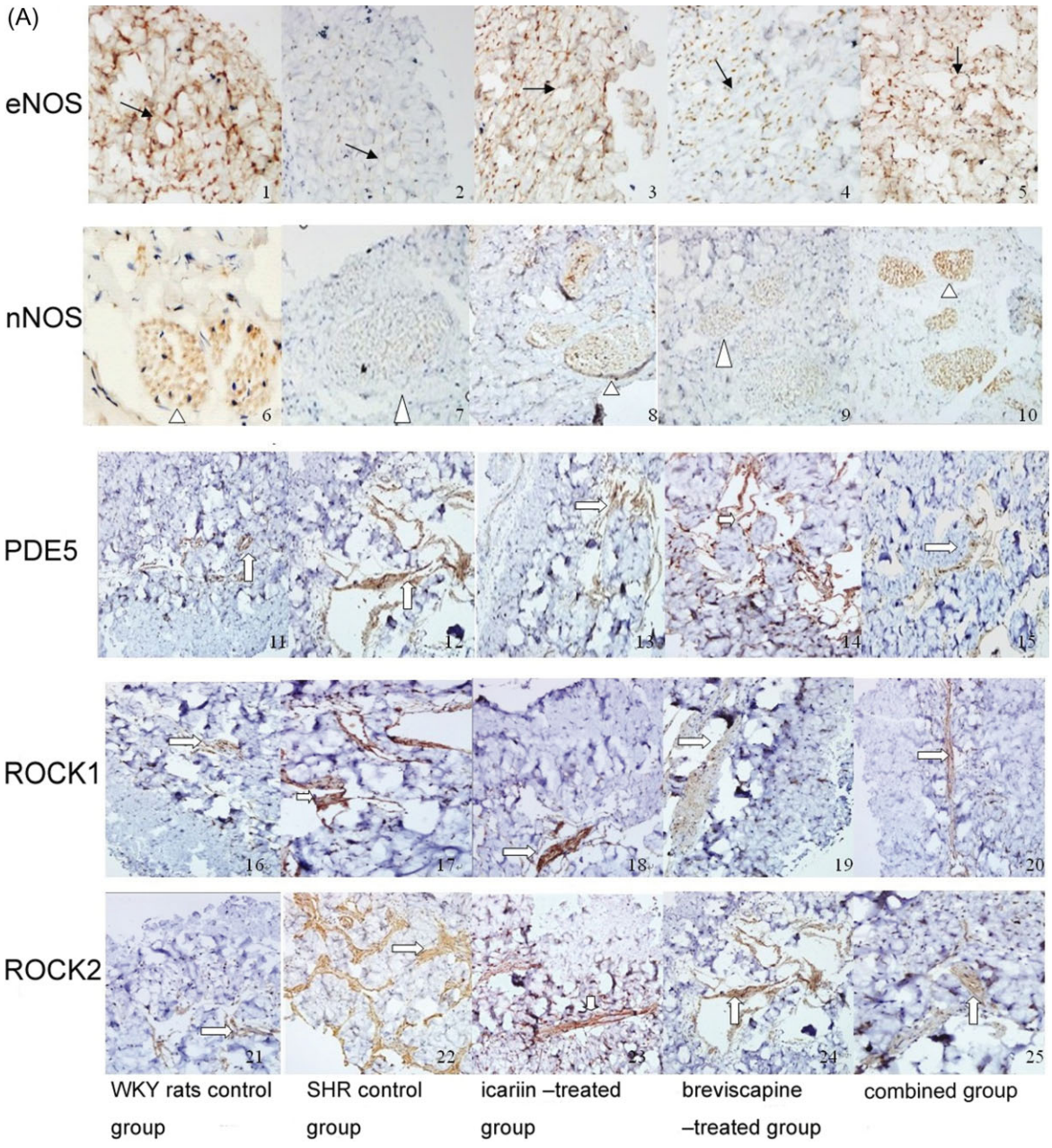
Figure 1A, B. The ICPmax/MAP in SHR control group was significantly less than in WKY rats control group, icariin-treated group, and breviscapine-treated group at 3 V and at 5 V stimulation voltage ($P < 0.05$). There was no significant difference of the ICPmax/MAP between icariin-treated group and breviscapine-treated group at 3 V ($P > 0.05$). However, the ICPmax/MAP in icariin-treated group was significantly less than in breviscapine-treated group at 5 V. The combined treatment group was significantly increased compared with SHR control group, icariin-treated group, and breviscapine-treated group at 3 V and 5 V, respectively. Meanwhile, the combined treatment group was significantly decreased compared with WKY rats control group at 3 V and 5 V, respectively.

Immunohistochemical Analysis of eNOS, nNOS, PDE5, ROCK1, and ROCK2 Expression

Expression of eNOS, nNOS, PDE5, ROCK1, and ROCK2 in penile tissue by immunohistochemistry were shown in Figure 2A, B.

eNOS is mainly expressed in the vascular and sinusoidal endothelial cells and cavernosal smooth muscle cells of penile tissue. nNOS is mainly expressed in the cytoplasts of the nerve cells. PDE5, ROCK1, and ROCK2 are mainly distributed in the cytoplasm of smooth muscle cells.

The protein expression of eNOS and nNOS was significantly higher in the icariin-treated group than in SHR control group ($P < 0.05$). There is no significant difference in the protein expression of eNOS and nNOS between breviscapine-treated


(A)
eNOS
nNOS
PDE5
ROCK1
ROCK2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
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20
21
22
23
24
25
WKY rats control group
SHR control group
icariin –treated group
breviscapine –treated group
combined group

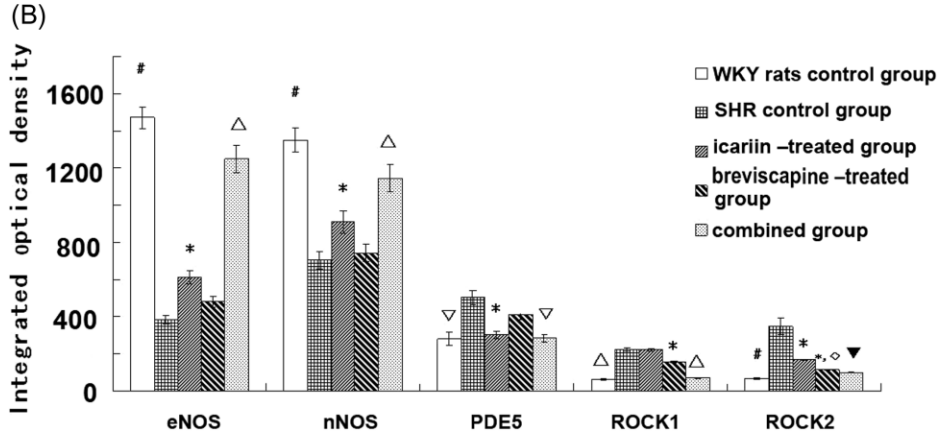
(B)
Integrated optical density
1600
1200
800
400
0
eNOS
nNOS
PDE5
ROCK1
ROCK2
WKY rats control group
SHR control group
icariin –treated group
breviscapine –treated group
combined group

Figure 2 Effect of icariin, breviscapine, and icariin combined with breviscapine on the expression of eNOS, nNOS, PDE5, ROCK1, and ROCK2 in penis of SHR and WKY was analyzed by immunolocalization. (A, 1–20). Immunolabeling of eNOS, nNOS, PDE5, ROCK1, and ROCK2 were stained in brown. eNOS is mainly expressed in the membrane of the vascular endothelial cell (solid arrow, ×400). nNOS is mainly expressed in neurons (empty triangle, ×400). ROCK1, ROCK2, and PDE5 is mainly expressed in cytoplasm of smooth muscle cell (empty arrow) (×200). (B) The levels of eNOS, nNOS, PDE5, ROCK1, and ROCK2 expression were quantitated by measurement of integral optical density (IOD) (N = 5). $^*P < 0.05$ vs. SHR control group. $\diamond P < 0.05$ vs. icariin-treated group. $\triangledown P < 0.05$ vs. SHR control group and breviscapine-treated group, respectively. $\blacktriangledown P < 0.05$ vs. SHR control group and icariin-treated group, respectively. $\triangle P < 0.05$ vs. SHR control group, icariin-treated group, and breviscapine-treated group, respectively. $\#P < 0.05$ vs. SHR control group, icariin-treated group, breviscapine-treated group, and combined treatment group, respectively.

group and SHR control group ($P > 0.05$). The protein expression of eNOS and nNOS was significantly higher in the combined treatment group than in SHR control group, icariin-treated group, and breviscapine-treated group ($P < 0.05$).The protein expression of PDE5 was significantly lower in icariin-treated group than in SHR control group ($P < 0.05$).There is no significant difference in the protein expression of PDE5 between SHR control group and breviscapine-treated group ($P > 0.05$). Among these groups, the protein expression of eNOS and nNOS was the strongest in WKY rat control group.

There is no significant difference in the protein expression of ROCK1 between SHR control group and icariin-treated group ($P > 0.05$). The protein expression of ROCK1 was significantly lower in breviscapine-treated group than in SHR control group ($P < 0.05$). The protein expression of ROCK1 was significantly lower in combined treatment group than in SHR control group, icariin-treated group, and breviscapine-treated group ($P < 0.05$). The protein expression of ROCK1 was significantly lower in breviscapine-treated group than in SHR control group ($P < 0.05$). Among these groups, the protein expression of ROCK1 was the lowest in SHR control group.

The protein expression of ROCK2 was significantly higher in SHR control group than in WKY rats control group, icariin-treated group, breviscapine-treated group, and combined treatment group ($P < 0.05$). The protein expression of ROCK2 was significantly lower in breviscapine-treated group than in icariin-treated group ($P < 0.05$). There is no significant difference in the protein expression of ROCK2 between combined treatment group and breviscapine-treated group ($P > 0.05$).

Western Blot Analysis of eNOS, nNOS, PDE5, ROCK1, and ROCK2 Expression

Expression of eNOS, nNOS, PDE5, ROCK1, and ROCK2 in penile tissue by western blot were shown in Figure 3. The results of the protein expression of eNOS, nNOS, PDE5, ROCK1, and ROCK2 determined by Western blot are consistent with those by immunohistochemical analysis.

Discussion

In this study, icariin increased ICPmax/MAP of SHR by upregulating the expression of eNOS and nNOS and downregulating the expression of PDE5 in the corpus cavernosum of SHR, consistent with the literature report [13]. Moreover, this is the first report that icariin had no effect on ROCK1 but significantly downregulated the overexpression of ROCK2 in SHR. Meanwhile, breviscapine had no effect on the expression of NOS and PDE5 but significantly increased ICPmax/MAP by downregulating overexpression of ROCK1 and ROCK2 in SHR. Although, the effect of ROCK inhibitors on the treatment of ED in humans has not yet been reported to date, these studies suggested that breviscapine may improve erectile function of SHR.

Icariin and breviscapine are extracts of Chinese herbs acting on different signal pathways. However, whether such combination may develop a synergistic therapeutic action remains unknown. In this study, combination therapy of icariin and breviscapine was significantly more efficacious in increasing ICPmax/MAP, upregulating the expression of eNOS and nNOS and downregulating the expression of ROCK1 and ROCK2 in corpus cavernosum of SHR than by either agent alone and without significant hypotensive reaction. With the crosstalk between the NO/cGMP and RhoA/Rho-kinase pathways on smooth muscle cellular functions in corpus cavernosum, the RhoA/Rho-kinase pathway plays a negative regulator role in the activity and expression of eNOS in endothelial cells. Activating the RhoA/Rho-kinase pathway would lead to dephosphorylation of eNOS while simultaneously suppressing its expression by decreasing its mRNA stability to achieve rapid and long-term decrease in NO production [29]. Data demonstrate that the downregulation activity of penile eNOS

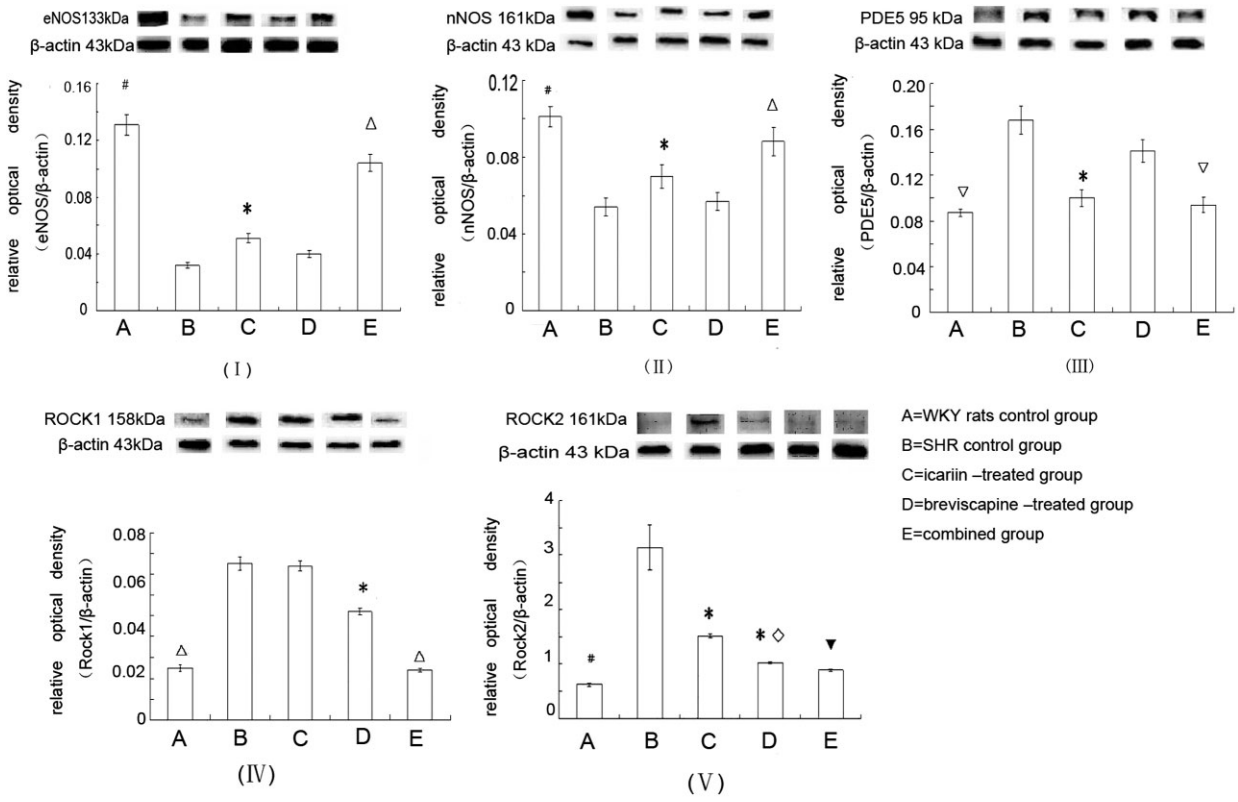

Figure 3 Effect of icariin, breviscapine, and icariin combined with breviscapine on the expression of eNOS, nNOS, PDE5, ROCK1, and ROCK2 in penis of SHR and WKY was measured by Western blot. (I–V) Expression of eNOS, nNOS, PDE5, ROCK1, and ROCK2 in corpus cavernosum of the five groups (N = 5). Data were presented as the relative density of each protein compared with that of β-actin. Each bar depicted the mean values ± standard deviation. $^*P < 0.05$ vs. SHR control group. $\diamond P < 0.05$ vs. icariin-treated group. $\triangledown P < 0.05$ vs. SHR control group and breviscapine-treated group, respectively. $\blacktriangledown P < 0.05$ vs. SHR control group and icariin-treated group, respectively. $\triangle P < 0.05$ vs. WKY rats control group, icariin-treated group, and breviscapine-treated group, respectively. ▌ $P < 0.05$ vs. SHR control group, icariin-treated group, breviscapine-treated group, and combined treatment group, respectively.

in diabetes is mediated by activation of the RhoA/Rho-kinase pathway. Inhibition of RhoA/Rho-kinase may improve eNOS protein content and activity, thus restoring erectile function in diabetes [30]. For example, Y-27632 significantly enhanced NO release in the corpus cavernosum [31]. Hydroxyfasudil, a ROCK inhibitor, ameliorates hypertension-associated dysfunction of NO-induced relaxation in corpus cavernosum smooth muscle possibly via inhibition of the RhoA/Rho-kinase pathway and activation of NO-eNOS pathway in the SHR [32]. The effect of breviscapine may be weaker than Y-27632 or hydroxyfasudil, and there was no significantly improved NOS pathway in SHR treated by breviscapine alone in the study. However, when used in combination, the inhibitory effect of breviscapine on RhoA/Rho-kinase pathway may strengthen the upregulatory role of icariin on NO pathway. On the other hand, the NO pathway phosphorylates RhoA at Ser-188, which prevents its translocation to the membrane and its activation [33]. Increasing NO/cGMP signal may suppress RhoA/Rho-kinase pathway in cavernous tissue [34,35]. Therefore, the effect of releasing NO from endothelial cells by icariin may inhibit the contraction mediated by RhoA/Rho-kinase pathway. This may be one of the mechanisms of icariin significantly downregulated the overexpression of ROCK2 in SHR. As a result, icariin-induced effect could thus potentiate the effect of breviscapine when used in combination. However, based on the results of our research, the synergistic effects are mainly occurred in the ROCK1 signal pathway and uninvolved in ROCK2 signal pathway.

Compared with WKY, we can ascertain ED occurred in these SHRs and the combined treatments partially reverse ED at least. Although a near complete normalization of signaling pathways changes except NOS by the combined administration, a marked reduction of erectile responses with

respect to WKY rats is present in SHR undergoing this combined treatment. Indeed, there are a few reasonable explanations for these findings. First, hypertension has primary effect on NOS and control of hypertension is an essential part of treatment for ED. Second, it indicates that there may be other molecular signaling pathways such as angiotensin II, carbon monoxide, and hydrogen sulfide involved in the erectile function of hypertensive rats, and this should be researched in the future [36–38]. Third, the change in erectile function after pharmacological treatment may be related to changes in the ultrastructure within the penis [27]. Early pharmacological treatment administered before the penile ultrastructure pathological changes develop may be more effective in improving ED in hypertensive patients [39].

The results of this study that proved the existence of a synergistic effect between icariin (as a stimulator of NOS expression and PDE5) [13] and breviscapine (as a RhoA/Rho-kinase inhibitor) [20], confirm our protocol, which might open a way to the development of a new efficacious treatment of ED in SHR and provide a solid foundation for treatment of refractory ED. It is important to distinguish the concepts between the expression and activity of proteins. Proteins usually exert their biological effects by means of biologically active form. NOS, RhoA/Rho-kinase, and PDE5 have their biologically active form, respectively. The limitation of this study is lack of data on the NOS, RhoA/Rho-kinase, and PDE5 activity and the ratio of smooth muscle to collagen content in penes of SHR and WKY. It may be necessary to examine the membrane-bound eNOS separately, too. We will add observation of a washout period in the future to realize whether the improvements in erectile function and molecular changes are due to the presence of the drug or as a result of the long-term changes from treatment. Even with these limitations, we believe that this is an important but a preliminary study and patients could benefit from the results of our study. In the future, we should compare the effect of icariin combined with breviscapine and PDE5 in treatment of ED. Large scale, randomized, placebo-controlled studies are also needed to further assess the long-term efficacy and safety of such combination therapy. Great value may be achieved from continued work in this field.

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Corresponding Authors: Rui Jiang, MD, PhD, Department of Urology, Affiliated Hospital, Luzhou Medical College, Taiping Road, Luzhou, Sichuan 646000, China. Tel: 86830-316-2521; Fax: 86830-239-2753; E-mail: jiangrui@126.com; Yanzheng He, MD, PhD, Department of Vascular Surgery, Affiliated Hospital, Luzhou Medical College, Taiping Road, Luzhou, Sichuan 646000, China. Tel: 86830-316-5001; Fax: 86830-239-2753; E-mail: Yanzheng_He@126.com

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Statement of Authorship

Category 1

(a) Conception and Design
Yongxian Li; Jun Jiang; Rui Jiang; Yanzheng He

(b) Acquisition of Data
Yongxian Li; Jun Jiang; Yong Cheng; Junxiang Liu; Zhongcai Fan

(c) Analysis and Interpretation of Data
Yongxian Li; Jun Jiang; Rui Jiang; Yanzheng He

Category 2

(a) Drafting the Article
Yongxian Li; Jun Jiang; Rui Jiang; Yanzheng He

(b) Revising It for Intellectual Content
Rui Jiang; Yanzheng He; Zhongcai Fan

Category 3

(a) Final Approval of the Completed Article
Rui Jiang; Yanzheng He

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