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Original Article

Smooth Muscle Relaxation by the Herbal Medicine *Ssanghwatang* associated with Nitric Oxide Synthase Activation and Nitric Oxide Production

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Ssanghwatang (SHT) has been known to prove effective in the treatment for erectile dysfunction (ED), and its modified formula is widely used in clinical practice. However, its fundamental mechanism of action is not clearly known. It is well known that endothelial cells can achieve the relaxation of vascular smooth muscles by the release of nitric oxide (NO). NO is synthesized by the enzyme NO synthase (NOS) from L-arginine and oxygen. It is widely accepted that NO plays an important role in the relaxation of corpus cavernous smooth muscle and vasculature. In addition, in terms of the penile erection, the NO/cGMP pathway is more potent than the PGE1
/cAMP pathway.
The main purpose of the present study was to investigate the mechanism of the erectile effects of SHT by focusing on its direct effects on corpus cavernous smooth muscle cells. We investigated the NOS activity, nitrite concentration and cGMP levels in rat corpus cavernous smooth muscle cell lines activated by SHT extracts. Furthermore, we evaluated the effect of SHT extracts on penile smooth muscle relaxation following oral administration of SHT extract powder to rats by the dosage of 1 g/kg over fifteen days.
As a result, we found that SHT stimulated NO release. NOS activity and cGMP levels were increased by SHT respectively. Furthermore, SHT relaxed the corpus cavernous smooth muscle. These results are consistent with the concept that penile erection by SHT is carried out through the NO/cGMP pathway.
In conclusion, the present study shows that SHT increases the NOS activity, synthesizes NO and augments the cGMP, which mediates penile erection. Further determination of the SHT mechanism related with the NO/cGMP pathway strongly indicates that SHT can be used as a remedy for erectile impotence.

Key Words : *Ssanghwatang*, smooth muscle relaxation, nitric oxide, penile erection, cGMP

Introduction

Ssanghwatang (SHT), a natural product and traditional herbal medicine, has been used for thousands of years as a therapeutic formula in Korea, China and Japan and is still widely so used. SHT has been known to be effective against erectile dysfunction (ED), and its modified formula is widely used in clinical practice. However, its fundamental mechanism of action is not clearly known. In the present study, we have investigated the mechanism of its erectile action by determining its direct action on penile corpus cavernous muscle and cultured cells.

Penile erection is the end result of smooth muscle relaxation in the penis. It is well established

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that the balance between contraction and relaxant factors controls the degree of tone of the penile vasculature and the smooth muscle of the corpus cavernosum, and determines the functional state of the penis: flaccidity or erection. ED is defined as the "inability to achieve or maintain an erection adequate for sexual satisfaction"[1]. ED may be due to inability of penile smooth muscle to relax. This inability can have multiple causes, including nerve damage, endothelial damage, alteration in receptor expression/function, or in the transduction pathways that are implicated in the relaxation and contraction of the smooth muscle.

It is well known that endothelial cells can achieve relaxation of vascular smooth by release of nitric oxide (NO). NO is synthesized by the enzyme NO synthase (NOS) from L-arginine and oxygen. An important role for NO in the relaxation of corpus cavernous smooth muscle and vasculature is widely accepted[2,3]. Also, in terms of the penile erection, the NO/cGMP pathway is more potent than the PGE1/cAMP pathway[4].

The aim of the present study was to estimate four hypotheses: 1) NO is involved in the mechanism of action of SHT; 2) SHT increases NOS activity; 3) SHT increases the cGMP level; 4) SHT has a direct effect on the corpus cavernous smooth muscle cell to relax. As a result, we found that SHT stimulated NO release, and NOS activity and cGMP level were increased by SHT. Furthermore, SHT relaxed the corpus cavernous smooth muscle. These results are consistent with the concept that penile erection by SHT is carried out through the NO/cGMP pathway.

Materials and methods

1. Materials

1) Herbal composition

SHT is a mixture of herbal drugs. The composition of the mixture is as follows (Table 1): *Paeonia Radix* (10g), *Rehmanniae Radix* (4g), *Astragali Radix* (4g), *Angelicae gigantis Radix* (4g), *Cnidii Rhizoma* (4g), *Cassiae Cortex* (3g), *Glycyrrhizae Radix* (3g), *Zingiberis Rhizoma* (4g), *Zizyphi inermis Fructus* (4g). All the herbal materials were purchased from Wonkwang University Kwangju Oriental Medical Center and identified by herbal experts.

Table 1. Composition of SHT

Scientific name	Weight (g)
Paeonia Radix	10
Rehmanniae Radix	4
Astragali Radix	4
Angelicae gigantis Radix	4
Cassiae Cortex	3
Glycyrrhizae Radix	3
Paeonia Radix	3
Cnidii Rhizoma	4
Zingiberis Rhizoma	4
Zizyphi inermis Fructus	4
Total	43

2) Reagents

Dulbecco's modified Eagle's medium (DMEM), sodium citrate, L-arginine, ethylene diamine tetra sodium salt (EDTA) and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was supplied by the HyClone (Logan, UT). Anti-eNOS antibodies were from BD Biosciences (San Jose, CA).

3) Animals

Eight-week-old male *Sprague-Dawley* white rats (250-300g) were provided from Damul Science (Daejeon, Korea) and housed under conditions of constant temperature and controlled illumination for 2 weeks. Food and water were available *ad libitum*.

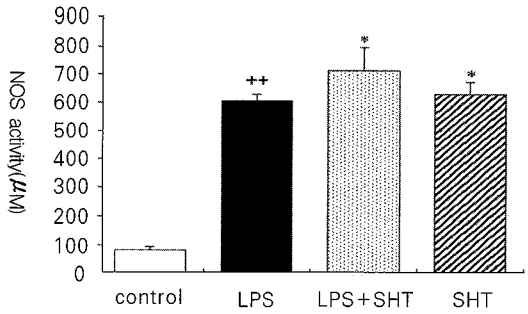

Fig 1. Effect of SHT on NOS. Cells were stimulated with LPS (100μg/ml) in the absence or presence of water extracts of SHT at 100mg/ml. After 24 hr incubation, NOS was measured. Data are mean ± SE. ++: significant difference between control and LPS group. *: significant difference between LPS and SHT group.

2. Methods

1) Preparation of extract and administration

For water extraction, SHT (400 g) was mixed with 3,000 mL of distilled water and extracted under reflux for 3 hr by boiling the formula. The extracts were filtered with a Whitman paper filter. The filtrate was concentrated with a rotary evaporator at 50℃ under vacuum and freeze-dried to dryness. The total amount of extract powder was 91.38 g. The extract powder was dissolve in 0.9% saline (2 mL) and administered orally to rats by the dosage of 1 g/kg for fifteen days.

2) Culture of rat corpus cavernous smooth muscle cells[5,6]

Rat corpus cavernous smooth muscle (CCSM) cells were obtained as sterile surgical specimens, the tissue was washed and cut into 1 to 2 mm pieces and placed into culture dishes with DMEM containing 20% FBS, penicillin (100 ㎍/mL), streptomycin (100 ㎍/mL) and 2 mM glutamine. After explants attached to the culture dish, usually 1 to 2 days, DMEM supplement with 10% FBS, penicillin, streptomycin, and glutamine were added. Smooth muscle cells

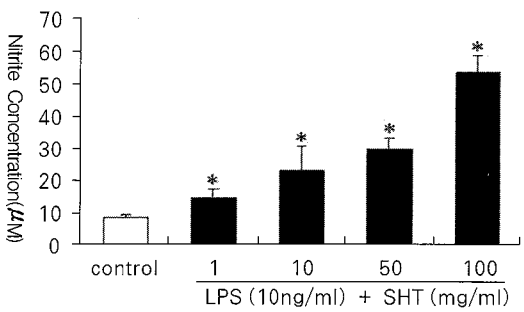

Fig 2. Effect of water extracts of SHT on NO production by LPS primed rat penile smooth muscle cells. Cells were activated with LPS (10ng/ml) in the absence or presence of SHT at indicated doses. After 48hr incubation, nitrite concentration was measured by Griess method. Each value represents the mean ± SE. * $p<0.05$ as compared with the control.

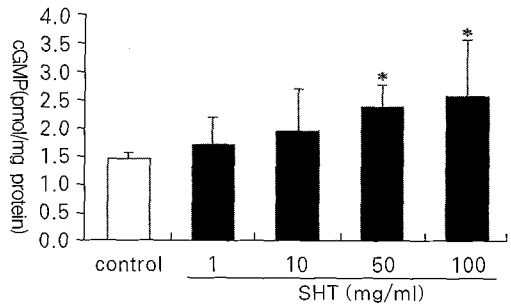

Fig 3. Effect of SHT on cGMP levels in rat penile smooth muscle cells. Each value represents the mean ± SE. * $p<0.05$ as compared with the control.

migrated out from the explants in 3-5 days. At this time, the explants were removed, and cells were allowed to achieve confluence. Cells were detached using 0.05% trypsin, 0.02% EDTA at 37℃ for 5 min to establish secondary cultures. Cultures were maintained for no more than four passages until the test.

3) Measurement of NOS activity

NOS activity in rat CCSM cells were measured by colorimetric assay (measurement of NADPH diaphorase activity) as was previously described by Schmidt[7]. In brief, the CCSM cells were stimulated with LPS in the absence or presence of water extracts of SHT at 37℃. After 24 hr incubation, 50 mM HEPES (pH 7.4) solution, L-arginine, NADPH, EDTA, CaCl2, dithiothreitol, calmodulin and nitro blue tetrazolium were added and reacted at 37℃ for 5 min. The colorimetric assay was carried out at a wave length of 585 nm.

4) Determination of nitrite production

To measure the production of nitrite, a stable end-product of NO oxidation, cells were grown in 96-well plates and incubated with cytokines, LPS (0-100 ㎍/mL), L-arginine analogs NG-monomethyl-L-arginine (L-NMA; Sigma) (10-1000 FM) or NG-nitro-L-arginine-methyl ester (L-NAME; Sigma) (1 mM), with or without added L-arginine (l-20 mM) or dexamethasone (1 × 10-8 M). Nitrite was measured according to the method described by Green and co-workers [8]. Briefly, 100 ul of medium was incubated with 100 ㎕ of Griess reagent consisting of 1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid for 10 min at room temperature. The absorbance was then measured at a wavelength of 540 nm using Titertek Multiscan MCC/340 (Flow Laboratories, Australia) and nitrite concentrations were determined from a standard curve made using a solution of sodium nitrite dissolved in medium.

5) Measurement of intracellular cyclic GMP content

Intracellular cyclic GMP concentrations in rat

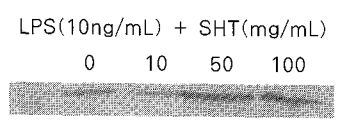

Fig 4. Western blot analysis of eNOS protein. Rat penile smooth muscle cells were stimulated with LPS (10ng/ml) in the absence or presence of SHT at indicated doses.

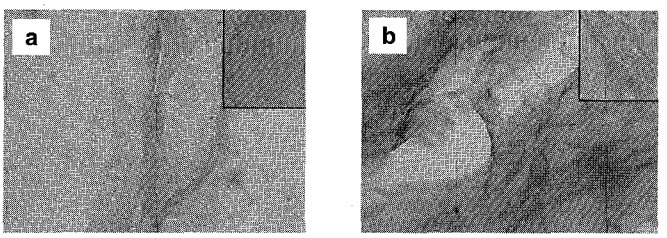

Fig 5. Immunohistochemistry of eNOS expression. Serial sections of rat penile smooth muscle after papaverine chloride injection shows more increased size of endothelium and upregulation of eNOS in SHT (1g/kg) treated group (b) than control (a). Inserts are cross section view at higher magnification (×400).

CCSM cells were assayed as previously described by Wu[9]. In brief, cells were finally grown in 24-well plates (105 cells well). At confluence, monolayer cells were washed with PBS and then incubated with SHT, CO2 5% at 37℃ for 10 min. Incubation was terminated by the addition of 6% trichloroacetic acid (TCA). Cell suspensions were sonicated, then centrifuged at 2,500 rpm for 15 min at 4℃. Then, the supernatants were added 10 ㎕/mL HCl, again centrifuged at 2500 rpm for 10 min. The coloring was measured at a wavelength of 405 nm by ELISA reader using cGMP kit (R&D Systems, Minneapolis, MN, USA).

6) Western Immunoblot[10]

Rat CCSM cells were centrifuged at 16,000 rpm in 8 volumes of ice-cold buffer containing 50 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 10% glycerol, and protease and phosphatase inhibitors (50 mM NaFe, 5 mM sodium pyrophosphate, 1 mM benzamidine, 1 M microcystin LR, 20 mM glycerol phosphate, 1 mM sodium vanadate, 2 g/mL aprotinin, 10 g/mL leupeptin, 10 g/mL trypsin inhibitor, 1 mM Pefabloc) for 30 min. It was separated on 4-16% gradient gels with protein sample buffer under non-reducing condition, transferred to nitrocellulose, reacted with anti-eNOS antibody, and washed. After washing, the secondary antibody was added and measured using DAB reaction.

7) Immunohistochemistry[11,12]

The animals injected with 1.5 mg of papaverine hydrochloride (Sigma) intracavernosally for erection were sacrificed and perfused with PBS followed by fixation with 4% paraformaldehyde. Then penile corpus cavernosa were removed and postfixed by chilled 4% paraformaldehyde for 18 hours at 4℃ and immersed in 30% sucrose solution for 24 hr. Coronal penile corpus cavernosum sections were sliced to 15 ㎛-thick with cryostat microtome and mounted on slide glasses. The section samples were washed in PBS and treated with anti eNOS antibody. This step was sometimes followed by washing in PBS and reaction with the secondary antibody. Coloring the sections was performed using DAB. Images of the section were observed using a microscope (Olympus, Japan).

8) Statistical evaluation of data

All values given in the text are expressed as mean ± SE. Differences between means were evaluated using ANOVA. Analysis of the data was done with the aid of software (the SAS System for

Windows V8 version). A p-value less than 0.05 was considered to be significant in all experiments.

Results

1. The effect of SHT on NOS activity

To evaluate the effect of SHT on NOS activity, the cultured CCSM cells were stimulated with LPS in the absence or presence of water extracts of SHT, and then NOS activity was measured. The NOS activity caused by LPS, LPS+SHT, and SHT was significantly greater than that of the control group. Furthermore, the NOS activity caused by LPS+SHT and SHT was significantly greater than that of the LPS group, but there was no significant difference between the LPS+SHT group and the SHT group (Fig. 1).

2. The effect of SHT on Nitrite production

We measured the nitrite concentration in the cultured CCSM cells which were stimulated with LPS and then activated by SHT for determining the NO formation in the cells. The nitrite production activated by each of the LPS and LPS+SHT (indicated doses) groups was significantly greater than that of the control group. The SHT increased the nitrite production in the CCSM cells dose-dependently (Fig. 2).

3. The effects of SHT on cGMP level

The level of cGMP was measured in the CCSM cells which were activated by SHT at indicated doses. The SHT increased the cGMP level in the CCSM cells dose-dependently. The cGMP level in the CCSM cells activated by SHT (50 mg/mL and 100 mg/mL) was significantly greater than that of the control group (Fig. 3).

4. The effects of SHT on eNOS expression

To evaluate the effects of SHT on eNOS expression in CCSM cells, we did Western blot to the cells which were stimulated with LPS in the absence or presence of SHT. The SHT increased the eNOS enzyme protein production in the CCSM cells dose-dependently at 50 and 100 mg/mL but showed lower expression at 10mg/mL (Fig. 4).

5. The effects of SHT on penile smooth muscle relaxation

To determine the effects of SHT on penile smooth muscle relaxation, the SHT extract powder was administered orally to rats by the dosage of 1 g/kg for fifteen days. Rat penile erection was accomplished by papaverine hydrochloride injection into the penile corpus cavernosum. The whole penis was removed, and then frozen specimens were prepared and visualized under the microscope. The penile smooth muscle relaxation of the rat group treated with SHT was augmented over that of the control group. Also, the eNOS expression in the endothelium of the rat group treated with SHT was increased over that of the control group (Fig. 5).

Discussion

Normal male sexual function requires an intact libido, the ability to achieve and maintain penile erection, and ejaculation. Penile tumescence leading to erection depends on increased blood flow into the lacunar network after complete relaxation of the arteries and corpus cavernous smooth muscle. The micro architecture of the corpus cavernosum is composed of a mass of smooth muscle which contains a network of endothelial-lined vessels. Subsequent compression of the trabecular smooth muscle against the fibroelastic tunica albuginea causes a passive

closure of the emissary veins and accumulation of blood in the corpus cavernosum. In the presence of a full erection and a competent valve mechanism, the corpus cavernosa become non-compressible cylinders from which blood does not escape.

Neural input to smooth muscle tone is crucial to the initiation and maintenance of an erection. There is also an intricate interaction between the corpus cavernous smooth muscle and its overlying endothelial cell lining. Nitric oxide (NO), which induces vascular relaxation, promotes erection. Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS), and is released from the non-adrenergic, non-cholinergic (NANC) autonomic nerve supply to act postjunctionally on smooth muscle cells. NO increases the production of cGMP, which induces relaxation of the smooth muscle. cGMP is gradually broken down by PDE5.

Erectile dysfunction (ED) may be due to inability of penile smooth muscle to relax. This inability can have multiple causes, including nerve damage, endothelial damage, alteration in receptor expression/function, or in the transduction pathways that are implicated in the relaxation and contraction of smooth muscle cells. Various diseases commonly associated with impotence can alter the mechanisms that control penile smooth muscle tone. Often, changes in the L-arginine/NO/cGMP system are involved.

A wide variety of drugs have been used for treatment of ED. Major advances have been made in our understanding of the mechanisms of drug action and of the mechanisms of penile erection, and presently, there seems to be a rational basis for a therapeutic classification of currently used drugs. Such a useful classification was suggested by Heaton et al.[13], in which ED treatments were divided into five major classes by their mode of action: 1) central initiators; 2) peripheral initiators; 3) central conditioners; 4) peripheral conditioners; and 5) other.

SHT, a natural product and a traditional herbal medicine, has been used for ED and other symptoms relating to weakness or colds, and its modified formulas used in clinical practice widely for the same reason. SHT is composed of *Paeonia Radix, Rehmanniae Radix, Astragali Radix, Angelicae gigantis Radix, Chuanxiong Rhizoma, Cassiae Cortex, Glycyrrhizae Radix, Zingiberis Rhizoma, Zizyphi inermis Fructus*. Among these, *Angelicae gigantis Radix* was reported to have vasorelaxation effect[14], *Astragali Radix* and *Chuanxiong Rhizoma* have protective effects on endothelial dysfunction[15], and *Paeonia Radix* has an endothelium-dependent vasodilator effect[16]. However, its fundamental mechanism of action is not clearly known. In the present study, we investigated the mechanism of its erectile action by determining its direct effect on penile corpus cavernous smooth muscle cells.

To evaluate the effect of SHT on NOS activity, the cultured CCSM cells were stimulated with LPS in the absence or presence of water extracts of SHT, and then NOS activity was measured. The NOS activity stimulated with LPS, LPS+SHT and SHT was significantly greater than that of the control group. Furthermore, the NOS activity treated with LPS+SHT and SHT was significantly greater than that of the LPS group, but there was no significant difference between the LPS+SHT and SHT groups (Fig. 1). Also, to evaluate the effects of SHT on eNOS expression in CCSM cells, we did Western blot to the cells which were stimulated with LPS in the absence or presence of SHT. SHT increased the eNOS enzyme protein production in the CCSM cells dose-dependently (Fig. 4). These results are consistent with Bloch et al.'s study[17]. They examined activities of NOS

enzymes in specimens of potent and impotent patients by means of light and electron microscopy using NADPH diaphorase staining and immunohistochemical eNOS-specific, smooth muscle actin-specific, and nNOS-specific markers. They found a distinct expression of eNOS in cavernous smooth muscle and in the small intracavernous helicine arteries.

We measured the nitrite concentration in the cultured CCSM cells which were stimulated with LPS and then activated by SHT for determining the NO formation in the cells. The nitrite production activated by LPS and LPS+ SHT was significantly greater than that of the control group. SHT increased the nitrite production in the CCSM cells dose-dependently (Fig. 2). NO's high chemical reactivity means that its life is short, so we evaluate NO formation by detecting nitrite production[18]. The result that SHT increased NO formation strongly indicates that SHT has potency for penile erection.

The level of cGMP was measured in the CCSM cells which were activated by SHT. SHT increased the cGMP level in the CCSM cells dose-dependently. The cGMP level in the CCSM cells activated by 50 and 100 mg/mL SHT was significantly greater than that of the control group (Fig. 3). Soluble guanylyl cyclase, when activated by NO, catalyzes the formation of cGMP from GTP, whereas PDEs catalyze the hydrosis of cGMP to GMP. Termination of signal transduction by hydrolysis of cGMP depends on the specificity and expression of PDE isozymes in the target tissues[19]. One such class of drugs is sildenafil, an inhibitor of cyclic GMP-specific PDE, for use in male erectile dysfunction[20]. In our study, SHT significantly raised the intracellular cGMP levels in a concentration-dependent manner in primary rat CCSM cells. These results further confirm that SHT activates the NO/sGC/cGMP pathway, and therefore elevates the intracellular cGMP levels leading to the CCSM relaxation.

In the in vivo study, to determine the effect of SHT on penile smooth muscle relaxation, the SHT extract powder was administered orally to rats in a dosage of 1 g/kg for fifteen days. Papaverine hydrochloride was injected intracavernosally for pharmacological penile erection, then frozen specimens were prepared and visualized under the microscope. The penile smooth muscle relaxation of the SHT treated group was augmented over that of the control group. Also, eNOS expression in the endothelium of the rat group treated with SHT increased over that of the control group (Fig. 5). Papaverine is believed to be a non-selective phosphodiesterase inhibitor that promotes smooth muscle relaxation directly[21]. Our findings indicate that oral administration of SHT increases rat penile smooth muscle relaxation and eNOS expression in the endothelium when the penis was stimulated by papaverine.

The present study shows that SHT increases the NOS activity, synthesizes NO, and augments the cGMP, which mediates penile erection. Clear determination of the SHT mechanism related with NO/cGMP pathway has therapeutic implications. SHT extracts might be a candidate for alleviating erectile dysfunction. However, the other mechanisms of SHT should be investigated hereafter.

Conclusion

The main purpose of the present study was to investigate the mechanism of the erectile effect of SHT by focusing on its direct effects on corpus cavernous smooth muscle cells. We investigated NOS activity, nitrite concentration and cGMP level in the rat corpus cavernous smooth muscle

cell lines activated by SHT extracts. Furthermore we evaluated the effect of SHT extracts on penile smooth muscle relaxation following oral administration of SHT extract powder to rats in the dosage of 1 g/kg for fifteen days. The result of our experiment is as follows:

1. The SHT extracts increased NOS activity in the rat corpus cavernous smooth muscle cell lines.

2. The SHT extracts increased nitrite concentration in the rat corpus cavernous smooth muscle cell lines.

3. The SHT extracts increased cGMP level in the rat corpus cavernous smooth muscle cell lines.

4. The SHT extracts increased eNOS expression in the rat corpus cavernous smooth muscle cell lines.

5. The SHT extracts augmented penile smooth muscle relaxation and eNOS expression in the endothelium of the rat.

In conclusion, the present study shows that SHT increases NOS activity, synthesizes NO and augments cGMP, which mediates penile erection. Further determination of the SHT mechanism related with NO/cGMP pathway strongly indicates that SHT can be used as a remedy for erectile impotence.

References

1. National Institutes of Health Consensus Statement. Impotence. J Am Med Assoc. 1993;123: 23-7.
2. Andersson K E, Wagner G. Physiology of penile erection. Physiol Rev. 1995;75:191-236.
3. Burnett A L. Nitric oxide in the penis: physiology and pathology. J Urol. 1997;157:320-4.
4. Trigo-Rocha F, Hsu G L, Donatucci C F, Lue T F. The role of cyclic adenosine monophosphate, cyclic guanosine monophosphate, endothelium and nonadrenergic, noncholinergic neurotransmission in canine penile erection. J Urol. 1993;149:872-7.
5. Liu X, Lin C S, Graziottin T, Resplande J, Lue T F. Vascular endothelial growth factor promotes proliferation and migration of cavernous smooth muscle cells. J Urol 2001;166(1):354-60.
6. Dahiya R, Sikka S, Hellstrom W J, Hayward S W, Narayan P, Tanagho EA, et al. Phenotypic and cytogenetic characterization of a human corpus cavernosum cell line (DS-1). Biochem Mol Biol Int 1993;30(3):559-69.
7. Schmidt H H H W, Murad F. Purification and characterization of a human NO synthase. Biochem. Biophys. Res. Commun. 1991;181:1372-7.
8. Green L C, Wanger D A, Glogowski J, Skipper P L, Wishnok J S, Tannenbaum S. R. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem. 1982;126(1):131-8.
9. Wu B N, Lin R J, Lin C Y, Shen K P, Chiang L C, Chen I J. A xanthine-based KMUP-1 with cyclic GMP enhancing and K+ channels opening activities in rat aortic smooth muscle. Br. J. Pharmacol. 2001;134:265-74.
10. Towbin H, Staehelin T, Gordon J. Electroniphoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA. 1979;76:4350-4.
11. Burnett A L, Nelson R J, Calvin D C, Liu J X, Demas G E, Klein S L, Kriegsfeld L J, Dawson V L, Dawson T M, Snyder S H. Nitric oxide-dependent penile erection in mice lacking neuronal nitric oxide synthase. Mol Med. 1996;2(3):288-96.
12. Rehman J, Christ G, Melman A, Fleischmann J. Intracavernous pressure responses to physical

and electrical stimulation of the cavernous nerve in rats. Urology. 1998;51(4):640-4.
13. Heaton JP, Adams MA, Morales A. A therapeutic taxonomy of treatments for erectile dysfunction: an evolutionary imperative. Int J Impot Res. 1997;9:115-21.
14. Kim SJ, Song BK, Lee EJ, Kim HK, Kim JK. Effects of Radix Angelicae Gigantis and Resina Ferulae on the Relaxation of Smooth Muscle and Expression of iNOS. J Korean Oriental Med. 2000;20(2):60-7.
15. Lu ZM, Yu YR, Tang H, Zhang XX. The protective effects of radix astragali and rhizoma ligustici chuanxiong on endothelial dysfunction in type 2 diabetic patients with microalbuminuria. Sichuan Da Xue Xue Bao Yi Xue Ban. 2005;36 (4):529-32.
16. Goto H, Shimada Y, Akechi Y, Kohta K, Hattori M, Terasawa K. Endothelium-dependent vasodilator effect of extract prepared from the roots of Paeonia lactiflora on isolated rat aorta. Planta Med. 1996;62(5):436-9.
17. Bloch W, Klotz T, Sedlaczek P, Zumbe J, Engelmann U, Addicks K. Evidence for the involvement of endothelial nitric oxide synthase from smooth muscle cells in the erectile function of the human corpus cavernosum. Urol Res. 1998;26:128-35.
18. Lowenstein CJ, Snyder SH. Nitric oxide, A novel biologic messenger. Cell. 1992;72:705-7.
19. Juilfs DM, Soderling S, Burns F, Beavo JA. Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases (PDEs). Rev Physiol Biochem Pharmacol. 1999;135:67-104.
20. Wallis RM, Corbin JD, Francis, SH, Ellis P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am. J. Cardiol. 1999;83:3C-12C.
21. Maggi M, Filippi S, Ledda F, Magini A, Forti G. Erectile dysfunction: from biochemical pharmacology to advances in medical therapy. Eur J Endocrinol. 2000;143:143-54.