Blackwell Publishing

Asian J Androl 2007; 9 (6): 795–800
DOI: 10.1111/j.1745-7262.2007.00321.x

AJA
www.asiaandro.com

· *Original Article* ·

Relaxation mechanisms of neferine on the rabbit corpus cavernosum tissue *in vitro*

Jun Chen[1,2,†], Jun Qi[1,†], Fang Chen[1], Ji-Hong Liu[2], Tao Wang[2], Jun Yang[2], Chun-Ping Yin[3]

[1]*Department of Urology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200092, China*
[2]*Department of Urology,* [3]*Department of Pharmacy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

Abstract

Aim: To investigate the relaxation mechanisms of neferine (Nef) on the rabbit corpus cavernosum tissue *in vitro*. **Methods:** Strips of rabbit corpus cavernosum were mounted in organ chambers. The effects of Nef were examined on isolated muscle strips precontracted with phenylephrine (PE) alone, in the presence of N^W-nitro-*L*-arginine (LNNA, a nitric oxide synthase inhibitor), 1-*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor), indomethacin (cyclooxygenase inhibitor), tetraethylammonium (Ca^{2+}-activated K^+ channel blocker), 4-aminopiridine (4-AP ,voltage dependent K^+ channel blocker) and glibenclamide (ATP sensitive K^+channel blocker). The effects of Nef on KCl-induced contraction of isolated muscle strips were also investigated. The procedure of calcium absence-calcium addition was designed to observe the effect of Nef on two components of the contractile responses to PE based on the source of Ca^{2+} (extracellular *vs.* intracellular). **Results:** Corpus cavernosum strips relaxed in response to Nef (10^{-9}–10^{-4} mol/L) in a concentration-dependent manner with an IC_{50} of 4.60×10^{-6} mol/L. However, they were not affected by LNNA, ODQ, indomethacin or K^+-channel blockers. Nef (10^{-6} mol/L, 10^{-5} mol/L) concentration dependently reduced the maximal contraction response of isolated strips induced by KCl to 79.3% ± 5.5% and 61.5% ± 3.2%, respectively ($P < 0.01$). In the calcium absence–calcium addition procedure, Nef 10^{-5} mol/L inhibited both intracellular calcium-dependent and extracellular calcium-dependent contraction induced by PE (2×10^{-5} mol/L) ($P < 0.05$). The inhibition ratios were 26.2% ± 5.4% and 48.3% ± 7.6%, respectively. **Conclusion:** The results of the present study suggest that Nef possesses a relaxant effect on rabbit corpus cavernosum tissues, which is attributable to the inhibition of extracellular Ca^{2+} influx and the inhibition of release of intracellular stored Ca^{2+}, but not mediated by the release of nitric oxide, prostaglandins or by the activation of potassium channels.*(Asian J Androl 2007 Nov; 9: 795–800)*

Keywords: neferine; corpus cavernosum; relaxation

Correspondence to: Prof. Fang Chen, Department of Urology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200092, China.
Tel: +86-21-6515-1555 Fax: +86-21-6579-5173
E-mail: chenfang007@hotmail.com
Prof. Jun Qi, Department of Urology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200092, China.
Tel: +86-21-6579-0000 ext.7805 Fax: +86-21-6579-5173
E-mail: jasonqi@sh163.net
†These two authors contributed equally to this work.
Received 2006-12-27 Accepted 2007-06-04

1 Introduction

Erectile dysfunction (ED) is a common problem affecting approximately 50% of men aged 40–70 years [1]. Current pharmacological treatment for ED includes the oral, intracavernosal and intraurethral administration of erectogenic drugs. Oral pharmacotherapy is the most effective therapy for ED with the highest patient preference. Oral phosphodiesterase type 5 (PDE5)-inhibitors (sildenafil, tadalafil and vardenafil) are superior in effectiveness to centrally acting drugs (apomorphine and yohimbine). Local pharmacotherapy (intracavernosal and intraurethral treatments) is a second line therapy in cases of failure or contraindica-tions for oral pharmacotherapy [2]. Although many drugs are now available for treating ED, finding a new drug for treating ED and understanding its mechanism of action is still important.

Many traditional Chinese medicines are effective as treatments for ED. Because of the complex chemical ingredients, it remains unclear which ingredients exactly, and by which mechanisms, have the chemical effect in the treatment of ED. Some extracts from traditional Chinese medicines, of the alkaloids, coumarin and saponin series, relax the smooth muscle of corpus cavernosum [3–8], which provides an open window for developing new drugs for the treatment of ED.

Neferine (Nef) is a bis-benzylisoquinoline alkaloid extracted (isolated) from the green seed embryo of Nelumbo nucifera Gaertn, which is effective in preventing the onset of reentrant ventricular tachyarrhythmias [9–10]. Nef can inhibit very low density lipoprotein oxidation [11] and platelet aggregation [12], protect vascular endothelial cells from damage induced by oxygen free radicals [13] and increase sensitivity to anticancer drugs [14]. In the course of our studies on the development of naturally occurring agents for the treatment of ED, we found that Nef induced relaxation on the phenylephrine (PE)-precontracted corpus cavernosum [6]. In the present study, the relaxation mechanisms of Nef on the isolated rabbit corpus cavernosum were investigated.

2 Materials and methods

All animal experiments were carried out with the approval of the Institute for Animal Care and Use Committee of Xinhua Hospital (Shanghai, China).

2.1 Materials

Nef was kindly provided by Prof. Jia-Ling Wang (Department of Pharmacology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China). The purity of Nef was greater than 99.8%. It was dissolved in HCl 0.1 mol/L, then diluted with Krebs' solution to the desired concentration. The following drugs were all obtained from Sigma Chemical Company (St. Louis, MO, USA): N^{W}-nitro-*L*-arginine (LNNA, a nitric oxide synthase inhibitor), 1-*H*-[1,2,4]oxadiazolo [4,3-α]quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor), indomethacin (cyclooxygenase inhibitor), tetraethylammonium (TEA, Ca^{2+}-activated K^+ channel blocker), 4-aminopiridine (4-AP, voltage dependent K^+ channel blocker) and glibenclamide (ATP sensitive K^+channel blocker). Phenylephrine (PE) was from Shanghai Harvest Pharmaceutical (Shanghai, China). The Krebs' solution (in mmol/L) consists of: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11 (pH 7.4). In calcium-free solution, $CaCl_2$ was omitted from the Krebs' solution with the addition of 0.05 mmol/L egtaic acid (EGTA).

2.2 Tissue procurement

Adult male (4–6 months) New Zealand white rabbits (2.5–3.0 kg) were killed with pentobarbital sodium (50 mg/kg). The penis was surgically removed *en bloc* with care being taken to keep the tunica albuginea intact in all animals. The corpus spongiosum and urethra were excised. The corpus cavernosum tissue was then carefully dissected free from the surrounding tunica albuginea. Each rabbit provided two strips of corpus cavernosum tissue. The strips of corpus cavernosum with dimensions of approximately 2 × 2 × 6 mm were mounted in 10 mL Organ-bath chambers containing Krebs buffer solution (pH 7.4) at 37ºC, and aerated with 95% oxygen and 5% CO_2. The strips were suspended with silk ties to a force-displacement transducer (ML T0201/D, AD Instruments, Sydney, Australia). Changes in isometric tension were measured by Powerlab/4sp (AD Instruments, Sydney, Australia). The strips of corpus cavernosum were stretched to a resting force of 2.0 g and were equilibrated for at least 60 min. During this period, the tissue was washed with fresh solution at 37ºC, for a consecutive 15 min, and tension was adjusted if necessary.

2.3 Organ bath experiments

2.3.1 Effects of Nef on PE-induced rabbit corpus

cavernosum smooth muscle responses alone, in the presence of LNNA, ODQ, indomethacin, TEA, 4-AP and glibenclamide

Strips were precontracted with 10^{-5} mol/L PE. After muscle strips were stabilized, relaxation responses to cumulative concentrations of Nef (10^{-9}–10^{-4} mol/L) were assessed. When the influence of antagonists on the Nef-induced relaxation was evaluated, inhibitors (LNNA 10^{-4} mol/L, ODQ 10^{-4} mol/L, indomethacin 10^{-4} mol/L, TEA 10^{-4} mol/L, 4-AP 10^{-4} mol/L or glibenclamide 10^{-5} mol/L) were added to the organ bath 20 min before addition of PE in different experiments.

2.3.2 Effects of Nef on KCl-induced rabbit corpus cavernosum smooth muscle responses

The addition of cumulative concentrations of KCl (2×10^{-2}, 4×10^{-2}, 6×10^{-2}, 8×10^{-2}, 10×10^{-2} mol/L) was performed and concentration response was recorded. After washout by Krebs solution and reequilibration, each cavernosal strip was incubated with different concentrations (10^{-6} mol/L, 10^{-5} mol/L) of Nef and equilibrated for 20 min. Cumulative concentration responses of KCl were repeatedly recorded.

2.3.3 Effects of Nef on the two contractile elements of isolated corpus cavernosum smooth muscle strips induced by PE

In Ca^{2+}-free medium, PE induced rapid transient contraction, presumably because of release of intracellular Ca^{2+}; and the sustained contraction when extracellular Ca^{2+} was present presumably the result of Ca^{2+} influx [15]. After being washed with Ca^{2+}-free Krebs solution three times and being equilibrated for 20 min, the strips were contracted by 2×10^{-5} mol/L PE (due to release of intracellular Ca^{2+}). After a stable higher tension baseline was established, 2.5×10^{-3} mol/L $CaCl_2$ was added, strips contracted again and a peak was reached (as a result of entry of Ca^{2+}). This was followed by thorough washout by Krebs solution and refilling of intracellular Ca^{2+} stores by repeated stimulation of 6×10^{-2} mol/L KCl in Ca^{2+} containing Krebs solution three times. After washing with Ca^{2+}-free Krebs solution, 10^{-5} mol/L Nef was added and incubated for 20 min, and then the above-mentioned experiment was repeated. The effects of Nef on the two contractile elements of isolated strips induced by PE were observed.

2.3.4 Data analysis

All data were expressed as mean ± SEM. Responses were expressed as a percentage of active muscle tone induced by PE or KCl. Statistical analysis was performed with one-way analysis of variance using SPSS 12.0 software (SPSS Ltd., Chicago, IL, USA). $P < 0.05$ was considered a significant difference.

3 Results

3.1 Effects of Nef on PE-induced rabbit corpus cavernosum smooth muscle responses alone, in the presence of LNNA, ODQ, indomethacin, TEA, 4-AP and glibenclamide

Corpus cavernosum strips relaxed in response to Nef (10^{-9}–10^{-4} mol/L) in a concentration-dependent manner with an IC_{50} of 4.60×10^{-6} mol/L ($n = 8$, $P < 0.05$) (Figure 1). However, the response of isolated muscle strips was not affected by nitric oxide synthase inhibitor (LNNA), guanylyl cyclase inhibitor (ODQ), cyclooxygenase inhibitor (indomethacin), Ca^{2+}-activated K^+ channel blockers (TEA), voltage dependent K^+ channel blocker (4-AP) and ATP sensitive K^+channel blocker (glibenclamide) ($n = 8$, $P > 0.05$) (Figure 2).

3.2 Effects of Nef on KCl-induced contraction of isolated corpus cavernosum smooth muscle strips

Nef (10^{-6} mol/L, 10^{-5} mol/L) concentration dependently reduced the maximal contraction response of isolated strips induced by KCl to 79.3% ± 5.5% and 61.5% ± 3.2%, respectively ($n = 8$, $P < 0.01$) (Figure 3).

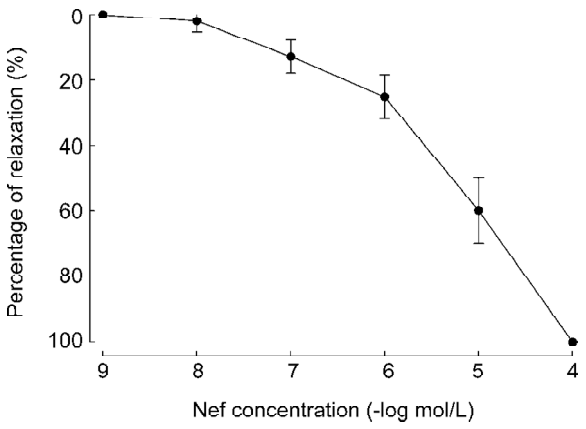

Figure 1. Relaxation effects of neferine (Nef) on phenylephrine-induced contraction of rabbit corpus cavernosum tissue *in vitro* ($n = 8$, the number of muscle strips).

3.3 Effects of Nef on the two contractile elements of isolated corpus cavernosum smooth muscle strips induced by phenylephrine

Nef 10^{-5} mol/L inhibited both intracellular calcium-dependent and extracellular calcium-dependent contraction induced by PE (2×10^{-5} mol/L) ($n = 6$, $P < 0.05$). The inhibition ratios were 26.2% ± 5.4% and 48.3% ± 7.6%, respectively (Figure 4).

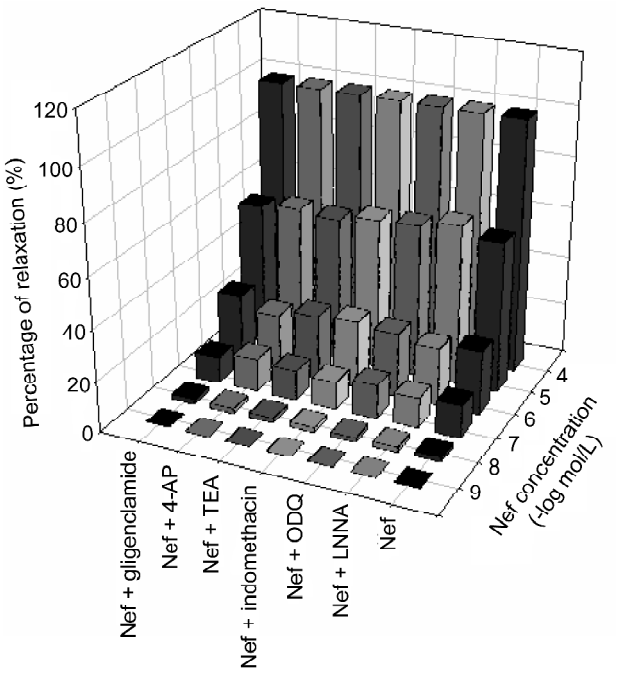

Figure 2. Relaxation effects of neferine (Nef) on phenylephrine-induced contraction of isolated rabbit corpus cavernosum tissue alone, in the presence of indomethacin, N^W-nitro-*L*-arginine (LNNA), 1-*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), tetraethylammonium(TEA), 4-aminopiridine (4-AP), and glibenclamide ($n = 8$, the number of muscle strips).

4 Discussion

A number of mechanisms have been identified for the local regulation of penile smooth muscle contractility and, therefore, penile erection. Molecules participating in these pathways can be considered targets for the development of new drugs to treat ED.

Many studies have demonstrated that nitric oxide (NO) is the most important factor for immediate relaxation of penile vessels and corpus cavernosum. During sexual stimulation, NO releases from the non-adrenergic non-cholinergic nerve terminals and endothelium within the erectile tissue of the penis. It activates guanylyl cyclase, resulting in an increased conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). cGMP in turn activates protein kinase G, which phosphorylates certain proteins and ion channels. This

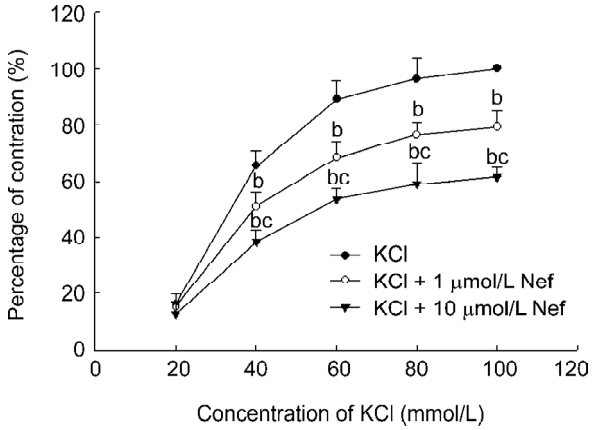

Figure 3. Relaxation effects of neferine (Nef) on KCl-induced contraction of rabbit corpus cavernosum tissue *in vitro* ($n = 8$, the number of muscle strips). $^bP < 0.01$, compared with the group of KCl. $^cP < 0.01$, compared with the group of KCl plus 1 μmol/L Nef.

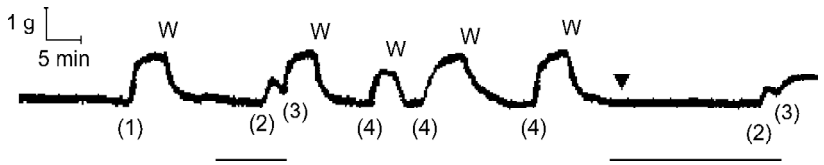

Figure 4. Effect of neferine on intracellular calcium dependent and extracellular calcium dependent contraction induced by 20 μmol/L phenylephrine (PE) ($n = 6$, the number of muscle strips). The horizontal lines indicate the change to Ca^{2+}-free medium. (1): precontraction by adding PE. (2): addition of PE (intracellular calcium-dependent). (3): addition of $CaCl_2$ (extracellular calcium-dependent contraction). (4): addition of KCl (for refilling of Ca^{2+} stores). W, washout; ▼: addition of Nef at desired concentrations.

process facilitates the reduction of intracellular calcium, enabling the smooth muscle to relax, resulting in erection [16, 17]. To investigate whether relaxation induced by Nef was attributable to an interaction with NO-cGMP pathway, corpus cavernosum tissues were pretreated with LNNA (a nitric oxide synthase inhibitor) and ODQ (a guanylyl cyclase inhibitor), respectively. The treatment of corpus cavernosum tissues with these inhibitors did not significantly affect the relaxant activity of Nef. These findings indicate that the relaxant action of Nef on the rabbit corpus cavernosum is not mediated by NO-cGMP pathway.

Prostaglandin (especially PGI_2 and PGE_1) is derived from arachidonic acid by the action of cyclooxygenases. Prostaglandin inhibits the release of noradrenaline from penile adrenergic nerves and increases intracellular cAMP levels in corpus cavernosum smooth muscle [18, 19]. This process promotes muscle relaxation through intracellular reduction of calcium concentration, resulting in erection. Therefore, the possibility that relaxation induced by Nef was a result of an interaction with prostaglandins was further investigated. However, the treatment of corpus cavernosum tissues with cyclooxygenase inhibitor (indomethacin) did not affect the relaxant activity of Nef. Therefore, the corpus cavernosum tissues relaxation effect of Nef was not related to prostaglandins.

Potassium channels (particularly Ca^{2+}-activated K^+ channel, voltage dependent K^+ channel, and ATP sensitive K^+channel) play an important role in the modulation of corpus cavernosum smooth muscle cell tone. Potassium channel opening is be expected to cause hyperpolarzation, inhibit Ca^{2+} channel activity, lower intracellular Ca^{2+} concentration and result in relaxation [20–23]. However, in the present experiment, we found that the treatment of corpus cavernosum tissues with TEA (Ca^{2+}-activated K^+ channel blocker), 4-AP (voltage dependent K^+ channel blocker) and glibenclamide (ATP sensitive K^+channel blocker) did not change the relaxant activity of Nef. These findings suggest that the relaxant effect of Nef is unrelated to Ca^{2+}-activated, voltage dependent and ATP sensitive K^+ channels.

It is understood that the contractile activity of corpus cavernosum smooth muscles is regulated by the concentration of free Ca^{2+} in the cytosol, which depends on voltage-dependent Ca^{2+} influx, receptor-operated Ca^{2+} influx, and release of intracellularly stored Ca^{2+} [24]. High extracellular K^+ induced the membrane depolarization rapidly, which opened the voltage-dependent Ca^{2+} channel and brought about the influx of the extracellular Ca^{2+}. PE elicited $[Ca^{2+}]_i$ elevation by: (i) activating α_1-adrenoceptor-operated Ca^{2+} channel to induce extracellular Ca^{2+} entry; and (ii) activating G protein and then facilitating the formation of IP3, which acts on IP3 receptor and induces intracellular Ca^{2+} release. In the present study, the contraction of corpus cavernosum tissues induced by PE or KCl was reduced by Nef. Furthermore, Nef also concentration-dependently relaxed PE-induced contraction of isolated muscle strips in a calcium-free solution. The results suggest that Nef relax corpus cavernosum tissues by inhibiting both extracellular Ca^{2+} influx and release of intracellular stored Ca^{2+}.

In conclusion, the results of the present study suggest that Nef possesses a relaxant effect on rabbit corpus cavernosum tissues, which is attributable to the inhibition of extracellular Ca^{2+} influx and the inhibition of release of intracellular stored Ca^{2+}, but is not mediated by the release of nitric oxide, prostaglandins or by the activation of potassium channels. Further experiments are required to establish whether cAMP signal transduction pathway, Rho-A/Rho-kinase pathway and cell-cell communication contributes to Nef induced relaxation or not. Because in the experiment on muscle strips *in vitro*, the influence of arterial inflow, venous outflow, autonomic innervation and a multitude of local and total body factors were negligible, further pharmacological research and toxicology tests are needed *in vivo* to clarify whether Nef can be developed into a drug for ED.

Acknowledgment

The authors thank Prof. Jia-Ling Wang for kindly supplying the Nef. The technical assistance from Mr Jun Pan (Department of Urology, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China) is also greatly appreciated. This study was sponsored in part by the National Natural Science Foundation of China (No. 30471736).

References

1 Feldman HA, Goldstein I, Hatzichristou DG, Krane RJ, McKinlay JB. Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. J Urol 1994; 151: 54–61.

2 Montorsi F, Salonia A, Deho' F, Cestari A, Guazzoni G, Rigatti P, *et al*. Pharmacological management of erectile dysfunction. BJU Int 2003; 91: 446–54.

3 Chen J, Liu JH. Advances in studies of Chinese herbs for improving relaxability of corporal smooth muscle. Zhonghua Nan Ke Xue 2003; 9: 615–8.
4 Xiao HJ, Liu JH, Wang SG, Chen Z, Li Y, Cao ZG. Effect of ligustrazine on the contraction of isolated rabbit corporal cavernosum tissues. Asian J Androl 2002; 4 (Suppl 3): 107.
5 Chen J, Liu JH, Xiao HJ, Wang T, Chen Z, Sun CL. The relaxation effects of tetrandrine on the corpus cavernosum tissue of rabbit *in vitro*. Zhonghua Nan Ke Xue 2003; 9: 234–6.
6 Chen J, Liu JH, Wang T, Wng SG, Yin CP, Xiao HJ, *et al*. The relaxation effects of six extracts from Chinese herbs on the corpus cavernosum tissue of rabbit *in vitro*. Zhonghua Nan Ke Xue. 2005; 11: 793–5, 799.
7 Liu JH, Chen J, Wang T, Liu B, Yang J, Chen XW, *et al*. Effects of tetrandrine on cytosolic free calcium concentration in corpus cavernosum smooth muscle cells of rabbits. Asian J Androl 2006, 8: 405–9.
8 Liu WJ, Xin ZC, Xin H, Yuan YM, Tian L, Guo YL. Effects of icariin on erectile function and expression of nitric oxide synthase isoforms in castrated rats. Asian J Androl 2005; 7: 381–8.
9 Guo Z. Electrophysiological effects of neferine against ischemic ventricular tachyarrhythmias. Zhonghua Xin Xue Guan Bing Za Zhi 1992; 20:119–22, 134.
10 Qian JQ. Cardiovascular pharmacological effects of bisbenzylisoquinoline alkaloid derivatives. Acta Pharmacol Sin 2002; 23:1086–92.
11 Feng Y, Wu J, Cong R, Wang C, Zong Y, Feng Z. The effect of neferine on foam cell formation by anti-low density lipoprotein oxidation. J Tongji Med Univ 1998; 18: 134–6.
12 Yu J, Hu WS. Effects of neferine on platelet aggregation in rabbits. Yao Xue Xue Bao 1997; 32: 1–4.
13 Wu YM, Jia JF, Li YJ. Protective effect of neferine on oxygen free radical-induced damage of vascular endothelial cell. Chin J Pharmacol Toxicol 1997; 11: 27.
14 Cao JG, Tang XQ, Shi SH. Multidrug resistance reversal in human gastric carcinoma cells by neferine. World J Gastroenterol 2004; 10: 3062–4.
15 Kwan CY, Chen YY, Ma MF, Daniel EE, Hui SC. Tetrandrine, a calcium antagonist of Chinese herbal origin, interacts with vascular muscle alpha 1-adrenoceptor. Life Sci 1996; 59: PL 359–64.
16 Saenz de Tejada I, Angulo J, Cellek S, Gonzalez-Cadavid N, Heaton J, Pickard R, *et al*. Pathophysiology of erectile dysfunction. J Sex Med 2005; 2: 26–39.
17 Dean RC, Lue TF. Physiology of penile erection and pathophysiology of erectile dysfunction. Urol Clin North Am 2005; 32: 379–95.
18 Moreland RB, Kim N, Nehra A, Goldstein I, Traish A. Functional prostaglandin E (EP) receptors in human penile corpus cavernosum. Int J Impot Res 2003; 15: 362–8.
19 Wespes E. Smooth muscle pathology and erectile dysfunction. Int J Impot Res 2002; 14 Suppl 1: S17–21.
20 Christ GJ. K^+ channels and gap junctions in the modulation of corporal smooth muscle tone. Drug News Perspect 2000; 13: 28–36.
21 Lee SW. Physiological roles and properties of potassium channels in corporal smooth muscle. Drugs Today (Barc) 2000; 36: 147–54.
22 Archer SL. Potassium channels and erectile dysfunction. Vascul Pharmacol 2002; 38: 61–71.
23 Christ GJ. K channels as molecular targets for the treatment of erectile dysfunction. J Androl 2002; 23: S10–9.
24 Liu J, Chen J. Ion channels and penile erection. Zhonghua Nan Ke Xue 2004; 10: 403–6, 410.

Edited by Dr Tibet Erdogru