Blackwell Publishing

Asian J Androl 2008; 10 (2): 307–312
DOI: 10.1111/j.1745-7262.2008.00342.x

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·*Original Article*·

Effects of plant extract neferine on cyclic adenosine monophosphate and cyclic guanosine monophosphate levels in rabbit corpus cavernosum *in vitro*

Jun Chen[1,†,*], Ji-Hong Liu[1,†], Tao Wang[1], Heng-Jun Xiao[2], Chun-Ping Yin[3], Jun Yang[1]

[1]*Department of Urology, Tongji Hospital,* [3]*Department of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[2]*Department of Urology, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China*

Abstract

Aim: To further investigate the relaxation mechanism of neferine (Nef), a bis-benzylisoquinoline alkaloid extracted (isolated) from the green seed embryo of Nelumbo nucifera Gaertn in China, on rabbit corpus cavernosum tissue *in vitro*. **Methods:** The effects of Nef on the concentrations of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in isolated and incubated rabbit corpus cavernosum tissue were recorded using ^{125}I radioimmunoassay. **Results:** The basal concentration of cAMP in corpus cavernosum tissue was 5.67 ± 0.97 pmol/mg. Nef increased the cAMP concentration in a dose-dependent manner ($P < 0.05$), but this effect was not inhibited by an adenylate cyclase inhibitor (cis-N-[2-phenylcyclopentyl]azacyclotridec-1-en-2-amine, MDL-12, 330A) ($P > 0.05$). The accumulation of cAMP induced by prostaglandin E_1 (PGE_1, a stimulator of cAMP production) was also augmented by Nef in a dose-dependent manner ($P < 0.05$). The basal concentration of cGMP in corpus cavernosum tissue is 0.44 ± 0.09 pmol/mg. Nef did not affect this concentration of cGMP, either in the presence or in the absence of a guanyl cyclase inhibitor (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, ODQ) ($P > 0.05$). Also, sodium nitroprusside (SNP, a stimulator of cGMP production)-induced cGMP production was not enhanced by Nef ($P > 0.05$). **Conclusion:** Nef, with its relaxation mechanism, can enhance the concentration of cAMP in rabbit corpus cavernosum tissue, probably by inhibiting phosphodiesterase activity. *(Asian J Androl 2008 Mar; 10: 307–312)*

Keywords: neferine; cyclic adenosine monophosphate; cyclic guanosine monophosphate; rabbit corpus cavernosum

Correspondence to:
Prof. Ji-Hong Liu, Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Tel: +86-27-8366-2278 Fax: +86-27-8360-8783
E-mail: jhliu@tjh.tjmu.edu.cn
and Dr Jun Chen, Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Tel: +86-21-6502-1292
E-mail: jchen121121@hotmail.com
Received 2006-12-22 Accepted 2007-07-26

1 Introduction

Erectile dysfunction (ED) is a common problem with a prevalence of approximately 50% in 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 and has 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 contraindications for oral pharmacotherapy [2]. Although many drugs are now available for treating ED, finding a new drug for treating ED and understand its mechanism of action are still important research goals.

Many traditional Chinese medicines are effective in treating ED. Because of the complex chemical ingredients, it remains unclear which ingredient exactly, and by which mechanisms, has a positive effect in treating ED. Recent studies have found that some extracts from traditional Chinese medicines, containing alkaloids, coumarin and saponin compounds, can relax the smooth muscle of corpus cavernosum [3–15], which provides an open window for developing new drugs for the treatment of ED.

Penile erection is the process that takes place under the regulation of the neural and endocrine system through the dilation of the penile arteries and corpus cavernosum, thereby increasing the blood flow into the penis. During sexual stimulation, nitric oxide (NO) is released, by the action of nitric oxide synthase (NOS), from non-adrenergic, non-cholinergic nerves, parasympathetic nerves and endothelium. Guanylate cyclase is activated by NO, which converts cyclic guanosine monophosphate (GTP) into cyclic guanosine monophosphate (cGMP). In addition, adenylate cyclase is also activated by receptor mechanisms (such as by prostacyclin, prostaglandin E1 and E2, and β-adrenoceptor agonists) or non-receptor mechanisms (such as activated by forskolin) leading to increases in cyclic adenosine monophosphate (cAMP) levels. By inhibiting calcium channels, both cGMP and cAMP decrease intracellular Ca^{2+}, causing the relaxation of cavernous smooth muscle, and thus erection [16–18]. Therapeutically, drugs that enhance cGMP and/or cAMP accumulation could be applied in the treatment of ED.

Neferine (Nef) is a bis-benzylisoquinoline alkaloid extracted (isolated) from the green seed embryo of *Nelumbo nucifera Gaertn* in China, which is effective in preventing from the onset of reentrant ventricular tachyarrhythmias [19, 20]. Moreover, Nef can inhibit very low density lipoprotein oxidation [21] and platelet aggregation [22], protect vascular endothelial cells from damage induced by oxygen free radicals [23], and increase sensitivity to anticancer drugs [24]. The empirical formula of Nef is $C_{38}H_{44}N_2O_6$, with a relative molecular weight of 624. The molecular structure of Nef is showed in Figure 1. 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 [14]. In the present study, the effects of Nef on cAMP and cGMP levels in isolated and incubated rabbit corpus cavernosum are measured using ^{125}I radioimmunoassay to clarify the relaxation mechanisms of Nef on the corpus cavernosum smooth muscle.

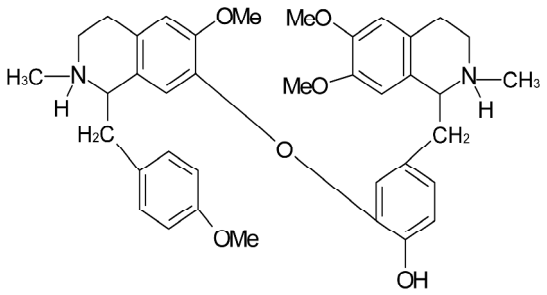

Figure 1. Molecular structure of neferine.

2 Materials and methods

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

2.1 Reagents

Neferine was kindly provided by Professor Jia-Ling Wang (Department of Pharmacology, Tongji Medical College, Huazhong University of Science and Technology, Wuha, China). The purity of Nef was greater than 98%. cGMP and cAMP ^{125}I radioimmunoassay kits were purchased from Shanghai Chinese Medicine University (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) was acquired from Gibco (Grand Island, NY, USA); MDL-12,330A (cis-N-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine) and ODQ (1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one) from Sigma Chemical; sodium nitroprusside (SNP) from the Board of Beijing Double-Crane Pharmaceuticals (Beijing, China) and PGE_1 (prostaglandin E_1) from Nanyang Pukang Groups (Nayang, China).

2.2 Rabbit corpus cavernosum preparation

Male New Zealand white rabbits (aged 4–6 months, approximately 2.5–3.0 kg body weight) were killed by air injection and their penises were excised soon afterwards. After the epidermis, connective tissue and albuginea were removed, and each penis was cut longitudinally into two strips and then transversely into eight segments. The segments of corpora cavernosa from all the rabbits were pooled and incubated in DMEM at 37ºC for 1 h with regular changes of medium to allow the tissues to recover from preparative handling. The solution was continuously gassed with 95% oxygen and 5% carbon dioxide (carbogen).

2.3 Pro-releasing of cAMP and cGMP

2.3.1 Effects of Nef on cAMP and cGMP levels in rabbit corpus cavernosum

The freshly isolated corpus cavernosum segments were incubated in 48-well plates with 5×10^{-1} mL DMEM at 37ºC for 1 h. After DMEM was removed, these segments were divided into three groups and the segments in each group were subdivided into four portions. (1) Nef group: 2×10^{-1} mL DMEM was added to the four portions. (2) MDL-12,330A (adenylyl cyclase [AC] inhibitor) + Nef group: 1.8×10^{-1} mL DMEM and MDL-12,330A (final concentration 3×10^{-5} mol/L) 2×10^{-2} mL solvent were added, respectively. (3) ODQ (Guanylate cyclase inhibitor) + Nef group: 1.8×10^{-1} mL DMEM and ODQ (final concentration 3×10^{-5} mol/L) 2×10^{-2} mL solvent were added, respectively. After pre-incubation of the tissue for 10 min at 37ºC, various concentrations of Nef 2×10^{-2} mL were added (final concentration 0, 10^{-7} mol/L, 10^{-6} mol/L, 10^{-5} mol/L), respectively. They were then incubated at 37ºC for another 30 min.

2.3.2 Effects of Nef on cAMP and cGMP levels in rabbit corpus cavernosum in the presence of sodium nitroprusside and PGE_1

Fresh corpus cavernosum segments were incubated in 48-well plates with 5×10^{-1} mL DMEM at 37ºC for 1 h. After DMEM was removed, 1.8×10^{-1} mL DMEM and 2×10^{-2} mL solvent with various concentrations of Nef (final concentration 0, 10^{-7}, 10^{-6}, 10^{-5} mol/L, respectively) were added. After pre-incubation of the tissue for 10 min at 37°C, these segments were incubated with 2×10^{-2} mL PGE_1 (final concentration 10^{-6} mol/L) or SNP (final concentration 10^{-6} mol/L) for 30 min at 37ºC.

2.4 Extraction of cAMP and cGMP

The supernatants were removed; the reaction was stopped by the addition of 5×10^{-1} mL of 1 mol/L perchloric acid, and then the tissues were crushed and homogenated with 1 mL acetic acid buffer (5×10^{-2} mol/L, pH 4.75) in an ice bath. After centrifugation at 3 000 × *g* for 15 min at 4ºC, the supernatants were collected, dried in an oven at 60ºC, and then stored at 4ºC.

2.5 cAMP and cGMP assay

The concentrations of cAMP and cGMP were measured using the ^{125}I radioimmunoassay kits according to the manufacturer's instructions.

2.6 Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's multiple comparisons test, and the paired *t*-test using SPSS software version 10.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered a significant difference.

3 Results

3.1 Effects of neferine on cAMP levels in the rabbit corpus cavernosum in vitro

The basal level of cAMP in the rabbit corpus cavernosum was 5.67 ± 0.97 pmol/mg. Nef could increase the contents of cAMP in corpus cavernosum tissue in a dose-dependent manner ($P < 0.05$), but MDL-12,330A did not affect this elevation of cAMP levels ($n = 6$, $P > 0.05$) (Figure 2).

In the presence of PGE_1, the cAMP level increased to 56.42 ± 7.26 pmol/mg, representing a 9.95-fold increase, and Nef dose-dependently increased the cAMP levels in corpus cavernosum tissue ($n = 6$, $P < 0.05$) (Figure 3).

3.2 Effects of Nef on cGMP levels in the rabbit corpus cavernosum in vitro

The basic level of cGMP in the rabbit corpus cavernosum was 0.44 ± 0.09 pmol/mg. Whether ODQ was present or not, Nef had no effect on the cGMP accumulation cavernosum tissue ($n = 6$, $P > 0.05$) (Figure 4).

In the presence of SNP, the cGMP level increased to 1.90 ± 0.70 pmol/mg, representing a 4.32-fold increase. However, Nef had no effect on SNP-induced elevation

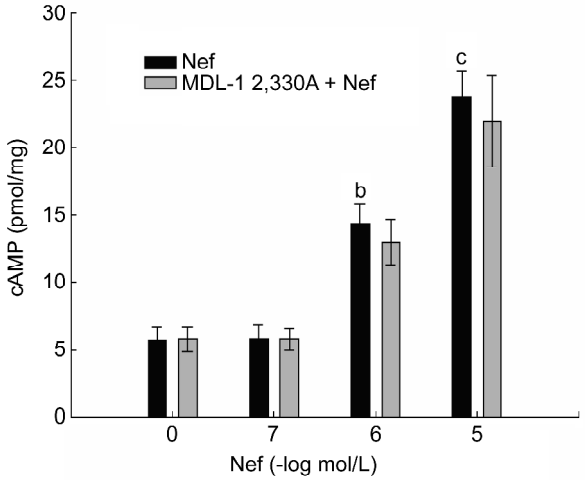

Figure 2. Effect of neferine (Nef) on cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) levels of corpus cavernosum smooth muscle (n = 6) in the absence and presence of adenylyl cyclase inhibitor MDL-12, 330A. $^{b}P < 0.05$, $^{b}P < 0.01$, compared with control group.

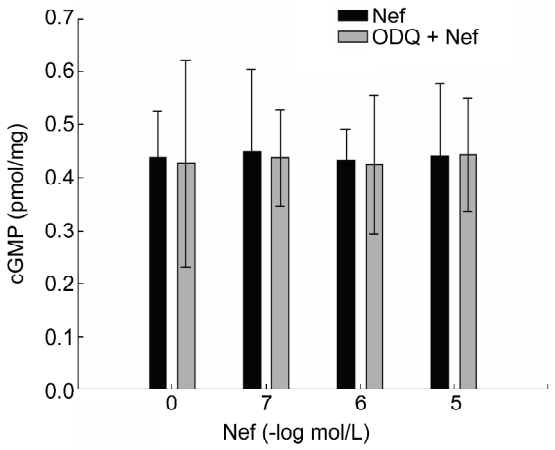

Figure 4. Effect of neferine (Nef) on cyclic guanosine monophosphate levels of corpus cavernosum smooth muscle in the absence and presence of guanylyl cyclase inhibitor ODQ (n = 6).

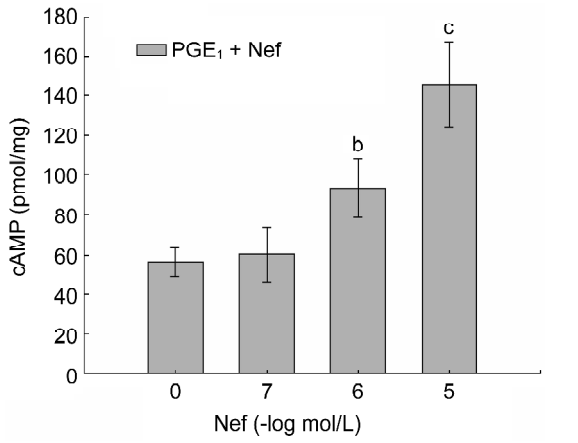

Figure 3. Effect of neferine (Nef) on prostaglandin (PGE_1)-induced cyclic adenosine monophosphate levels of corpus cavernosum smooth muscle (n = 6). $^{b}P < 0.05$, $^{c}P < 0.01$, Compared with control group.

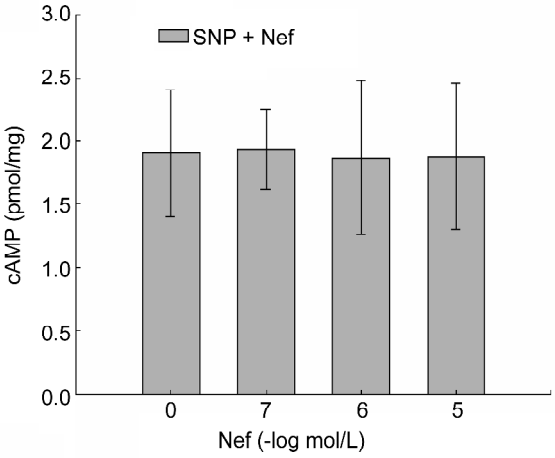

Figure 5. Effect of neferine (Nef) on sodium nitroprusside (SNP)-induced cyclic guanosine monophosphate (cGMP) levels of corpus cavernosum smooth muscle (n = 6).

of cGMP levels (n = 6, $P > 0.05$) (Figure 5).

4 Discussion

Using ^{125}I radioimmunoassay, we found that Nef had no effect on the cGMP accumulation in isolated and incubated corpus cavernosum tissue, whether or not the guanyl cyclase inhibitor (ODQ) or stimulator of cGMP production (SNP) was present. This indicated that the NO-cGMP pathway had nothing to do with the effect of Nef on the relaxation of the corpus cavernous smooth muscle.

In the present study, we found that Nef enhanced cAMP accumulation in the corpus cavernosum. These results demonstrate that the rise of cAMP levels might be one of the mechanisms of Nef-induced relaxation.

cAMP is produced from adenosine triphosphate (ATP) by AC, and is degraded to 5'-AMP by phosphodiesterases (PDE). The levels of cAMP are regulated by the AC and PDE activities. In the absence of any stimulation, the basal level of cAMP in the rabbit corpus cavernosum *in vitro* was 5.67 ± 0.97 pmol/mg. However, the incubation in the presence of 3×10^{-5} mol/L MDL-12,330A (AC inhibitor) had no effect on the basal level of cAMP. This indicated that the ability of producing cAMP by the rabbit corpus cavernosum *in vitro* was very weak when there was not any stimulation. However, in the absence of any stimulation, Nef could increase the contents of cAMP in corpus cavernosum tissue in a dose-dependent manner, and MDL-12,330A did not affect this elevation of cAMP levels. Moreover, Nef dose-dependently increased cAMP levels in corpus cavernosum tissue in the presence of PGE1 (a stimulator of cAMP production). This indicated that Nef increased cAMP in the corpus cavernosum probably by inhibiting PDE activity, without activating AC.

PDE is a superfamily enzyme system, 11 types of which have been reported. Some of these isoenzyme families contain more than one gene (isogenes) and some genes are alternatively spliced [25–27]. In early 1990s, using anion exchange chromatography, Stief and coworkers [28] reported the separation of hydrolytic activities of PDE isoenzymes 3, 4 and 5 from cytosolic supernatants prepared from human cavernous smooth muscle, whereas others reported the presence of PDE 2, 3 and 5 [29]. Currently, the presence of mRNAs specific for 14 different human PDE isoenzymes and isoforms in human cavernous tissue has been shown by means of reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. The expression of the following genes were detected: PDE1A, PDE1B, PDE1C, PDE2A and PDE10A, which hydrolyze both cAMP and cGMP; the cAMP specific PDEs PDE3A, PDE4A-D, PDE7A and PDE8A, and the cGMP specific PDEs PDE5A and PDE9A [26, 27]. However, the mere expression of an enzyme does not yield any information regarding its functional relevance. PDE3 and 5 are the predominant isoenzymes in the degradation of cNMP in human corpus cavernosum musculature [26–32]. Because a PDE isoenzyme can be expressed in several organs or tissues, there might be side effects associated with the systemic administration of a PDE inhibitor. For example, PDE5 is not only expressed in corpus cavernosum smooth muscle, but also in the vascular and central nervous system and the gastrointestinal tract. Therefore, the main adverse events of sildenafil include headache, visual disturbances, flushing and dyspepsia [33]. Meanwhile, the distributions of PDE isozymes in the corpus cavernosum vary from species to species. In rabbit corpus cavernosum, the PDE is PDE5, 2 and 1. PDE3, which contributes significantly to the total PDE activity in human corpus cavernosum, is apparently lacking in rabbit corpus cavernosum [34]. Therefore, it is important to perform further research to determine whether Nef enhances cAMP accumulation by inhibiting PDE1, 2, 3, 4, 7, 8 and/or by inhibiting PDE10 in corpus cavernosum and other organs or tissues in different species.

Acknowledgment

The authors thank Prof. Jia-Ling Wang for kindly supplying the neferine. The technical support from Prof. Bo-Hua Shu is also greatly appreciated. This study was sponsored by the National Natural Science Foundation of China (No. 30471736) and China Postdoctoral Science Foundation (No. 20070410176).

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Edited by Dr Premendu Prakash Mathur