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·Original Article·

# Effects of tetrandrine on cytosolic free calcium concentration in corpus cavernosum smooth muscle cells of rabbits

Ji-Hong Liu[1,†], Jun Chen[1,†], Tao Wang[1], Bo Liu[1], Jun Yang[1], Xiao-Wen Chen[1], Shao-Gang Wang[1], Chun-Ping Yin[2], Zhang-Qun Ye[1]

*[1]Department of Urology and [2]Department of Pharmacy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

**Abstract**

**Aim:** To study the relaxation mechanisms of tetrandrine (Tet) on the corpus cavernosum smooth muscle. **Methods:** The corpus cavernosum smooth muscle cells from New Zealand white rabbits were cultured *in vitro*. $[Ca^{2+}]_i$ was measured by Fluorescence Ion Digital Imaging System, using Fluo-2/AM as a $Ca^{2+}$-sensitive fluorescent indicator. **Results:** Tet (1, 10 and 100 μmol/L) had no effect on the resting $[Ca^{2+}]_i$ ($P > 0.05$). In the presence of extracellular $Ca^{2+}$ (2.5 mmol/L), Tet (1, 10 and 100 μmol/L) inhibited $[Ca^{2+}]_i$ elevation induced by high $K^+$ and phenylephrine (PE) in a concentration-dependent manner ($P < 0.05$). In calcium free solution containing egtaic acid, Tet (1 and 10 μmol/L) had no inhibitory effects on $[Ca^{2+}]_i$ elevation induced by PE ($P > 0.05$). However, Tet (100 μmol/L) inhibited $[Ca^{2+}]_i$ elevation induced by PE ($P < 0.05$). **Conclusion:** Tet inhibited the $Ca^{2+}$ influx from the extracellular site via voltage-activated $Ca^{2+}$ channel and $\alpha_1$-adrenoceptor-operated $Ca^{2+}$ channel. At a high concentration, Tet might inhibit the cytosolic calcium pool release in cultured corpus cavernosum smooth muscle cells. This inhibitory action on $[Ca^{2+}]_i$ might be one of the relaxation mechanisms of Tet on the corpus cavernosum smooth muscle. (*Asian J Androl 2006 Jul; 8: 405–409*)

**Keywords**: tetrandrine; penis; smooth muscle cell; free calcium; corpus cavernosum; erectile dysfunction

## 1 Introduction

Erectile dysfunction (ED) is a common problem with a prevalence of approximately 50% in men aged 40 to 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 PDE5 inhibitors (sildenafil, tadalafil and vardenafil) are superior in effectiveness to centrally acting drugs (apomorphin and yohimbine). Lo-

Correspondence to: Dr 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
Dr Jun Chen, Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Tel: +86-27-8360-5073, Fax: +86-27-8360-8783
E-mail: jchen121@sohu.com
†Drs Liu and Chen contributed equally to this work.
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cal 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 understanding its mechanism of action is still important.

It is well known that many traditional Chinese medicines are effective as a treatment 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 alkaloids, chromoc, coumarin and saponin series, have been found to relax the smooth muscle of corpus cavernosum [3–13], which provides an open window for developing new drugs for the treatment of ED.

Tetrandrine (Tet) is a bis-benzylisoquinoline alkaloid isolated from the Chinese medicinal herb-root of *Stephania tetrandra* S Moore, which was traditionally used as an anti-inflammatory, antipyretic and analgesic herb in Chinese medicine. Tet is the active principle of the root of *S. tetrandra*. The empirical formula of Tet is $C_{38}H_{42}O_8N_2$, with a relative molecular weight of 622 kDa [14]. In the course of our studies on the development of naturally occurring agents for the treatment of ED, we found that Tet induced relaxation on the phenylephrine (PE)-precontracted corpus cavernosum [13]. In the present study, the effects of Tet on $Ca^{2+}$ influx from the extracellular site and $Ca^{2+}$ release from the cytosolic calcium pool in cultured corpus cavernosum smooth muscle cells are investigated, to clarify the relaxation mechanisms of Tet on the corpus cavernosum smooth muscle.

## 2 Materials and methods

All animal experiments were carried out with the approval of the Institute for Animal Care and Use Committee of Tongji Hospital.

### *2.1 Materials*

Tet was kindly provided by Prof. Jia-Ling Wang (Department of Pharmacology, Tongji Medical College, Huazhong University of Science and Technology, China). The purity of Tet was greater than 99.8%. It was dissolved in HCl 0.1 mol/L, then diluted with Krebs' solution to the desired concentration. Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL Grand Island, NY, USA) was obtained from Gibco, Fluo-2/AM from Merck (Darmstadt, Germany), and phenylephrine (PE) from Shanghai Harvest Parmaceutical (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 1 mmol/L egtaic acid (EGTA). Fluorescence Ion Digital Imaging System (FIDIS) was used for measurement (TILL Photonics, München, Germany).

### *2.2 Cell culture*

Adult male (4–6 months) New Zealand white rabbits (2.5–3.0 kg) were killed with pentobarbital sodium (50 mg/kg). The penises were rapidly excised and put into fresh phosphate-buffered saline (PBS) solution (penicillin 100 μg/mL, streptomycin 100 μg/mL) to isolate the corpus cavernosum. Then the tissues were washed and cut longitudinally into 1–3 mm pieces. These pieces were disaggregated for a period of 12–18 h with DMEM containing 0.1% (w/v) collagenase and 20% (v/v) fetal bovine serum (FBS) at 37ºC. The tissues were then dispersed into single cells. The primary smooth muscle cells were washed with 0.01 mol/L PBS solution (pH 7.4), and then cultured in DMEM with 20% FBS, $10^5$ U/L penicillin-streptomycin and 2 mmol/L glutamine. The medium was changed after 24 h for the first time, and thereafter every 2 days. Cells reached confluence in approximately 5 days and were passaged every 5–6 days. A single cell suspension from the third to fourth passages was cultured in a culture dish with a glass cover slip on its bottom for 2 days before the start of the measurements.

### *2.3 $Ca^{2+}$ staining and $[Ca^{2+}]_i$ measurement*

The cells were incubated in DMEM, with a final concentration of Fluo-2/AM of 3 μmol/L. After being incubated at 37ºC in the dark for 45 min, they were rinsed three times with Krebs solution or $Ca^{2+}$-free Krebs solution. Cells with intact plasmalemma and homogeneous endochylema were chosen for measurements, one cell in each slide. The effect of Tet on the intracellular $[Ca^{2+}]_i$ was observed with the FIDIS. The fluorescence intensity was observed in the fluorescence inverted microscope at $\lambda_{ex}$=340 nm/380 nm, $\lambda_{em}$=510 nm. After the signals were collected by the charged couple device system and managed by Till Vision software (TILL Photonics, München, Germany), the ratio value of the fluorescence intensity at 340 and 380 nm was recorded (F340/F380, R). The $[Ca^{2+}]_i$ changes were represented by changes

of $R$-value. The experiments were repeated six times independently with reproducible results.

*2.4 Statistical analysis*

Data were expressed as mean ± SEM. Statistical analysis was performed with ANOVA by means of SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant differences.

## 3 Results

*3.1 Effect of tetrandrine on resting $[Ca^{2+}]_i$*

With extracellular $Ca^{2+}$ 2.5 mmol/L, the $R$-value of resting $[Ca^{2+}]_i$ was 741.7 ± 35.2. After the preincubation with Tet (1, 10 and 100 μmol/L) for 5 min, the $R$-values of resting $[Ca^{2+}]_i$ were 743.0 ± 40.0, 741.7 ± 59.1 and 738.2 ± 30.8, respectively. No significant difference was found ($n = 6$; $P > 0.05$). In the absence of extracellular $Ca^{2+}$, the $R$-value of resting $[Ca^{2+}]_i$ was 354.4 ± 50.1. After the preincubation with Tet (1, 10 and 100 μmol/L) for 5 min, the $R$-values of resting $[Ca^{2+}]_i$ were 352.6 ± 37.6, 338.0 ± 28.8 and 340.3 ± 23.7, respectively. No significant difference was found ($n = 6$, $P > 0.05$).

*3.2 Effect of tetrandrine on KCl-induced $[Ca^{2+}]_i$ elevation*

In the presence of extracellular $Ca^{2+}$ 2.5 mmol/L, when KCl 40 mmol/L was added, the fluorescence intensity of intracellular calcium increased rapidly; $R$-value increased by 1 002.0 ± 48.3. Tet (1, 10 and 100 μmol/L) inhibited the KCl-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner. The resultant increases in $R$-values were 781.6 ± 37.1, 591.0 ± 28.2 and 270.7 ±12.7 ($n = 6$; $P < 0.05$), respectively (Figure 1).

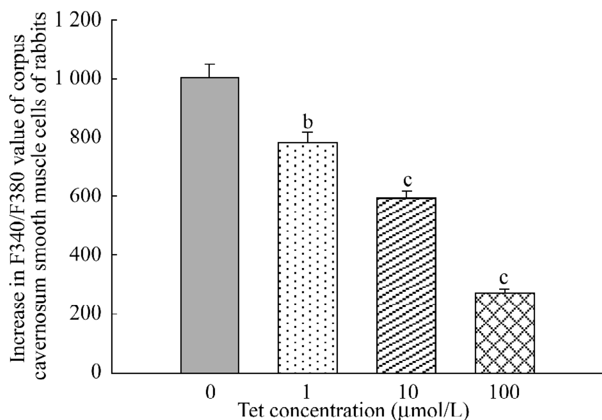


Figure 1. Effect of tetrandrine (Tet) on elevation of $[Ca^{2+}]_i$ induced by KCl in the presence of extracellular 2.5 mmol/L $Ca^{2+}$, $n = 6$. $^bP < 0.05$ and $^cP < 0.01$ compared with Tet (0 μmol/L).

*3.3 Effect of tetrandrine on phenylephrine-induced $[Ca^{2+}]_i$ elevation*

In the presence of extracellular 2.5 mmol/L $Ca^{2+}$, when 10 μmol/L PE was added, the fluorescence intensity of intracellular calcium increased rapidly, $R$-value increased by 489.5 ± 23.3. Tet (1, 10 and 100 μmol/L) inhibited the PE-induced $[Ca^{2+}]_i$ elevation in a concentra-

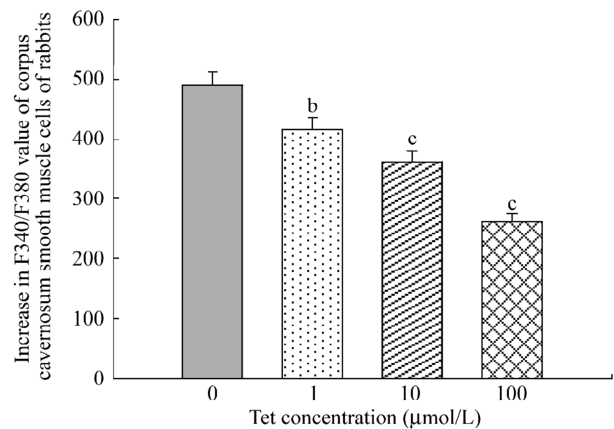


Figure 2. Effect of tetrandrine (Tet) on elevation of $[Ca^{2+}]_i$ induced by phenylephrine (10 μmol/L) in the presence of extracellular 2.5 mmol/L $Ca^{2+}$, $n = 6$. $^bP < 0.05$ and $^cP < 0.01$ compared with Tet (0 μmol/L).

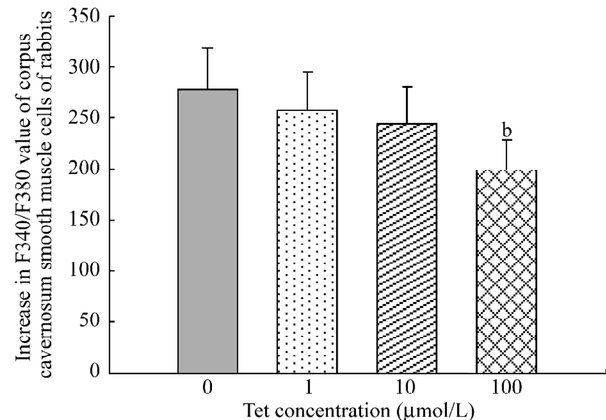


Figure 3. Effect of tetrandrine (Tet) on elevation of $[Ca^{2+}]_i$ induced by phenylephrine (10 μmol/L) in the absence of extracellular $Ca^{2+}$ containing egtaic acid, $n = 6$. $^bP < 0.05$ compared with Tet (0 μmol/L).

tion-dependent manner. The resultant increases in *R*-values were 416.2 ± 19.8, 360.5 ± 19.2 and 261.2 ± 13.9 ($n$ = 6; $P$ < 0.05), respectively (Figure 2).

In the absence of extracellular $Ca^{2+}$, when 10 μmol/L PE was added, the fluorescence intensity of intracellular calcium increased rapidly, *R*-value increased by 277.4 ± 41.1. Tet (1 and 10 μmol/L) had no significant effect on $[Ca^{2+}]_i$ and *R*-value increased by 257.5 ± 37.3 and 243.3 ± 36.5 ($n$ = 6; $P$ > 0.05), respectively. However, 100 μmol/L Tet significantly inhibited PE-induced $[Ca^{2+}]_i$ elevation, the resultant increases in *R*-value was 199.0 ± 29.4 ($n$ = 6; $P$ < 0.05) (Figure 3).

## 4 Discussion

Corpus cavernosum smooth muscle is an important factor in regulating penile erection, and changes in $[Ca^{2+}]_i$ directly regulate relaxation/contraction of corpus cavernosum smooth muscle. Various kinds of neurotransmitters, hormones or other influencing factors have been found to decrease the concentration of $[Ca^{2+}]_i$ in the cytoplasm, related to relaxation of the smooth muscle of corpus cavernosum and an increase in penis blood flow to keep it erect [15]. Consequently, detecting the effects of Tet on cytosolic $[Ca^{2+}]_i$ plays an important role in understanding its mechanism on relaxation of corpus cavernosum.

At the quiescent condition, the level of $[Ca^{2+}]_i$ in the cytoplasm depends on a series of transport mechanisms of calcium on the cell membrane, and extracellular $Ca^{2+}$ enters the cell by passive diffusion. In the present study, Tet had no effect on the resting $[Ca^{2+}]_i$, so it is possible that Tet had no effect on the passive diffusion of $Ca^{2+}$ through the cell membrane of the corpus cavernosum smooth muscle.

High extracellular $K^+$ induced the membrane depolarization rapidly, which opened the voltage-dependent $Ca^{2+}$ channel (VOC) and brought about the influx of the extracellular $Ca^{2+}$. Wang *et al.* [16] and Li *et al.* [17] reported that Tet inhibits high $K^+$-induced $[Ca^{2+}]_i$ elevation in the vascular smooth muscle cell. Ai *et al.* [18], Li *et al.* [19] and Sun *et al.* [20] reported that Tet inhibits high $K^+$-induced $[Ca^{2+}]_i$ elevation in myocardial cells. The present study shows that Tet can inhibit the high $K^+$-induced $[Ca^{2+}]_i$ elevation in corpus cavernosum smooth muscle cell. This result suggests that Tet exerted a VOC blocking effect in corpus cavernosum smooth muscle cells, which decreased the high $K^+$-induced $Ca^{2+}$ influx and relaxed corpus cavernosum smooth muscle.

Phenylephrine elicited $[Ca^{2+}]_i$ elevation in two ways: (i) activating $\alpha_1$-adrenoceptor-operated $Ca^{2+}$ channel to induce extracellular $Ca^{2+}$ entry; (ii) activating G protein and then facilitating the formation of IP3, which acts on IP3 receptor and induces intracellular $Ca^{2+}$ release. The present study shows that Tet concentration-dependently inhibits PE-induced $[Ca^{2+}]_i$ elevation in the presence of extracellular $Ca^{2+}$, whereas in the absence of extracellular $Ca^{2+}$ Tet in high concentration also has the inhibitory effect. This result indicates that Tet decreased $[Ca^{2+}]_i$ by inhibiting $\alpha_1$-adrenoceptor-operated $Ca^{2+}$ channel and $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores.

Our study suggests that Tet inhibited $[Ca^{2+}]_i$ in corpus cavernosum smooth muscle cells by blocking VOC, $\alpha_1$-adrenoceptor-operated $Ca^{2+}$ channel and $Ca^{2+}$ release from intracellular $Ca^{2+}$ pool. This might be one of the mechanisms of Tet on the relaxation of corpus cavernosum smooth muscle. However, whether Tet relaxed corpus cavernosum smooth muscle by blocking other types of adrenoceptors, 5-HT-receptor or Ang-receptor-sensitive $Ca^{2+}$ channel in the plasma membrane, requires further studies.

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