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Research article

In vitro and *in vivo* evaluation of tissue-cultured mountain ginseng on penile erection



Ho Sung Lee¹, Young Joo Lee¹, Yoon Hee Chung², Moo Yeol Lee³, Sung Tae Kim¹,
Sung Kwon Ko⁴, Mariko Momoi⁵, Yutaka Kondoh⁵, Fumio Sasaki⁵, Ji Hoon Jeong^{1,*}

¹ Department of Pharmacology, College of Medicine, Chung-ang University, Seoul, Korea

² Department of Anatomy, College of Medicine, Chung-ang University, Seoul, Korea

³ Department of Physiology, College of Medicine, Chung-ang University, Seoul, Korea

⁴ Department of Oriental Medical Food and Nutrition, Semyung University, Jecheon, Korea

⁵ Omnica Co., Tokyo, Japan

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ABSTRACT

Background: Progressed tissue culture techniques have allowed us to easily obtain mass products of tissue-cultured mountain ginseng over 100 yr old (TCMG-100). We investigated the effects of TCMG-100 extract on erectile function using *in vitro* and *in vivo* studies.

Methods: To examine the relaxation effects and mechanisms of action of TCMG-100 on rabbit cavernosal strips evaluated in an organ bath. To investigate the long-term treatment effect of TCMG-100, 8-wk administration was performed. After administration of TCMG-100, intracavernosal pressure, cyclic guanosine monophosphate and nitric oxide (NO) levels of cavernosal tissue, serum testosterone level, histological observation of collagen fiber, endothelium, smooth muscle cell, and transforming growth factor- β 1 were investigated.

Results: TCMG-100 extract displayed dose-dependent relaxation effects on precontracted rabbit corporal smooth muscle. The TCMG-100-induced relaxation was significantly reduced by removing the endothelium, and treatment with an NO synthase inhibitor or NO scavenger. Eight weeks of TCMG-100 administration increased intracavernosal pressure in a rat model. The levels of cyclic guanosine monophosphate and NO in the corpus callosum and serum testosterone level were also increased by TCMG-100 treatment. Furthermore, histological evaluation of collagen, smooth muscle, and endothelium showed increases in endothelium and smooth muscle, and a decrease in transforming growth factor- β 1 expression.

Conclusion: These relaxation effects on corporal smooth muscle and increased erectile function suggest that TCMG-100 might be used as an alternative herbal medicine to improve erectile function.

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1. Introduction

Erectile dysfunction (ED) has become an important health issue in terms of quality of life and the transition to an aging society. It has been reported that about 50% of men older than 40 yrs experience ED [1]. With increasing lifespan, the chance of having erectile problems increases. ED can have both physical and psychological causes including hypertension, high cholesterol, diabetes, hormonal problems, anxiety, and depression [2–4]. These

causes interfere with the complicated erection process that involves contributions from nerves, muscles, blood vessels, and spongy tissue in the penis.

The pathogenesis of ED is complex. Various pathophysiological mechanisms of ED have been studied, such as cavernous nerve dysfunction, reduced production of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP), and smooth muscle or collagen degradation [5]. Among them, the reduction in levels of NO and cGMP are known to play an important role in penile ED [6].

* Corresponding author. Department of Pharmacology, College of Medicine, Chung-ang University, Heuk-seok 06974, Dong-jak gu, Seoul, Korea.
E-mail address: jhjeong3@cau.ac.kr (J.H. Jeong).

Therefore, pharmacological treatment of ED includes phosphodiesterase-5 (PDE5) inhibitors, which act as intracellular NO signal amplifiers by slowing the degradation of cGMP by PDE5, resulting in subsequent penile smooth muscle relaxation. However, recent reviews and studies reported the effectiveness of 5-PDE inhibitors in ED irrespective of etiology [7–10]. More attention has recently been directed toward natural alternatives to synthetic pharmaceuticals [11,12], because it seems that many people prefer to use phytotherapies rather than pharmaceutical drugs for their health.

Mountain ginseng, one of the most well-known traditional herbs, has been widely used for the healing of various disorders [13]. Since the first report of the clinical efficacy of Korean Red Ginseng for ED [14], various types of studies have demonstrated that whole-ginseng extracts or purified ginsenosides from ginseng could improve ED [15,16]. In particular, ginsenosides protect the vascular endothelium against free radical-induced injury and have a relaxing effect on vascular smooth muscle associated with NO release from the vascular endothelium [17,18]. Furthermore, ginseng has an effect on corpus cavernosal smooth muscle with no side effects [14].

Traditionally, the cultivative or economic efficacy in red ginseng production has led to the popularity of ginseng products. However, rarely gathered mountain ginseng has not gained popularity, even though mountain ginseng has unique or strong effects. Tissue culture techniques for mountain ginseng have made bioreactor technology a useful tool for large-scale production of root biomass [19]. Thus, tissue-cultured mountain ginseng over 100 yrs old (TCMG-100) is obtained from wild mountain ginseng species. Furthermore, TCMG-100 contains a specific type of ginsenoside, Re [20], different from red ginseng-contained ginsenosides, Rb1 or Rg3 [21]. The types of cells in TCMG-100 are equal to wild mountain ginseng and have genetic equivalence. However, little has been reported on the differences between wild and cultivated ginseng in the treatment of ED. Thus, we aimed to evaluate whether TCMG-100 might be an alternative to current PDE5 inhibitors for ED. We investigated the effects of TCMG-100 on isolated rabbit corpus cavernosal smooth muscle. Rats were administered TCMG-100 orally and the effect on erectile function and related parameters were subsequently assessed.

2. Materials and methods

2.1. Animals

For *in vitro* experiments, 30 male New Zealand white rabbits (2.5 ± 0.5 kg) were anesthetized with pentobarbital sodium (50 mg/kg). The rabbits' penises were surgically removed with the tunica albuginea intact. The corpus cavernosum (CC) tissue was dissected free from the tunica albuginea. The strips of CC tissues were studied in separate organ chambers. We used 50 7-wk-old Sprague-Dawley rats for *in vivo* experiments. They were allowed to adapt for 1 wk before use and were permitted access to food and water *ad libitum*.

The animals were purchased from Koa-tech (Seoul, Korea) and maintained at a temperature of $20 \pm 2^\circ\text{C}$, humidity of $45 \pm 10\%$, and a 12-h light/dark cycle. Experimental animals were handled according to principles outlined in the Guide to the Care and Use of Experimental Animals Prepared by the Chung-Ang University Committee of Animal Ethics (Seoul, Korea).

2.2. Preparation of TCMG-100 extract

TCMG-100 was kindly provided from Omnica (Tokyo, Japan) and cultured by JIN-SANSAMBIO (Seoul, Korea). Briefly, callus, induced

from wild mountain ginseng tissue, was cultivated to the adventitious root. The adventitious roots were selected and cultivated in a sterile bioreactor for about 90 d. The cultivated TCMG-100 was qualified using a UV method of saponin-component ratio quantification (> 40 mg/g). The dried TCMG-100 was extracted with 70% ethanol and the concentrated under rotary evaporator. All extracts were freeze-dried cycle to yield of extract powders. Thus, extracted powder was used to carry out *in vivo* or *in vitro* experiments.

2.3. High-performance liquid chromatography analysis

The high-performance liquid chromatography conditions were based on those described by Kanazawa et al [22], which provided satisfactory resolution of major ginsenosides including Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1. The ginsenoside separation was conducted on a Eurospher C18 $5 \mu\text{m}$ 200×3 analytical fractions column (Altmann Analytik, München, Germany) using the following gradient system: 0–25 min, 17% acetonitrile, and 83% distilled water (DW); 25–50 min, 25% acetonitrile, and 75% DW; 50–105 min, 40% acetonitrile, and 60% DW; 105–125 min, 100% acetonitrile; 125–135 min, 17% acetonitrile, and 83% DW. The flow rate was 0.8 mL/min, and the ginsenosides were monitored at 203 nm. The sample injection quantity was 10 μL , and the temperature of the column was sustained at 30°C . The ginsenoside peaks were monitored, with the peak areas corresponding to samples matching authentic ginsenoside standards purchased from ChromaDex (Santa Anna, CA, USA).

2.4. *In vitro* experiments

Organ bath tissue experiments were performed using the methods described by Choi et al [23]. Briefly, the strips of rabbit CC measuring approximately $2 \text{ mm} \times 2 \text{ mm} \times 6 \text{ mm}$ were mounted longitudinally in a 20 mL organ bath chamber containing Krebs buffer solution with 95% oxygen and 5% CO_2 gas. The CC was stretched for 1 h and the optimal resting isometric tension for contraction was determined. The tissue was contracted with phenylephrine (PHE) $5 \times 10^{-6}\text{M}$ after every stretch (0.5 g, tension/stretch). Each strip was used for up to four separate rounds of testing, washed three times with Krebs solution, and allowed to equilibrate for 30 min between rounds.

Relaxation was studied in muscle strips precontracted with PHE. After muscle strips precontracted with PHE were stabilized, they were treated with solutions of TCMG-100 in increasing concentrations from 5 mg/mL. The mechanism of muscle relaxation

Table 1

Contents of ginsenosides in the tissue-cultured mountain ginseng over 100 yr old (w/w%)

Ginsenosides	TCMG-100
Rb1	0.703 ± 0.084
Rb2	0.683 ± 0.036
Rc	0.802 ± 0.067
Rd	0.345 ± 0.037
Re	2.066 ± 0.036
Rf	0.211 ± 0.003
Rg1	0.059 ± 0.030
Rg2	0.178 ± 0.030
Rg6	0.006 ± 0.005
Rh1	0.050 ± 0.008
F1	0.046 ± 0.020
Total ginsenosides	5.670
Diol/triol	0.969

Data are presented as mean \pm SD.

TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

induced by TCMG-100 was studied in muscles pretreated with N-nitro-L-arginine (NNA, NO synthase inhibitor), methylene blue (guanylate cyclase antagonist), pyrogallol (NO scavenger), glibenclamide (K^+ adenosine triphosphate channel inhibitor), or indomethacin (cyclooxygenase-2 inhibitor). The endothelium (D-endo) was disrupted by rubbing the cavernosal tissue strips between the thumb and index finger for about 20 s. After rinsing in chilled Krebs solution, tissue strips were gently rolled across a dry paper towel to generate shear forces across the endothelial surfaces of the lacunar spaces. NNA, methylene blue, pyrogallol, glibenclamide, and indomethacin were added to the muscle strips precontracted with PHE and incubated for 15–20 min prior to the addition of TCMG-100. Pyrogallol was added to the muscle strips precontracted with PHE (5×10^{-6}) for 5 min before the addition of TCMG-100. In order to evaluate the effect of TCMG-100 on PHE-induced contraction, PHE induced contractions were assessed after pretreatment with TCMG-100 from 5 mg/mL.

2.5. In vivo experiment

The 50 rats were divided into the following three groups: control (saline, $n = 10$), TCMG-100 250 mg/kg ($n = 20$), and TCMG-100 500 mg/kg ($n = 20$). All groups were orally administered their respective daily treatment for 8 wk and doses were equal across groups (0.5 mL/d). After 8 wk, the rats were anaesthetized with sodium pentobarbital (40 mg/kg) to enable evaluation of maximal intracavernosal pressure (MIP)/maximal arterial pressure (MAP).

Briefly, the bladder and prostate were exposed via a transperitoneal midline incision. The wall of the prostate was incised, and the cavernosal nerve was isolated. A platinum electrode was placed on the cavernosal nerve and connected to an electric stimulator. After incising the penile skin, the CC was isolated. To measure the MIP, a 26 G needle was inserted into the CC. To simultaneously monitor MAP, a 22 G catheter was inserted into the carotid artery and connected to a transducer and polygraph system. The outputs for MAP and MIP were connected to a sequential

amplifier. MIP was continuously measured under cavernous nerve stimulation at low voltage (voltage 2 V; frequency 12 Hz; pulse-width 1 ms; duration 1 min). To minimize the influence of cavernous nerve stimulation on the blood pressure, which would artificially raise the MIP, the data were presented as the ratio of MIP/MAP. Sildenafil (2 mg/kg) was used to confirm erectile status and was administered intraperitoneally 30 min before inserting the catheter. These methods are described in detail in a previous report [24].

After this experiment, rats were euthanized in order to evaluate other erectile-related parameters. To assess testosterone content, blood was collected from the posterior vena-cava. Serum was collected using centrifugation of blood at $1,800 \times g$ for 15 min. Serum testosterone was analyzed using a commercial enzyme-linked immunosorbent assay kit (Elabscience Biotechnology Co., Ltd, Beijing, China). The CC was then minced with scissors and homogenized in lysis buffer solution (pH 7.4). The homogenization was carried out in a Teflon-glass homogenizer to obtain a 1:10 (w/v) dilution. The homogenates were used to analyze cGMP levels by enzyme-linked immunosorbent assay (Abcam, Cambridge, MA, USA) or NO levels by colorimetric excitation-emission methods (Abcam). All biochemical methods were performed according to the manufacturer's instructions.

The rest of the detached CC was immediately fixed in 10% formalin phosphate buffer solution before embedding in paraffin. The sections were deparaffinized and hydrated by sequential incubation in xylene and ethanol. Collagen fibers were stained with Masson's trichrome. For immunohistochemical analysis, tissue sections (20 μ m) were incubated with antibody to factor VIII (Abcam) or antibody to smooth muscle α -actin (Abcam) at 4°C overnight. After several washes with phosphate-buffered saline, the sections were incubated with mouse antibody to immunoglobulin-G for 1 h at room temperature. Signals were visualized and digital images were obtained with an Olympus microscope BX-50 (Olympus Optical Co., Ltd, Tokyo, Japan). Quantitative analysis of collagen, smooth muscle, and endothelium in CC

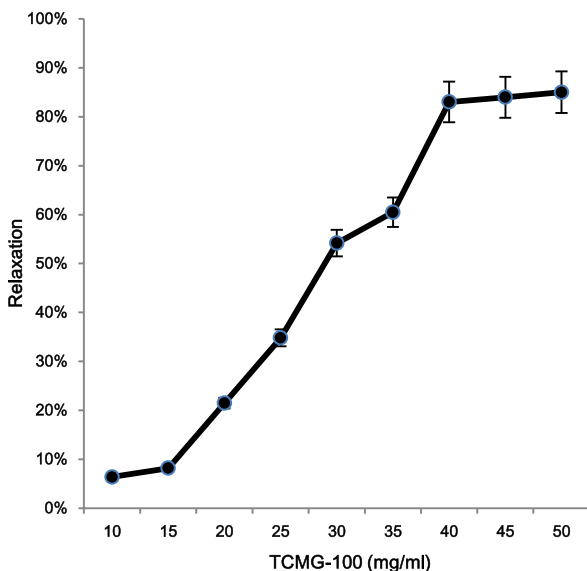


Fig. 1. Representative tracing of tissue-cultured mountain ginseng over 100 yr old effects on isolated rabbit corpus cavernosum muscle strips submaximally precontracted by phenylephrine. Tissue-cultured mountain ginseng over 100 yr old relaxed precontracted muscle strip in a dose-dependent fashion. Values represent the mean \pm SD of mean and are expressed as percent relaxation ($n = 8$). TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

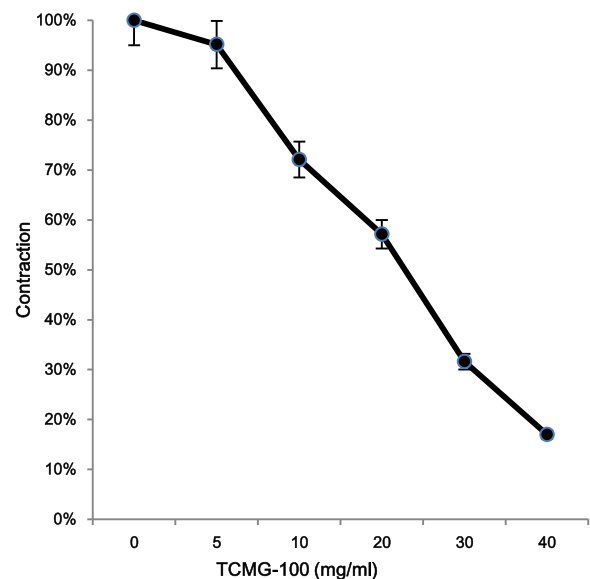


Fig. 2. Effects of tissue-cultured mountain ginseng over 100 yr old pretreatment on phenylephrine (PHE)-induced contraction in rabbit corpus cavernosum muscle strips. The contractile response of the isolated cavernosal muscle strips to PHE was significantly inhibited by tissue-cultured mountain ginseng over 100 yr old pretreatment in a dose-dependent fashion. Values represent mean \pm SD of mean and are expressed as the percentage of the initial contraction induced by PHE ($n = 7$). TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

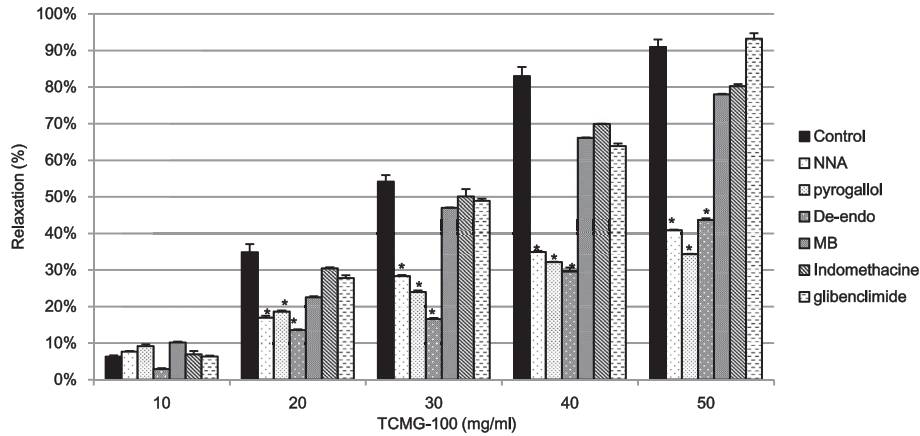


Fig. 3. Effects of various treatments on tissue-cultured mountain ginseng over 100 yr old-induced relaxation in the phenylephrine-induced precontracted muscle strips. The relaxation effects of tissue-cultured mountain ginseng over 100 yr old were significantly inhibited by endothelial disruption and by pretreatment with N-nitro-L-arginine (nitric oxide synthase inhibitor) or pyrogallol (nitric oxide scavenger). Values represent the mean \pm SD of mean and are expressed as percentage of the relaxation. * $p < 0.05$ compared with the control group ($n = 8$). De-endo, endothelial disruption; MB, methylene blue; NNA, N-nitro-L-arginine; TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

tissue was carried out with an image analyzer Paint.NET (dotPDN LLC., WA, USA).

2.6. Assessment of transforming growth factor- β 1 expression in CC homogenate

CC tissue homogenates were used for Western blotting performed according to standard procedures. Briefly, homogenates were lysed in lysis buffer containing 50mM Tris-HCl (pH 8.0). These homogenates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane blocked with 5% skim milk, and hybridized with primary antibodies. Transforming growth factor- β 1 (TGF- β 1) antibody and monoclonal β -actin antibody were

purchased from Abcam. After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemiluminescent electrogenerated chemiluminescence assay kit (Abcam) according to the manufacturer's instructions.

2.7. Statistical analysis

The data are expressed as mean \pm standard deviation. Statistical differences between means were analyzed by Student t test or Tukey's multiple comparison test after analysis of variance using sigma plot version 11.2 (Systat Software Inc., San Jose, CA, USA). Values of $p < 0.05$ were taken to indicate statistical significance.

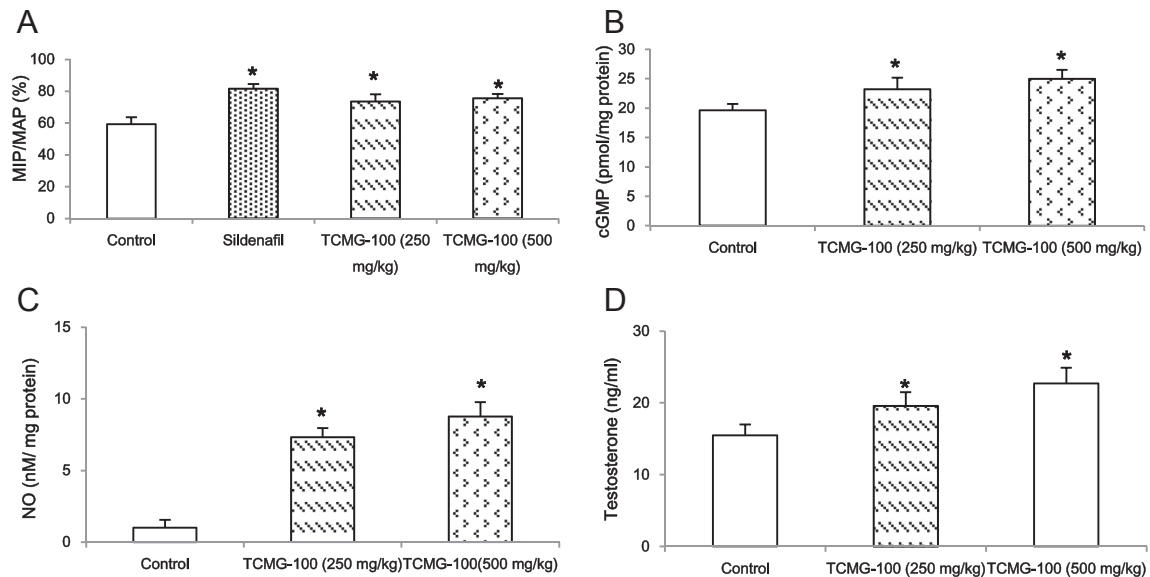


Fig. 4. Effects of tissue-cultured mountain ginseng over 100 yr old (TCMG-100). (A) Effects on maximal intracavernosal pressure/maximal arterial pressure ratio. (B) Effects on cyclic guanosine monophosphate. (C) Effects on nitric oxide levels. (D) Effects on serum testosterone. The ratio of maximal intracavernosal pressure/maximal arterial pressure with cavernosal nerve electro-stimulation after 8 wk of TCMG-100 administration was significantly increased compared with control group. The cyclic guanosine monophosphate levels and nitric oxide levels in the TCMG-100 treatment groups were significantly increased compared with those in the control group. The serum TT levels in the TCMG-100 treatment groups were significantly increased. Data are expressed as the mean \pm SD. * $p < 0.05$ compared with the control group ($n = 8$). cGMP, cyclic guanosine monophosphate; MIP/MAP, maximal intracavernosal pressure/maximal arterial pressure; NO, nitric oxide; TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

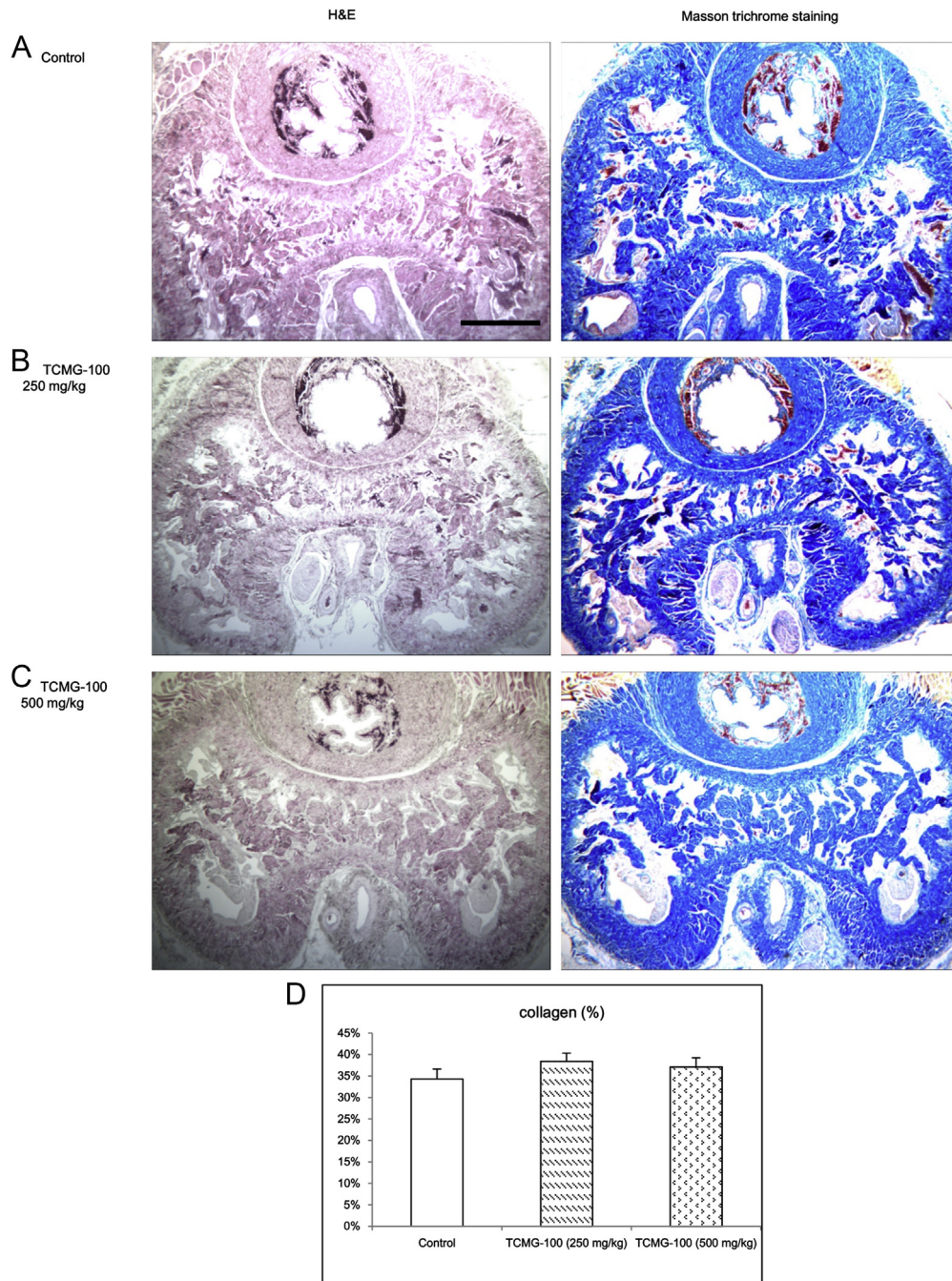


Fig. 5. The distribution of collagen fibers in hematoxylin and eosin or Masson's trichrome stained cavernous tissue. (A) Control group. (B) Tissue-cultured mountain ginseng over 100 yr old (250 mg/kg). (C) tissue-cultured mountain ginseng over 100 yr old (500 mg/kg). (D) Quantitative analysis of collagen (blue) in the corpus cavernous body was performed with an image analyzer. Scale bars = 50 μ m. Data are expressed as the mean \pm SD. H&E, hematoxylin and eosin; TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

3. Results

3.1. Determination of ginsenosides in TCMG-100

The proposed method determined 11 ginsenosides in TCMG-100 (Table 1). Ginsenosides Re and Rc were the two main ginseng saponins in TCMG-100. The amounts of Re were greater than those of other saponins found in TCMG-100. Total amount of ginsenosides was 5.67%, whereas the ratio of diol/triol was 0.969 (Table 1).

3.2. Effects of TCMG-100 on the submaximally precontracted muscle strips with PHE

TCMG-100 began to exert a relaxing effect on the isolated rabbit CC smooth muscle strips submaximally precontracted with PHE (5×10^{-6} M) at a concentration of 10 mg/mL, and the muscle strips reached $84.21 \pm 2.01\%$ relaxation at a concentration of 50 mg/mL (Fig. 1).

3.3. Effects of TCMG-100 pretreatment on PHE-induced contraction

The contractile responses of the isolated-cavernosal muscle strips to PHE (5×10^{-6} M) were inhibited by TCMG-100 pretreatment up to 17.22% of the control. TCMG-100 pretreatment significantly inhibited PHE-induced contraction in a dose-dependent fashion (Fig. 2).

3.4. TCMG-100-induced muscle relaxation with the NO pathway

TCMG-100-induced relaxation was partially reduced by removing the endothelium and inhibited by pretreatment with an NO synthase inhibitor (NNA, $41.85 \pm 0.27\%$ at a concentration of 50 mg/mL) and an NO scavenger (pyrogallol, $34.30 \pm 0.32\%$ at a concentration of 50 mg/mL) compared with the control group ($91.23 \pm 1.07\%$ relaxation). However, relaxation effects of TCMG-100 were not induced by indomethacin (10^{-4} M), methylene blue (guanyl cyclase inhibitor; 5×10^{-4} M) or a glibenclamide (K^+ adenosine triphosphat channel inhibitor, 10^{-4} M; Fig. 3).

3.5. In vivo evaluation of the effects of TCMG-100 on MIP/MAP

The MIP/MAP ratio was increased in the TCMG-100 treatment groups. After 8 wks of treatment, the ratio of the control group was $59.34 \pm 3.34\%$. However, the ratios of the TCMG-100 treatment groups (250 mg/kg and 500 mg/kg) were significantly increased compared with the control group at $73.51 \pm 5.54\%$ and $71.55 \pm 3.78\%$, respectively (Fig. 4A).

3.6. Evaluation of the cGMP and NO levels in CC tissues

The association of cGMP and NO levels with erectile function of CC tissue was evaluated (Figs. 4B, 4C). The cGMP levels in the TCMG-100 treatment groups (250 mg/kg, 500 mg/kg) were significantly increased (23.23 ± 1.93 pmol/mg or 24.99 ± 1.51 pmol/mg protein, respectively) compared with that in the control group (19.63 ± 1.09 pmol/mg protein). The NO levels in the TCMG-100 treatment groups (250 mg/kg, 500 mg/kg) were significantly increased (7.32 ± 0.85 nM/mg and 8.76 ± 1.02 nM/mg protein, respectively) compared with that in the control group (1.01 ± 0.53 nM/mg protein).

3.7. Effect of TCMG-100 treatment on serum testosterone levels

Serum testosterone (TT) levels are displayed in Fig. 4D. The serum TT levels were significantly increased in the TCMG-100 groups (20.58 ± 1.89 ng/mL and 21.69 ± 2.19 ng/mL) compared with TT levels of the control group (15.47 ± 1.52 ng/mL).

3.8. Effect of TCMG-100 treatment on collagen, smooth muscle, and endothelium in CC tissue

The collagen fibers of the CC penis were observed using Masson's trichrome. The collagen fibers were dyed blue, and were regularly arranged (Fig. 5A–5C). According to the histomorphological image analysis, TCMG-100 treatment (250 mg/kg: 38.43%, 500 mg/kg: 37.13%) slightly increased the collagen ratio compared with that in the control group (34.29%), but the difference was not

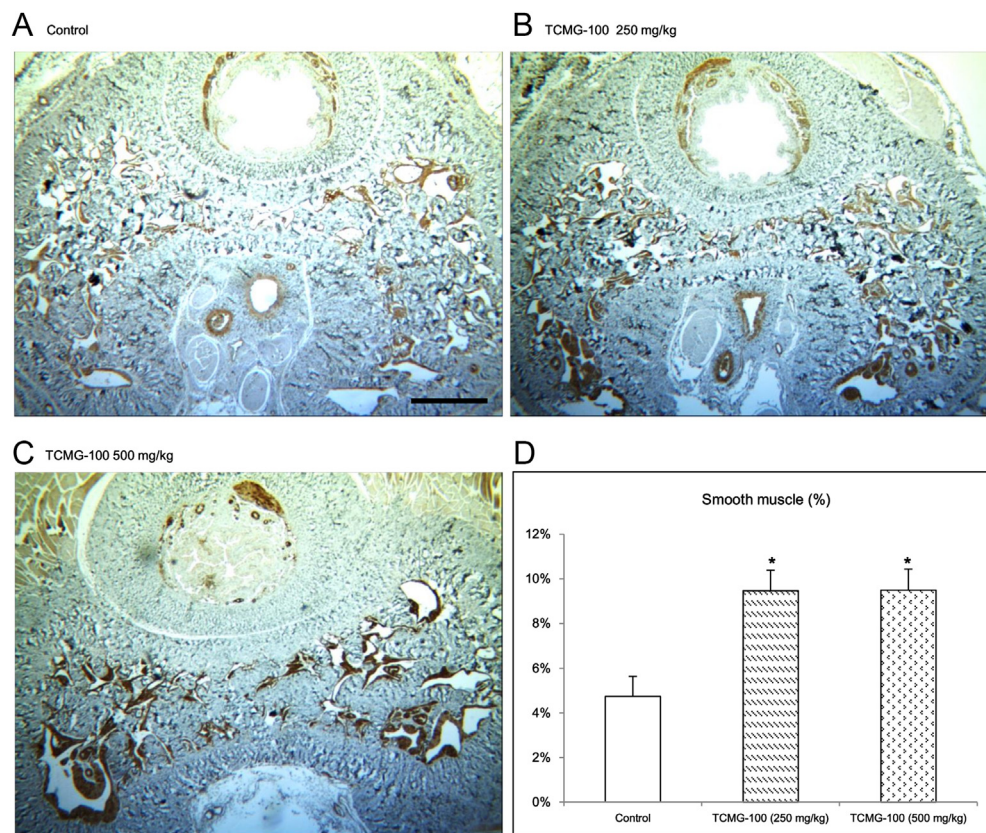


Fig. 6. Immunohistochemical localization of smooth muscle cells in cavernous tissue. (A) Control group. (B) Tissue-cultured mountain ginseng over 100 yr old (250 mg/kg). (C) Tissue-cultured mountain ginseng over 100 yr old (500 mg/kg). (D) Quantitative analysis of smooth muscle (brown) in the corpus cavernosum body was performed with an image analyzer. Data are expressed as the mean \pm SD. Scale bars = 50 μ m. * $p < 0.05$ compared with the control group. TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

significant (Fig. 5D). Immunohistochemistry was performed to assess the smooth muscle cells and endothelial cells in the CC (Figs. 6 and 7). TCMG-100 groups (250 mg/kg: 9.47%, 500 mg/kg: 9.50%) had a significantly increased proportion of smooth muscle ($p < 0.05$) compared with the control group (4.73%) after 8 wks of treatment (Fig. 6D). The endothelium ratio of the control group was 2.43%, and TCMG-100 treatment significantly increased the endothelium ratio (250 mg/kg: 4.74%, 500 mg/kg: 5.39%, $p < 0.05$ compared with the control group, respectively) after 8 wks of treatment (Fig. 7D).

3.9. TGF- β 1 expression in CC tissue homogenates

Western blotting was used to evaluate the effects of TCMG-100 on TGF- β 1 levels in rat CC homogenates. TGF- β 1 expression in CC tissue was decreased in the treatment groups compared with that in the control group (Fig. 8). The relative expression levels of TGF- β 1 (44 kDa) were significantly decreased in TCMG-100 groups (250 mg/kg: 0.55 ± 0.07 mg/kg, 500 mg/kg: 0.53 ± 0.09 mg/kg, $p < 0.05$ compared with the control group, respectively). The relative levels of the cleaved mature form (13 kDa) were also

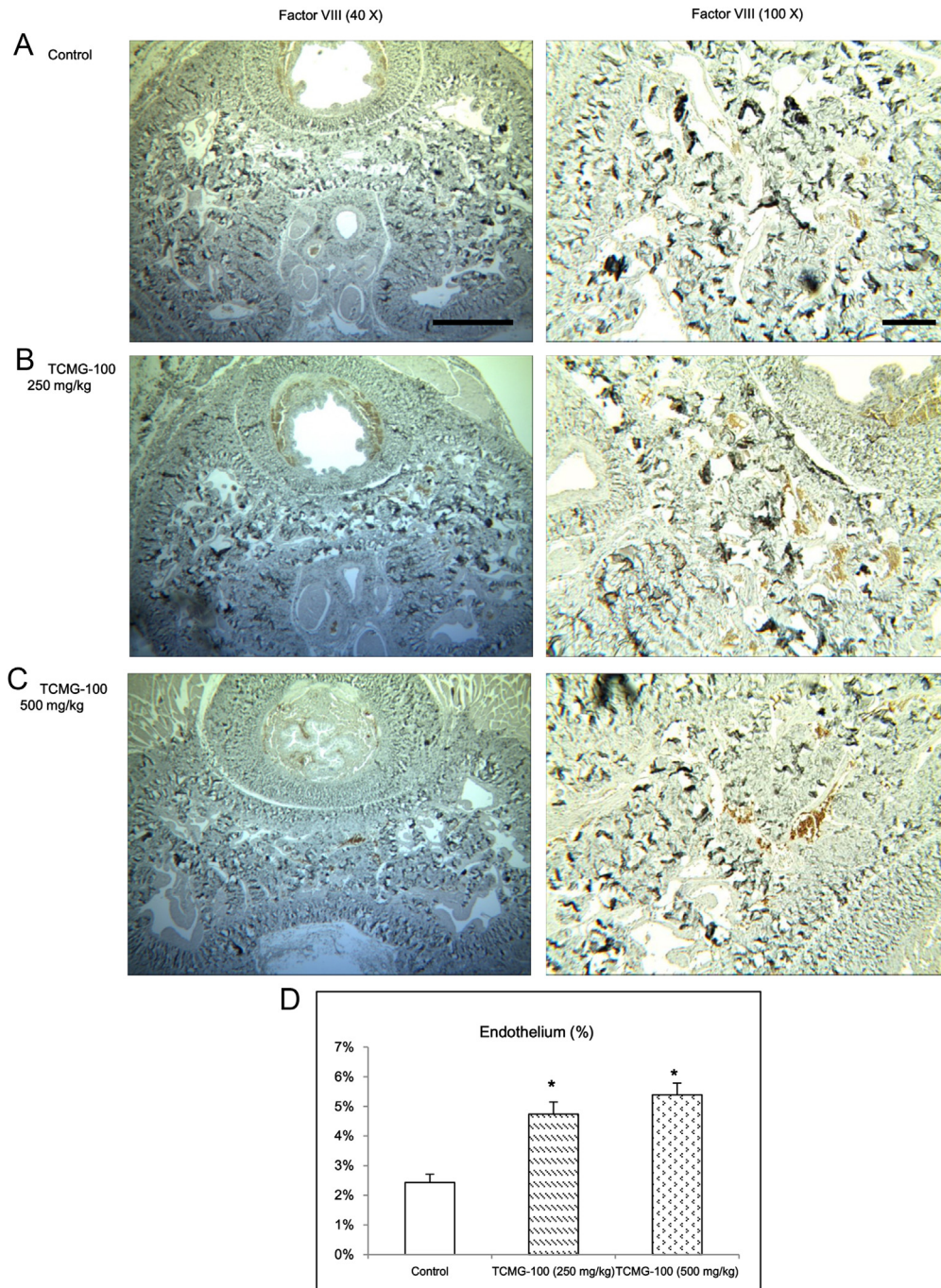


Fig. 7. Immunohistochemical localization of endothelial cells in cavernous tissue. (A) Control group. (B) Tissue-cultured mountain ginseng over 100 yr old (250 mg/kg). (C) Tissue-cultured mountain ginseng over 100 yr old (500 mg/kg). (D) Quantitative analysis of endothelium (brown) in corpus cavernous body was performed with an image analyzer. Data are expressed as the mean \pm SD. Scale bars = 50 μ m (left side) or 20 μ m (right side). * $p < 0.05$ compared with the control group. TCMG-100, tissue-cultured mountain ginseng over 100 yr old.

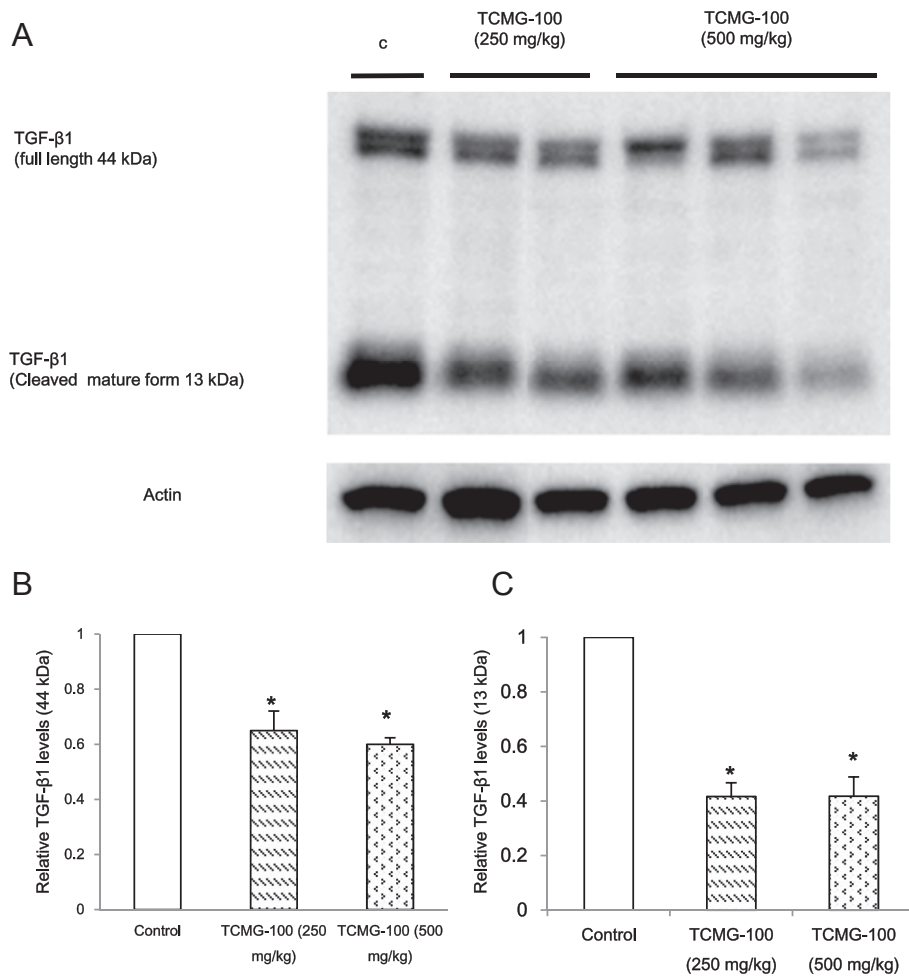


Fig. 8. Effects of tissue-cultured mountain ginseng over 100 yr old on transforming growth factor- β 1 expression in corpus cavernosum tissue. The relative or fold-increases of transforming growth factor- β 1 expression (44 kDa or 13 kDa) were significantly decreased in the tissue-cultured mountain ginseng over 100 yr old treatment groups compared with control group. Data are expressed as the mean \pm SD. * $p < 0.05$ compared with the control group. TCMG-100, tissue-cultured mountain ginseng over 100 yr old; TGF- β 1, transforming growth factor- β 1.

significantly decreased in the TCMG-100 groups (250 mg/kg: 0.61 ± 0.05 mg/kg, 500 mg/kg: 0.54 ± 0.07 mg/kg, $p < 0.05$ compared with the control group, respectively).

4. Discussion

Erectile function is correlated with relaxation of penile smooth muscle and dilatation of the arteries, leading to increased blood flow to lacunar spaces. In this study, PHE-induced contraction of rabbit corporal smooth muscle was alleviated by TCMG-100 treatment. After 8 wk of TCMG-100 treatment in rats, there were significant increases in MIP/MAP, cGMP and NO levels in CC tissue, and serum TT levels, suggesting that TCMG-100 may improve erectile function. Furthermore, histological and biochemical evaluation of smooth muscle, endothelium, and TGF- β 1, also showed its effect on erectile function.

TCMG-100 treatment in an organ bath relaxed CC muscle strips precontracted by PHE, and pretreatment with TCMG-100 inhibited PHE-induced contraction. The present study showed that removing endothelium (De-endo) as well as treatment with an NO synthesis inhibitor (NNA) or an NO scavenger (pyrogallol) inhibited the relaxation effects of TCMG-100. These results are consistent to previous studies reporting that various ginsenosides or different varieties of ginseng had a relaxation effect on vascular smooth

muscle and these effects were correlated with the L-arginine-NO-cGMP pathway of the endothelium [17,25,26]. Treatment of red ginseng [23] or ginseng berry [24] caused muscle relaxation by NO production and consequent guanylyl cyclase activation and cGMP production. Unexpectedly, the guanylyl cyclase inhibitor (methylene blue) only partially inhibited the relaxation effect of TCMG-100 in this study. As the intracellular cGMP concentrations are regulated not only by soluble guanylyl cyclase, but also by PDE, it is possible that TCMG-100 can slow the degradation of cGMP by PDE, resulting in smooth muscle relaxation. Hence, our results suggest that the relaxation effect of TCMG-100 may be mediated through the induction of cGMP, as a consequence of the increase of NO synthesis, reinforcing the notion that NO-cGMP pathway is important.

In *in vivo* experiments, the MIP/MAP level was increased after 8 wk of treatment with TCMG-100. Intracavernosal pressure is related to erectile function [27]. Thus, an increased MIP/MAP ratio in the TCMG-100-treated group supports the notion that it increases vasodilation-related factors or TT levels. CC smooth muscle relaxation is mediated by NO, which is released from the endothelium in arteries through the formation of cGMP [6,28]. The cGMP and NO levels in CC tissue were increased after TCMG-100 administration in this study, and these results indicate that long-term TCMG-100 treatment might stimulate vasodilation factors

and increase capillary capacity in the CC. Furthermore, a previous study demonstrated that TT had a direct role in erection through an effect on NO synthase within the CC [29]. In a clinical trial, low serum TT level was associated with sexual dysfunction [30]. Therefore, our results suggest that TCMG-100 might improve ED.

The collagen fiber, endothelial, and smooth muscle cells of the CC are essential for erectile function. Collagen fibers were not significantly different between the control and TCMG-100-treatment groups, whereas the ratio of endothelial and smooth muscle cells in CC tissue was increased after 8 wk of TCMG-100 treatment. A previous study demonstrated that an increase in endothelial cells induced by an intracavernosal injection of vascular endothelial growth factor improved erectile function [31]. Impairment of endothelial or smooth muscle cells, such as in dyslipidemia or obesity, is regarded as a pathological cause of ED [32]. Furthermore, patients with vasculogenic ED have a particular pathological point at which trabecular smooth muscle decreases and the cavernous extracellular matrix or connective tissue content increase [33,34]. Thus, our results showing increases in endothelial and smooth muscle cells suggest that administration of TCMG-100 might alleviate or improve ED.

Chronic accumulation of collagen caused by ischemia of the CC smooth muscle followed by increased production of extracellular matrix proteins is characteristic of the progression of fibrosis [5]. A previous study reported that increased TGF- β 1 expression resulted in increased fibrosis in the CC and treatment with a TGF- β 1 antagonist peptide enhanced the erectile response via amelioration of CC fibrosis [35]. TGF- β 1 induces fibroblast aggregation and promotes the generation of collagenous connective tissues [33]. In the present study, there were no differences in the areas of stained collagen fibers between treated groups and the control group. However, TGF- β 1 expression was decreased in TCMG-100-treated groups, which may contribute to improve erectile function by preventing fibrosis. As we used healthy young animals in this study, further research using an animal model of ED such as diabetes mellitus or hypercholesterolemia, is needed to explain this result.

In conclusion, TCMG-100 treatment in an organ bath study relaxed precontracted CC muscle strips and inhibited contraction entirely through NO- and NO synthase-mediated processes. In animal experiments, TCMG-100 increased intracavernosal pressure, cGMP and NO levels of CC tissues, serum TT level and the ratios of endothelial and smooth muscle. TCMG-100 has economic advantages over red ginseng which is made from 6-yr-old ginseng plants, as it can be easily and continuously produced from tissue culture. Therefore, these results suggest that TCMG-100 can be used as an alternative medicine to improve penile erectile function. However, further studies using an animal model of ED are required to elucidate its exact mechanism.

Conflicts of interest

All authors have no conflicts of interest to declare.

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