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Effects of *Schisandra chinensis* fruit extract and gomisin A on the contractility of penile corpus cavernosum smooth muscle: a potential mechanism through the nitric oxide - cyclic guanosine monophosphate pathway

Bo Ram Choi[1*], Hye Kyung Kim[2*] and Jong Kwan Park[1§]

[1]Department of Urology, Chonbuk National University and Research Institute of Clinical Medicine of Chonbuk National University-Biomedical Research Institute and Clinical Trial Center of Medical Device of Chonbuk National University, 567, Baekje-daero, Deokjin-gu, Jeonju-si, Jeonbuk 54896, Korea
[2]College of Pharmacy, Kyungsung University, Busan 48434, Korea

BACKGROUND/OBJECTIVES: This study evaluated the effects and molecular mechanisms of the *Schisandra chinensis* fruit extract (SC) and its major compound gomisin A (GA), on the contractility of rabbit penile corpus cavernosum smooth muscle (PCCSM).
MATERIALS/METHODS: PCCSM was exposed to SC or GA after appropriate pretreatment with nitric oxide synthase (NOS) blocker, guanylate cyclase blocker, adenylyl cyclase blocker or protein kinase A blocker. Subsequently, we evaluated the cyclic nucleotide in the perfusate by radioimmunoassay, protein expression level of neuronal NOS (nNOS) and endothelial NOS (eNOS) by western blot, and the interaction of SC or GA with udenafil and rolipram.
RESULTS: Both SC and GA induce PCCSM relaxations in a concentration-dependent manner. Pretreatment with NOS blocker, guanylate cyclase blocker, adenylyl cyclase blocker or protein kinase A blocker result in significantly decreased relaxation. SC and GA also induce the levels of cyclic nucleotide in the perfusate in a concentration-dependent manner. Perfusion with GA also showed significantly higher levels of eNOS protein. Furthermore, the udenafil and rolipram induced relaxations of PCCSM were enhanced after exposure to SC and GA. Our results indicate that SC and GA induce the relaxation of PCCSM via the nitric oxide (NO)-cGMP and cAMP signaling pathways.
CONCLUSIONS: The SC and GA are potential alternative treatments for men who want to consume natural products to ameliorate erectile function, or who do not respond to the commercially available medicines.

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INTRODUCTION

Schisandra chinensis Baillon is traditionally used in oriental medicine and possesses many biological activities [1]. Lignans such as schisandrin, γ-schisandrin, deoxyschisandrin and gomisin A are the major components of the *S. chinensis* fruit [2]. *S. chinensis* and its active component, gomisin A, has numerous pharmacological properties including cardioprotective effects, antioxidative, anti-inflammatory and antihepatotoxic properties [3-6]. As previously demonstrated by numerous studies, schisandrols have a relaxant effect on the isolated corpus cavernous smooth muscle, and the efficacy is exerted due to the direct effect on smooth muscles via inhibition of the Ca^{2+} influx [7,8].

Erectile dysfunction (ED) is defined as the disability to attain or maintain penile erection sufficient for sexual satisfaction [9]. The prevalence of ED increases with age, and ranges from 23.3% in men aged 30-39 years to 85.8% in men aged 60-69 years [10]. ED is a disease of organic origin such as vascular, hormonal, neurogenic and mixed etiologies, with most ED involving a vascular etiology [11]. Nitric oxide (NO) is the predominant neurotransmitter responsible for cavernosal smooth muscle relaxation, and is recognized to play an important role in penile erection [12].

NO is formed from the precursor amino acid L-arginine, and has received increasing attention as a neurotransmitter. Three isoforms of nitric oxide synthase (NOS) have been confirmed: inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) [13]. Although all three isoforms are found in the penis, only nNOS and eNOS are related to erectile function [14]. NO activates the soluble guanylyl cyclase which catalyzes the conversion of guanosine-5′-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in smooth muscle cells [15]. An increase in

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§ Corresponding Author: Jong Kwan Park, Tel. 82-63-250-1510, Fax. 82-63-273-2382, Email. rain@chonbuk.ac.kr
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* These two authors contributed to this work equally.

cGMP elicits the relaxation of smooth muscle cells by decreasing the intracellular calcium ion concentration [16]. Adenylate cyclase directly activates inducible cyclic adenosine monophosphate (cAMP), resulting in relaxing the penile corpus cavernosum [17].

Previous studies have shown that the relaxation of rat aortic rings due to an aqueous extract of *S. chinensis* is mediated partially by an endothelium-dependent mechanism [18]. This study was undertaken to investigate the relaxant effect of the ethanol extract of *S. chinensis* fruit and the active component gomisin A on penile corpus cavernosum smooth muscle (PCCSM), and to further evaluate their potential mechanism of action in ED.

MATERIALS AND METHODS

Preparation of crude extract

In September 2016, we purchased the *S. chinensis* fruits from Jangsu, Korea, which were identified and confirmed by one of the authors (Dr. H. K. Kim). We deposited a voucher specimen at the Herbarium Unit, University Kyungsung, bearing voucher number Herbarium KHK-SC-1. Dried *S. chinensis* fruits (50 g) were extracted with 100% ethanol using an ultrasonic bath (Danbury, CT, USA) for 3 h. Prior to use, the concentrated *S. chinensis* fruit extract (SC) was freshly dissolved in HEPES buffer.

Corpus cavernosum strip preparation

Animal protocols were approved by the Chonbuk National University Hospital Laboratory Animal Centre, South Korea, and were as per the guidelines recommended by the NIH guide for care (IACUC, cuh-IACUC-2016-12). Sexually mature male New Zealand white rabbits were anesthetized by injecting ketamine (45 mg/kg) and xylazine (20 mg/kg) into the ear vein. Based on the *ex vivo* method proposed by Zhao *et al*. [19], the penis was surgically excised by removing the urethra. The cannulated penis (PE50 polyethylene tubing) was mounted vertically in the organ chamber and perfused with SC or gomisin A (GA) for measuring the cyclic nucleotide concentrations (cGMP and cAMP). Another penis was perfused with SC or GA for molecular biology experiments (western blot).

Organ bath studies

Phenylephrine (Phe, 10^{-5} M) was added after stabilization of the strip was equilibrated for 60 min with several adjustments of length until a baseline force stabilized at 1 g. Once a stable contraction plateau was reached, the relaxation effect of SC and GA were evaluated at final concentrations of 0.1-2 mg/mL and 10^{-7}-10^{-4} M, respectively. To investigate the SC and GA-mediated relaxation pathway, we examined the effect of SC and GA on the Phe-induced tone by comparing the effect of relaxation before and after incubation of PCCSM with NOS blocker Nω -Nitro-L-arginine methyl ester hydrochloride (L-NAME, 10^{-3} M), guanylate cyclase blocker ODQ (10^{-5} M), adenylyl cyclase blocker MDL 12330A (10^{-5} M) and protein kinase A (PKA) blocker H-89 (10^{-4} M) for 30 min.

Radioimmunoassay (RIA) of cAMP and cGMP concentrations

cAMP and cGMP were measured by the radioimmunoassay method of Cui *et al*. [20]. Briefly, 100 μL of perfusate was mixed with trichloroacetic acid for 15 min at 37°C. The resulting supernatant (100 μL) was extracted thrice with diethyl ether and then dried. The sample was treated with sodium acetate buffer and used for measuring the cAMP and cGMP levels.

Western blot for NOS expression level

Tissue homogenate was prepared by homogenizing the corpus cavernosum smooth muscle in RIPA buffer mixed with a protease inhibitor tablet (Indianapolis, IN, USA). Proteins were quantified using the Bradford protein assay (Hercules, CA, USA). Proteins (20 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride membrane (PVDF). The membrane was blocked for 30 min with 5% skim milk TBS-T buffer. Membranes were then exposed to the appropriately diluted primary antibodies, as per the recommended protocol of the manufacturer. As secondary antibodies, anti-mouse IgG-HRP for eNOS and anti-goat IgG-HRP for nNOS (1:2000 dilution; Zymed Laboratories, San Francisco, CA, USA) were reacted at ambient temperature for 1 h, and the membrane was washed again with TTBS six times with 5 min intervals between each washing. Chemiluminescence was detected using ECL western blotting detection reagents. Beta actin was used for the control.

Interaction of SC or GA with phosphodiesterase type 5 (PDE5) and phosphodiesterase type 4 (PDE4) inhibitors on PCCSM

The PCCSM was precontracted with Phe and treated with the PDE5 and PDE4 inhibitors, 10^{-7} M udenafil and 10^{-6} M rolipram, respectively, following which they were exposed to 1 mg/mL SC or 10^{-5} M GA. In another reverse experiment, the precontracted PCCSM was first pretreated with SC or GA, with subsequent addition of PDE5 and PDE4 inhibitors.

Statistical analysis

For the control, contractions induced by Phe alone were considered as the 100% values. The relaxant response is expressed as the percent reversal of Phe-induced contractions. The relaxation is expressed as a percentage decrease from the maximal contractile tension induced by 10^{-5} M Phe. All results are presented as means ± standard derivations (SD), and n refers to the number of tissue samples examined. The statistical analysis includes the one-way analysis of variance (ANOVA) for multiple comparisons, using the SPSS statistical software and Student's paired *t*-test. A value of $P < 0.05$ is considered to be significant.

RESULTS

Cumulative effects of SC or GA on PCCSM

SC had a concentration-dependent relaxant effect (Fig. 1), with maximum effect obtained at 2 mg/mL. Pretreatment with ODQ (Fig. 1A), L-NAME (Fig. 1B), MDL 12330A (Fig. 1C) or H-89 (Fig. 1D) significantly decreased the relaxations induced by SC. The SC induced relaxation at high concentrations (1 and 2 mg/mL) was significantly blocked by ODQ and L-NAME ($P < 0.05$), and efficiently and significantly inhibited by MDL 12330A and H-89 ($P < 0.01$).

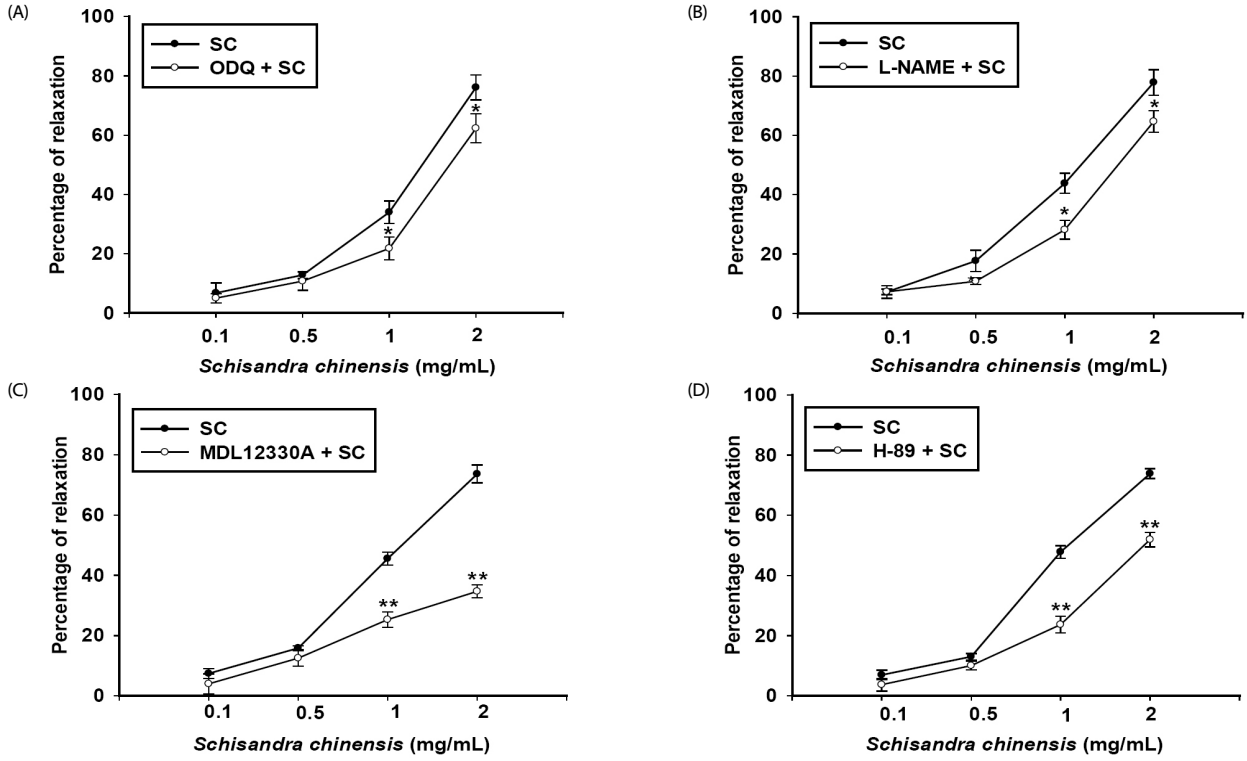

Fig. 1. Relaxation effect of SC in Phe-induced contraction (n = 4). PCCSM contracted by Phe (10^{-5} M) was pretreated with ODQ (A, 10^{-5} M), L-NAME (B, 10^{-3} M), MDL 12330A (C, 10^{-5} M) or H-89(D, 10^{-4} M), and subsequently exposed to four concentrations of SC (0.1, 0.5, 1 and 2 mg/mL). SC: *Schisandra chinensis*; Phe: L-phenylephrine; PCCSM: penile corpus cavernosum smooth muscle; ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; L-NAME: Nω-Nitro-L-arginine methyl ester hydrochloride; MDL 12330A: MDL 12330A hydrochloride; H-89: H-89 dihydrochloride hydrate. Values are mean ± SD. */** Statistically significant from SC. * $P < 0.05$, ** $P < 0.01$.

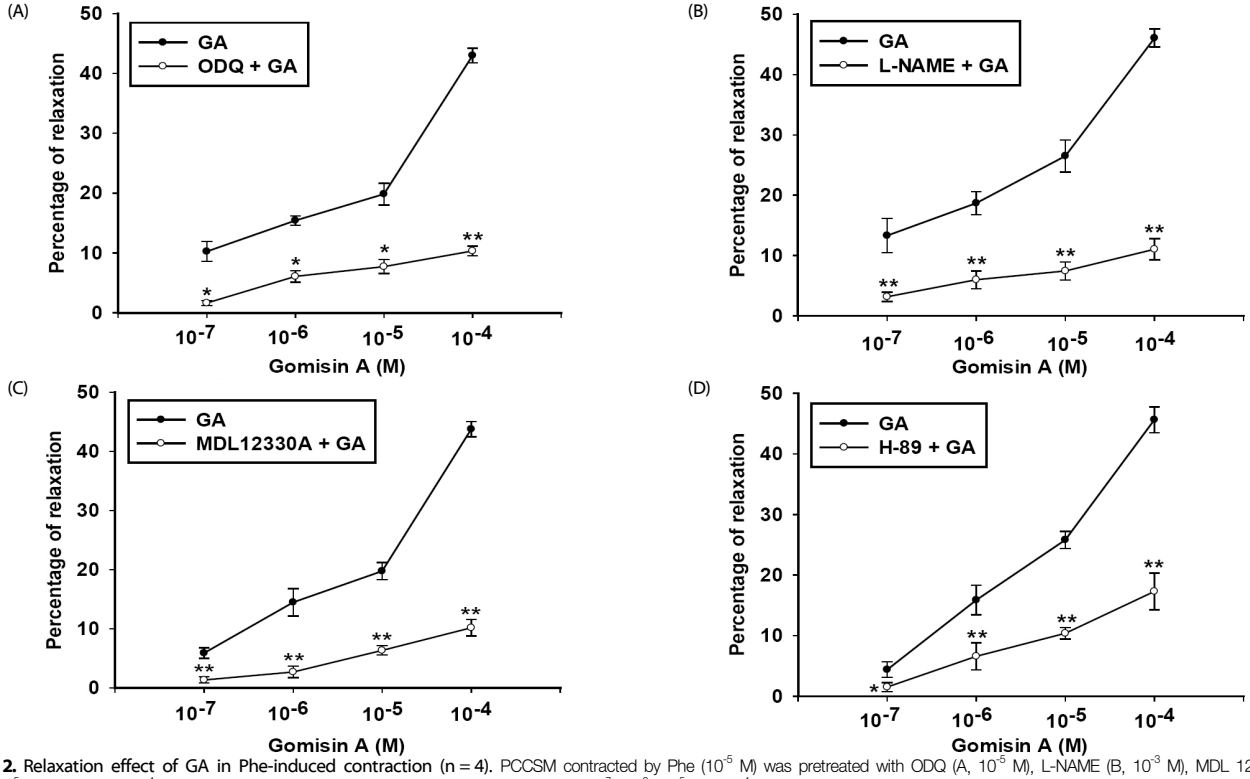

Fig. 2. Relaxation effect of GA in Phe-induced contraction (n = 4). PCCSM contracted by Phe (10^{-5} M) was pretreated with ODQ (A, 10^{-5} M), L-NAME (B, 10^{-3} M), MDL 12330A (C, 10^{-5} M) or H-89(D, 10^{-4} M), and subsequently exposed to four concentrations of GA (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M). GA: gomisin A; Phe: L-phenylephrine; PCCSM: penile corpus cavernosum smooth muscle; ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; L-NAME: Nω-Nitro-L-arginine methyl ester hydrochloride; MDL 12330A: MDL 12330A hydrochloride; H-89: H-89 dihydrochloride hydrate. Values are mean ± SD. */** Statistically significant from GA. * $P < 0.05$, ** $P < 0.01$.

Table 1. Cyclic nucleotides in perfusate with SC and GA

Samples	Concentrations	Cyclic nucleotides	
		cAMP (fM/mg)	cGMP (fM/mg)
SC (mg/mL)	Control	1,304.80 ± 39.30	163.00 ± 6.74
	0.1	1,402.30 ± 36.60**	220.90 ± 5.70*
	0.5	1,535.10 ± 36.60**	304.70 ± 7.20**
	1	1,688.30 ± 74.10**	330.80 ± 8.10**
	2	1,977.70 ± 51.30**	449.30 ± 10.00**
GA (M)	Control	1,234.60 ± 62.50	184.40 ± 3.80
	10^{-7}	1,242.60 ± 60.20*	204.00 ± 6.60
	10^{-6}	1,347.70 ± 37.30*	211.40 ± 6.80
	10^{-5}	1,494.00 ± 41.60**	224.80 ± 5.70*
	10^{-4}	1,773.00 ± 90.80**	238.40 ± 5.70*

SC: *Schisandra chinensis* fruit extract, GA: gomisin A; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate. Values are mean ± SD. **** Statistically significant from control concentrations. * $P < 0.05$, ** $P < 0.01$.

GA also demonstrated a significant and concentration-dependent relaxation (Fig. 2). Pretreatment with ODQ (Fig. 2A), L-NAME (Fig. 2B), MDL 12330A (Fig. 2C) or H-89 (Fig. 2D) significantly decreased the relaxations induced by GA. ODQ, L-NAME, MDL 12330A or H-89 significantly blocked the GA induced relaxation at 10^{-4} M ($P < 0.01$).

Effect of SC or GA on cAMP and cGMP concentrations

The results of cAMP and cGMP concentrations are presented in Table 1. Exposure to 2 mg/mL of SC resulted in a significant increase in cAMP and cGMP concentrations at 1,977.7 ± 51.3 fM/mg and 449.3 ± 10.0 fM/mg, respectively. The maximum levels of cAMP and cGMP achieved by GA treatment were 1,773.0 ± 90.8 fM/mg and 238.4 ± 5.7 fM/mg, respectively.

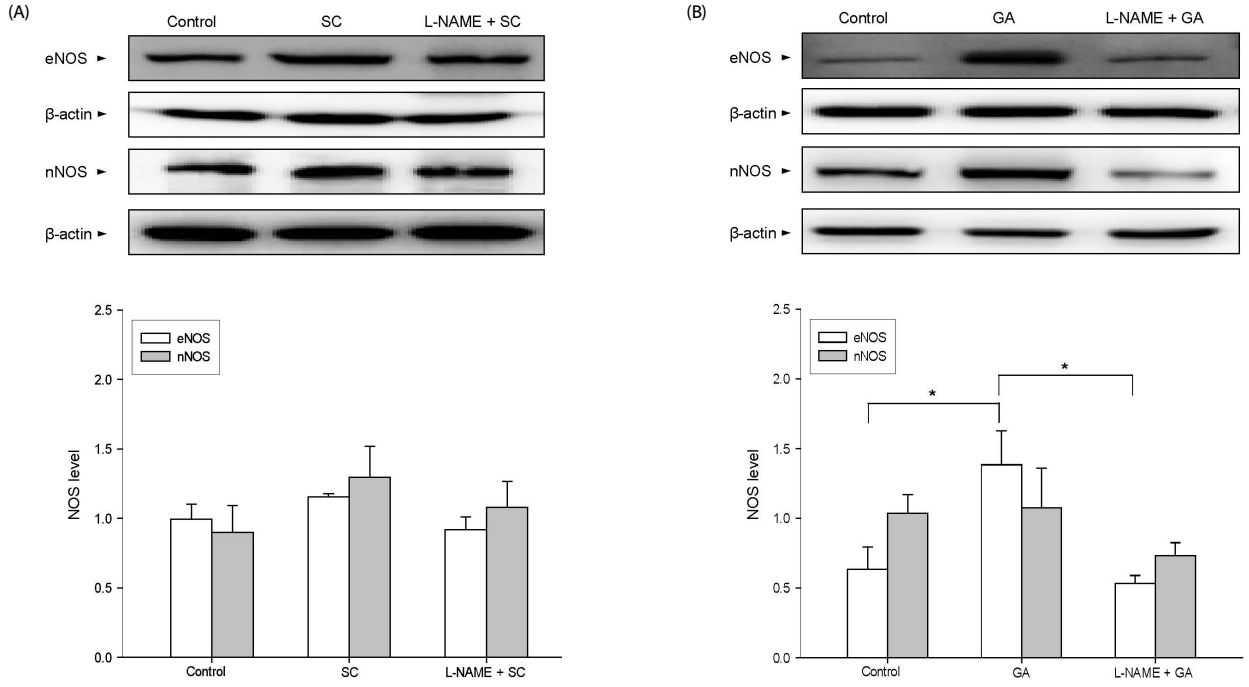

Fig. 3. Effects of L-NAME (10^{-3} M) in SC (A, 1 mg/mL) or GA (B, 10^{-5} M) induced eNOS and nNOS protein in the PCCSM by western blotting (n = 4). L-NAME: Nω -Nitro-L-arginine methyl ester hydrochloride; SC: *Schisandra chinensis*; GA: gomisin A; eNOS: endothelial nitric oxide synthase; nNOS: neuronal nitric oxide synthase; PCCSM: penile corpus cavernosum smooth muscle. Values are mean ± SD. * Statistically significant from GA, $P < 0.05$.

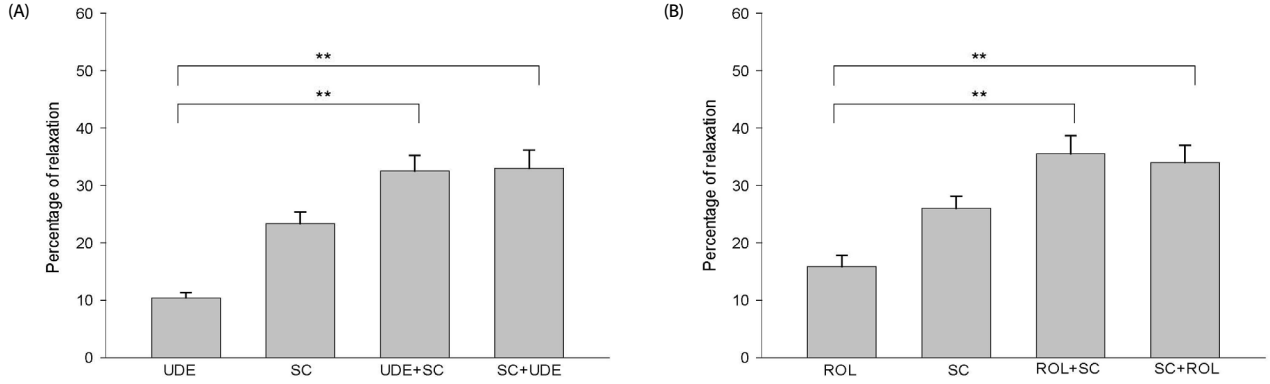

Fig. 4. Interaction of SC (1 mg/mL) with UDE (10^{-7} M) or Rol (10^{-6} M) (n = 4). SC: *Schisandra chinensis*; UDE: udenafil; Rol: rolipram; UDE + SC: SC induced relaxation in the UDE-pretreated PCCSM (A); SC + UDE: UDE induced relaxation in the SC-pretreated PCCSM (A); Rol + SC: SC induced relaxation in the Rol-pretreated PCCSM (B); SC + Rol: Rol induced relaxation in the SC-pretreated PCCSM (B). Values are mean ± SD. ** Statistically significant from Ude or Rol, $P < 0.01$.

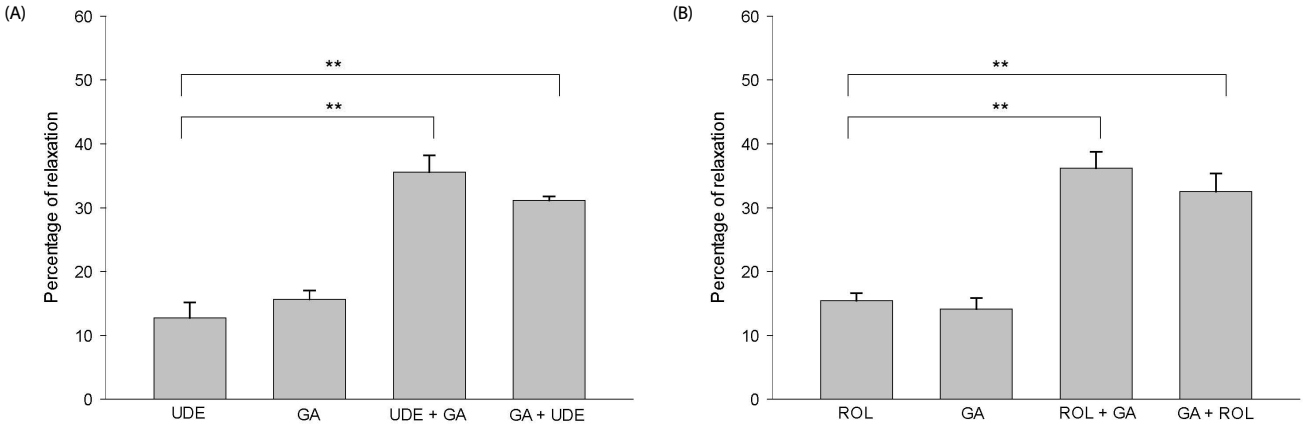

Fig. 5. Interaction of GA (10^{-5} M) with UDE (10^{-7} M) or Rol (10^{-6} M) (n = 4). GA: gomisin A; UDE: udenafil; Rol: rolipram; UDE + GA: GA induced relaxation in the UDE-pretreated PCCSM (A); GA + UDE: UDE induced relaxation in the GA-pretreated PCCSM (A); Rol + GA: GA induced relaxation in the Rol-pretreated PCCSM (B); GA + Rol: Rol induced relaxation in the GA-pretreated PCCSM (B). Values are mean ± SD. ** Statistically significant from Ude or Rol, $P < 0.01$.

The expression of eNOS and nNOS on PCCSM

After treatment with SC (2 mg/mL) for 2 h, the nNOS level of PCCSM increased by approximately 1.4-fold (Fig. 3A). Pretreatment with L-NAME slightly inhibited the levels of eNOS and nNOS, which were increased after SC exposure. Our results show that exposure to GA significantly increased the eNOS level by more than 2-fold (Fig. 3B, $P < 0.05$). The expression of eNOS level was significantly decreased by pretreatment with L-NAME on PCCSM, compared to incubation with GA (10^{-5} M) ($P < 0.05$).

Effect of SC on PCCSM pretreated with udenafil or rolipram

Udenafil effectively increased the Phe-precontracted PCCSM at 10^{-8} M concentration (10.43 ± 0.85%, Fig. 4A). Conversely, the application of SC (1 mg/mL) induced the relaxation by 23.36 ± 2.03%. The mixed relaxation value of udenafil and SC was 32.50 ± 2.79% on udenafil-pretreated PCCSM, and 33.02 ± 3.13% on the SC-pretreated PCCSM. Rolipram effectively increased the Phe-precontracted PCCSM in 10^{-6} M concentration (15.84 ± 1.95%, Fig. 4B). The mixed relaxation value of rolipram and SC were 35.53 ± 3.17% on rolipram-pretreated PCCSM and 34.01 ± 3.00% on the SC- pretreated PCCSM.

Effect of GA on PCCSM pretreated with udenafil or rolipram

The relaxation induced by udenafil on Phe-precontracted PCCSM was 12.71 ± 2.47%, and GA (10^{-5} M) induced relaxation of 15.63 ± 1.37% (Fig. 5A). The mixed relaxation value of udenafil and GA was 35.55 ± 2.66% on udenafil- pretreated PCCSM, and 31.17 ± 0.60% on GA-pretreated PCCSM. Rolipram effectively increased the Phe-precontracted PCCSM (15.46 ± 1.15%, Fig. 5B). The relaxation of GA was 14.13 ± 1.70% on Phe-contracted PCCSM. The mixed relaxation value of rolipram and GA was 36.17 ± 2.58% on rolipram-pretreated PCCSM and 32.55 ± 2.81% on GA-pretreated PCCSM.

DISCUSSION

Results of the present study indicate that SC and GA significantly relax the rabbit PCCSM in a concentration-dependent response. Also, exposure to SC or GA significantly increases the cAMP and cGMP concentrations. This result is supported by the findings that SC and GA may affect the penile erection and possibly be associated with the NO-cGMP and cAMP pathway. Additionally, schisandrol A, a lignan isolated from *S. chinensis*, also shows significant relaxation effect on PCCSM at concentrations of 10^{-7}-10^{-4} M [8,21]. Previous researches by the author have demonstrated that schisandrol A and gomisin A are the most potent relaxants among the lignans [21]. The relaxant effect of compounds with a hydroxyl group (schisandrol A and gomisin A) was higher than compounds without hydroxyl group [21]. Thus, it seems credible to conduct a study about the structure-activity correlation of principal ingredients of *S. chinensis*.

The major effective ingredients of *S. chinensis*, lignans (including gomisin A) induce vascular relaxation [22,23]. In this study, we investigated the *ex vivo* effects of *S. chinensis* on PCCSM in a rabbit. Previous studies have shown that the *n*-hexane fraction of SC causes significant relaxation of PCCSM as compared to the ethyl acetate and *n*-butanol fractions [21]. Hence, GA isolated from *n*-hexane fraction of *S. chinensis* may be the active component that exerts the relaxant effect on PCCSM.

Normal erectile function is a complex neurovascular process induced by both restricted venous outflow and increased intracavernosal blood flow, resulting from PCCSM relaxation [24]. Nitric oxide (NO) is released during nonadrenergic and noncholinergic neurotransmission, and is believed to be the endothelial derived relaxation factor in the corpora cavernosa [25]. NO generated from the eNOS and nNOS in the penile tissue triggers penile erection in the rat [26]. In the present study, eNOS expression level was significantly increased in PCCSM treated with GA.

NO stimulates the guanylyl cyclase in the smooth muscle cell, converting GTP to the intracellular second messenger cGMP [27]. An increase in cGMP produces a decrease in intracellular calcium, resulting in the relaxation of PCCSM [12]. The results of the present study revealed that L-NAME and ODQ significantly reduce the SC or GA induced relaxation. These results suggest that the relaxation by SC or GA on PCCSM is mediated

by the NO-cGMP signaling pathway. This finding is constant with results attained by Park *et al*. [28], which reported that the hexane extract of *S. chinensis* causes endothelium-denuded aortas vasorelaxation by mediating the endothelium dependent NO pathway.

Multiple approaches such as hormones, intraurethral suppositories, intracavernosal injections, surgery and PDE5 inhibitors have been used to treat ED [29]. Avanafil, mirodenafil, sildenafil, tadalafil, udenafil and vardenafil are classified as PDE5 inhibitors, but adverse side effects have been reported, such as visual disorder, headache, facial flushing, rhinitis and dyspepsia [30,31]. Our results indicate that SC and GA efficiently enhance the PDE5 inhibitor-induced relaxation by more than 3-fold. Many clinicians and researchers are considering alternative medicines to ameliorate erectile function. Since SC and GA enhance the PDE5 inhibitor-induced relaxation, they may therefore aid in ameliorating ED in patients who do not respond to the available PDE5 inhibitors.

Rolipram represents a novel class of phosphodiesterase type 4 inhibitors which exert its pharmacological action through an increase of the cAMP levels in vascular tissue [32]. Intracellular cAMP concentrations are produced by adenylyl cyclase and are inhibited by cyclic nucleotide phosphodiesterase [33]. The present study revealed that SC and GA efficiently increase the rolipram induced relaxation.

In conclusion, both SC and GA significantly relaxed the PCCSM, and showed an increasing effect on udenafil or rolipram induced relaxation. The increased cAMP and cGMP concentrations, increased level of eNOS, and the inhibition of SC or GA relaxation with ODQ, L-NAME, MDL 12330A and H-89 suggests that SC and GA may be associated with erectile function via the NO-cGMP and cAMP pathways. Our findings provide the possibility that SC and GA are potential candidates of alternative medicines, or supplements for men who do not respond to the available medicines for ED.

CONFLICT OF INTEREST

The authors declare no potential conflicts interests.

ORCID

Bo Ram Choi: https://orcid.org/0000-0002-0203-7080
Hye Kyung Kim: https://orcid.org/0000-0001-5982-5005
Jong Kwan Park: https://orcid.org/0000-0001-9682-2081

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