ISSN (Print) : 0974-6846
Indian Journal of Science and Technology, *Vol 9(39), DOI: 10.17485/ijst/2016/v9i39/103234, October 2016* ISSN (Online) : 0974-5645

Relaxing Mechanism of Gami-Shinkiwhan in Corpus Cavernosum Smooth Muscle

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Abstract

Objectives: The purpose of this study was to investigate the relaxation effects and mechanisms of Gami-Shinkiwhan (GS) in corpus cavernosum smooth muscle. **Methods/Statistical Analysis:** We treated isolated corpus cavernous strips with the water extract of GS and also with 1 mm of calcium chloride (Ca2+), after pretreatment with GS in a Ca2+-free Krebs-Ringer solution. Contraction strips were pretreated with Nω-nitro-L-arginine (L-NNA) before GS. Nitric oxide (NO) concentration in human umbilical vein endothelial cells (HUVECs) was measured by Griess reagent. Endothelial NO syntheses (eNOS) and phosphodiesterase5 (PDE5) production was investigated by histochemical and immunohistochemical staining. **Findings:** GS significantly affected the relaxation of cavernous strips. GS-induced relaxation was inhibited by pretreatment with L-NNA. Contraction induced by the addition of Ca2+ was decreased by treatment with the GS extract, compared to the non-treatment with the GS extract in Ca2+-free solution. NO concentration in HUVECs was increased by pretreatment of GS. When GS was applied to the corpus cavernosum penis of rats, the ratio of smooth muscles to collagen fibers decreased. The formation of eNOS around the corpus cavernosum smooth muscle increased but PDE5 formation decreased. **Improvements/Applications:** The smooth muscle relaxation effects of GS on the corpus cavernosum is regulated by suppressing the influx of extracellular Ca2+ through the production of NO and suppression of PDE5.

Keywords: Corpus Cavernosum, Endothelial Nitric Oxide Synthase (eNOS), Gami-Shinkiwhan (GS), Nitric Oxide (NO), Phosphodiesterase Type 5 (PDE5)

1. Introduction

Erectile dysfunction is a very common condition in male adults above the age of 40. It is defined as the inability to develop or maintain an erection, which leads to unsatisfactory sexual relationships[1].

Although erectile dysfunction is not life threatening, it hinders intimacy in couples and reduces the confidence of sufferers. It can negatively affect sex life, psychologically deteriorate a man's self-esteem, and in extreme cases, produce social problems[2].

It is estimated that 2 million males suffer from erectile dysfunction in Korea. A 2007 study by [3]reported that the prevalence of erectile dysfunction in Korean males above 46 years of age was 41.5%. Furthermore, they reported that moderate and severe erectile dysfunction occurs in 5.3% of Korean males between the ages of 45-54 years and it increases to 87.3% in males above 75 years of age.

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With the recent improvement in economy, interest on erectile dysfunction treatments is increasing due to the increase in people's interest on their quality of life. Since the elderly population is increasing due to the extended life span thanks to the developments in medicine, it is expected that the number of erectile dysfunction patients will increase further.

Some of the causes of erectile dysfunction are cardiovascular disease, cerebrospinal disease, nerve damage during abdominal-pelvic surgery, endocrine disease, systemic disease and drug use, among others[4]. Most cases of erectile dysfunction in males above the age of 50 are caused by aging or other diseases. The most common cause of erectile dysfunction is vascular disease within the penis[5]. Therefore, vascular surgery, penile prosthesis or vascular dilator injections within the corpus cavernosum, and vasodilators, are commonly used to treat erectile dysfunction[6]. Invasive procedures such as surgery or self-injections can cause severe side effects[7], while non-invasive procedures such as orally administered drugs, which are cost-effective, can cause vasomotor side effects such as facial flushing and headaches[8]. Therefore, the demand for effective treatments with minimal side effects is increasing as well as the interest in oriental medicinal treatments.

The most representative medication is Shinkiwhan. A study by[9] on the effects of Shinkiwhan on erectile dysfunction and sexual function reported that Shinkiwhan causes relaxation of the corpus cavernosum smooth muscles. Similarly[10] reported that the administration of Shinkiwhan not only improves physical and psychological functions but also improves sexual function.

Shinkiwhan, made of Yookmijihwangwon and schisandra, is a treatment for fatigue of the body and the kidney described in the Dongui Bogam. Since shinkiwhan treats kidney fatigue[11], it can be inferred that it could improve sexual function.

Eucommia[12], amomi amari fructus[13], batryticatus bombycis[14], acanthopanax[15], rubus coreanus[16], and lycii fructus[17] are herbal medicines that improve vasodilation and erections. Therefore, the addition of these herbal medicines in shinkiwhan would further improve body restoration and erectile function.

In the present study, we use a mixture of Gami-Shinkiwhan (GS) with eucommi, amomi amari fructus, batryticatus bombycis, acanthopanax, rubus coreanus, and lycii fructus on experimental animals. Organ bath studies and histochemical/immunohistochemical studies were conducted on corpus cavernosum fragments of experimental animals to investigate the effects and the mechanism of GS on penile erections.

2. Materials and Methods

2.1 Experimental Animals

Male New Zealand white rabbits (Samtako, Korea) that weigh about 2 kg and 10-week-old Spague Dawley rats (Samtako, Korea) were used in this study. The experimental animals were allowed to adapt to the lab environment for 1 week prior to the study with sufficient feed and water.

This study was conducted after getting the approval from the Semyung University Institutional Animal Care and Use Committee (smecac 15-06-01).

2.2 Preparation of GS Extract

For the extract, 658 g of GS as shown in Table 1 and 3000 ml of distilled water were mixed. The mixture was heated at 100°C for 3 hours in a round flask and extracted. The extract was concentrated under reduced pressure using a rotator evaporator (Eyela, Japan) and was dried. We obtained 31.6 g of extract powder.

Table 1. Prescription of Gami-Shinkiwhan (GS) per Pack

Name of Herb	Pharmacognostic Name	Weight (g)
Sukjihwang	Rhizoma Rehmanniae	16
Sanyak	Rhizoma Discoreae	8
Sansuyu	Fructus Corni	8
Omija	Schisandra chinensis Fructus	8
Taeksa	Rhizoma Alismatis	6
Mokdanpi	Cortex Moutan Radicis	6
Baekbokryung	Poria Cocas	6
Duchoong	Eucommiae Cortex	6
Ikjiin	Amomi Amari Fructus	6
Baekgamjang	Bombycis Batryticatus	6
Ogapi	Acanthopanax Cortex	6
Bokbunja	Rubus Coreanus	6
Gugija	Lycii Fructus	6
Total		94

2.3 Measurement of Corpus Cavernosum Relaxation

2.3.1 Preparation of Cavernosum Fragments

The rabbits were placed under anesthesia using urethane (0.6g/kg, intravenous injection) and their penises were removed. The removed penis tissues were placed in a modified krebs-ringer bicarbonate solution (NaCl 125.4, KCl 4.9, $CaCl_2$ 2.8, $MgSO_4$ 1.2, $NaHCO_3$ 15.8, KH_2PO_4 1.2, glucose 12.2mM; pH 7.4) at 4°C to remove the adipose tissues and tunica albuginea. 2×2×6 mm penis fragments were prepared.

2.3.2 Measurement of Isotonic Contractions

Modified krebs-ringer bicarbonate solution saturated with compound gas (95% O_2 and 5% CO_2) at 37 °C was transferred to an organ bath (1.5ml in volume) using a peristaltic pump at a rate of 3 ml/min. One extremity of the penis fragment was fixated on the organ bath while the other extremity was connected to a force transducer. The tension of the fragment was measured using a physiograph (PowerLab, Australia).

The penis fragment was allowed to stabilize for 1 hour prior the experiment. Using a micromanipulator (Narishige N2, Japan), 1.5g of passive tension was applied to the fragment. The fragment was then allowed to stabilize for another hour prior to the next trial. The fragments were always allowed 1 hour to stabilize between the experimentations.

2.3.3 Verification of the Effects and Mechanism of GS on Relaxation

Penis fragments were placed in phenylephrine (PE, 10^{-6}M) to induce contraction. GS extracts of different concentrations (0.01, 0.03. 0.1, 0.3, and 1.3mg/ml) were added to the contracted penis fragments. The changes in contractions were recorded.

The penis fragments, which were pretreated with Nω-nitro-$_L$-arginine ($_L$-NNA, 10^{-4}M), were contracted with PE, and changes in contraction were recorded after GS extracts at different concentrations (0.01, 0.03. 0.1, 0.3, and 1.3 mg/ml) were added. The mechanism of relaxation was investigated by comparing the changes in contraction of the penis fragments that were pretreated with $_L$-NNA with those that were not pretreated with $_L$-NNA.

To confirm the effects of the influx of extracellular Ca^{2+} on the relaxation mechanism of the corpus cavernosum, a Ca^{2+} solution (1mM) was added to the PE-penis fragment mixture in Ca^{2+}-free kreb solution and the changes in contraction were recorded. The same penis fragment was then allowed to rest for an hour before being treated with GS extract (3mg/ml) for 10 minutes. A Ca^{2+} solution (1mM) was added to the penis fragment treated with GS and the changes in contraction were recorded. The changes in contraction of the treated penis fragments were compared to the untreated penis fragments.

2.4 Measurement of the Histological Changes in Corpus Cavernosum

2.4.1 Preparation of Corpus Cavernosum Tissue Samples

The rats were placed under anesthesia using ether and their lower abdomens were incised to remove their penises, which were then kept in a modified krebs-ringer bicarbonate solution (NaCl 125.4, KCl 4.9, $CaCl_2$ 2.8, $MgSO_4$ 1.2, $NaHCO_3$ 15.8, KH_2PO_4 1.2, glucose 12.2mM; pH 7.4) at 4°C. Adipose tissues on the penis were removed and they were prepared into 2 mm thick fragments. The penis fragments were divided into the control group, without any treatment; PE group, where the samples were only treated with PE (10^{-6} M); and GS group, where the samples were treated with both PE (10^{-6} M) and GS (3mg/ml). The prepared tissue samples were placed on 10% NBF for 24 hours at 36.5°C. The tissue samples were embedded with paraffin and were sliced into 3μmthin films.

2.4.2 Histochemistry of Corpus Cavernosum

Masson's trichrome staining was conducted to observe the distribution of smooth muscles and collagen fibers in corpus cavernosum. The penis was mordanted in Bouin solution at 50-60°C and picric acid was removed using 70% ethanol. The sample was then placed in Weigert iron hematoxylin for 10 minutes to dye the nucleus, followed by 15 minutes in Biebrich scarlet-acid fuchsin and phosphomolybdic-phosphotungstic acid. Finally, the sample was treated with aniline blue for 5 minutes to stain the smooth muscles (red) and the collagen fiber (blue). Image analysis was conducted on the images obtained from an optical microscope (BX50. Olympus Japan determines the proportion of smooth muscles and collagen fibers.

2.4.3 Immunohistochemistry of Corpus Cavernosum

To investigate the distribution of endothelial nitric oxide synthase (eNOS) and phosphodiesterase type 5 (PDE5) in corpus cavernosum, immunohistochemical staining was conducted. Penis fragments were placed in proteinase K (20μg/ml) for 5 minutes to undergo proteolysis and then 10% normal goat serum, a blocking serum, was added. The sample was then reacted with primary antibodies mouse anti-eNOS (1:100, Santa Cruz Biotec) and rabbit anti-PDE5 (1:100, Santa Cruz Biotec) in a humidified chamber for 72 hours at 4°C . The sample was then linked to a secondary antibody, biotinylated goat anti-mouse IgG_{2a} (1:100, DAKO, USA), for 24 hours at 37°C followed by a reaction with the avidin biotin complex kit (Vector Lab, USA) for an hour at 37°C. Finally, the sample was dyed in a 0.05 M tris-HCl buffer (pH 7.4) that contained 0.05% 3.3'-diaminobenzidine and 0.01% HCl, followed by a hematoxylin contrast dye. The magnitude of positive eNOS and PDE5 reactions were measured using image analysis.

2.5 Nitric Oxide (NO) Measurement

2.5.1 Measurement of Cytotoxicity

Human umbilical vein endothelial cells (HUVECs), which were cultivated using endothelial cell basal medium-2 (EBM-2) for 24 hours at 37□ and 5% CO_2, were treated with different concentrations of GS (100, 300, 500 and 1000 μg/ml). The samples were allowed 24 hours to culture after GS treatment and the cell viability was measured using an MTT assay[18]. Then, 20 μl of MTT was added to the cultured cells and they were left in the cell incubator at 36.5□ for 2 hours. The supernatant was removed and the formazan precipitates were dissolved for 15 minutes using 200 μl additions of DMSO. The resulting solution was placed in an ELISA microplate reader to measure its absorptivity at 540 nm, which was then used to calculate the cell viability.

2.5.2 NO Measurement

The amount of NO synthesis was determined by measuring the amount of nitrite formed in the medium, which serves as an indicator of NO synthesis, in a microplate reader. The cultivated cells were treated with GS over a period of 24 hours and 50μl of Griess reagent[19] (1% sulfanilamide, 0.1% naphtylethylenediamide and 2.5% phosphate) was added to a 50μl sample of the cultured cells. The compound was then allowed to react for 10 minutes at 37°C before measuring its absorptivity at 550 nm.

NO synthesis was determined by using a standard curve made with known concentrations of sodium nitrite samples. The amount of synthesized NO was converted to μM. The control group only used the culture solution.

2.6 Image Analysis and Statistic Processing

The average change in contraction of the corpus cavernosum was reported along with its standard deviation, and the observed contractions were expressed as percentages of the maximum contraction, which was measured by treating the tissue with PE. In order to quantify the results of histochemical and immunohistochemical stainings, image analyses were conducted using Image pro Plus (Media Cybernetic, USA). The comparison of the results was conducted using Student's *t*-test from sigma plot 2000 (Sigma).

3. Results

3.1 Relaxing Effects of GS on Corpus Cavernosum

GS solutions (0.3, 1, and 3 mg/ml) caused significant relaxation of corpus cavernosum fragments that were previously contracted using PE in Table 2, Figure 1.

Table 2. The Effects of Gami-Shinkiwhan (GS) in isolated strips of rabbit corpus cavernosum smooth muscle precontracted with phenylephrine (PE)

Treatment	Contraction (g)	Relaxation (%)
PE	1.22±0.17	0
PE + GS 0.01	1.20±0.16	1.8± 4.1
PE + GS 0.03	1.12±0.18	8.3± 6.0
PE + GS 0.10	1.10±0.16	9.9± 6.7
PE + GS 0.30	1.05±0.16*	13.8± 6.3
PE + GS 1.00	0.94±0.14***	23.0± 7.8
PE + GS 3.00	0.68±0.22***	44.5±14.8

Values are mean ± standard deviation (n = 12). GS extract-induced relaxation was expressed as percentage of PE-contraction. GS, Gami-Shinkiwhan extract (mg/*ml*) ;*$p < 0.05$, ***$p < 0.001$ compared with PE.

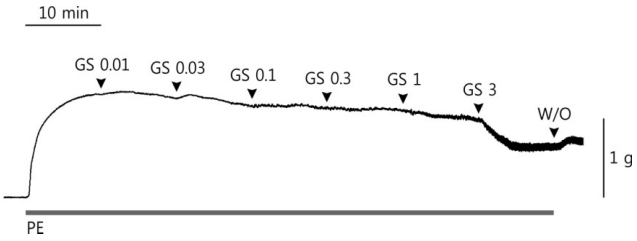

Figure 1. The concentration-response curve of Gami-Shinkiwhan (GS) in isolated strips of rabbit corpus cavernosum smooth muscle pre-contracted with phenylephrine (PE).

3.2 Effects of L-NNA Pretreatment on GS-Induced Relaxation of Corpus Cavernosum

When different concentrations of GS extracts were applied on the penis fragments that were contracted using PE, there was a significant relaxation effect with 1 and 3mg/ml GS extracts when the fragments were not pretreated with $_{L}$-NNA. However, only the application of 3mg/ml GS extract showed significant relaxation when the penis fragments were pretreated with $_{L}$-NNA in Table 3, Figure 2.

Furthermore, the relaxing effects of 0.3, 1, and 3 mg/ml GS extracts were more significantly inhibited when the fragments were pretreated with $_{L}$-NNA.

3.3 Change in Ca^{2+}-Induced Contractions with GS Treatment

The contraction of the penis fragment was measured to be 0.94±0.13 g when 1mM of Ca^{2+} was added, after contraction was induced using PE in a Ca^{2+}-free krebs-ringer solution. When the samples were pretreated with GS, following PE-induced contraction and the addition of 1mM of Ca^{2+}, 0.74±0.14 g of contraction was observed.

Table 3. Effects of pretreatment with Nω-nitro-L-arginine(L-NNA) on the relaxation effects of Gami-Shinkiwhan (GS) in isolated rabbit corpus cavernosum smooth muscle.

Treatment	Non-treatment with $_{L}$-NNA		Treatment with $_{L}$-NNA	
	Contraction (g)	Relaxation (%)	Contraction (g)	Relaxation (%)
PE	1.12±0.17	0	1.42±0.28	0
PE + GS 0.1	1.06±0.16	5.3±4.1	1.41±0.26	0.5±6.5
PE + GS 0.3	0.99±0.16	11.6±4.3	1.39±0.27	1.8±6.8##
PE + GS 1.0	0.92±0.16*	17.5±6.5	1.32±0.25	6.7±7.4##
PE + GS 3.0	0.72±0.17***	36.2±7.9	0.09±0.25*	23.1±8.4##

Values are mean ± standard deviation (n = 10). GS extract-induced relaxation was expressed as percentage of phenylephrine (PE)-contraction. *p< 0.05, ***p < 0.001 compared with PE; ##p < 0.005 compared with PE+GS in non-treatment of L-NNA.

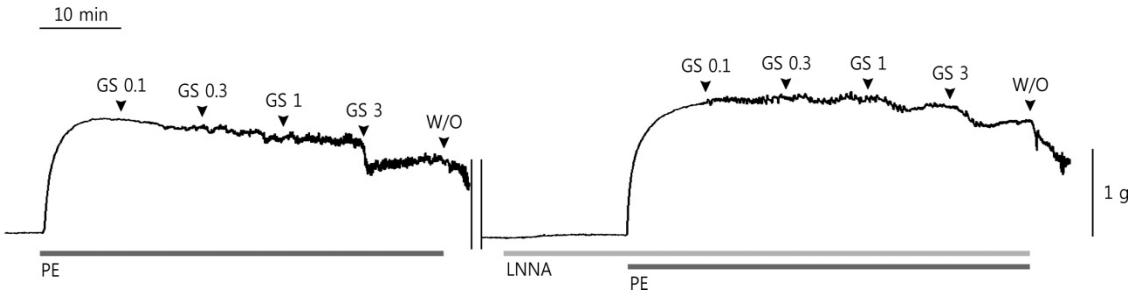

Figure 2. Effects of pretreatment with Nω-nitro-L-arginine(L-NNA) on the relaxation effects of Gami-Shinkiwhan (GS) in isolated rabbit corpus cavernosum smooth muscle.

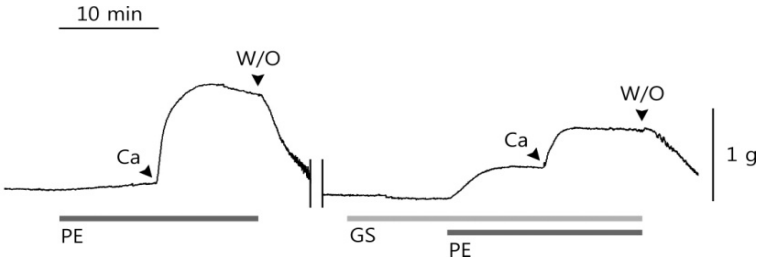

Figure 3. Effects of pretreatment of Gami-Shinkiwhan (GS) on calcium-induced contraction of corpus cavernosal strip in calcium-free media.

The GS extract significantly inhibited Ca^{2+}-induced increases in contraction in Table 4, Figure 3.

Table 4. Effects of pretreatment of Gami-Shinkiwhan (GS) on calcium-induced contraction of corpus cavernosal strip in calcium-free media

Treatment	Non-treatment with GS	Treatment with GS
	Contraction (g)	Contraction (g)
PE	0.04±0.02	0.28±0.07
PE + Ca	0.94±0.13	0.74±0.14#

Values are mean ± standard deviation (n = 6). GS, Gami-Shinkiwhan extract 3mg/ml; Ca, calcium chloride 1mM; #p < 0.05 compared with PE+Ca in non-treatment of GS.

3.4 Changes in Distribution of Smooth Muscles in Corpus Cavernosum

Histochemical analysis revealed that collagen fibers were observed in the central part of the corpus cavernosum and the around the tunica albuginea. The smooth muscles were observed between the collagen fibers within the corpus cavernosum. The smooth muscles were more abundant between the collagen fibers in the central part of the corpus cavernosum in the GS group as shown in Figure 4.

Image analysis revealed that the ratio of collagen fibers to smooth muscles within the corpus cavernosum was 1:0.071 in the control group, 1:0.034 in the PE group, and 1:0.305 in the GS group.

3.5 Changes in eNOS Distribution in Corpus Cavernosum

Histochemical analysis revealed that eNOS was positive in the smooth muscles around the central part of the corpus cavernosum. Image analysis revealed that positive eNOS significantly increased in the GS group compared to the PE group as shown in Table 5, Figure 5.

Table 5. Analysis of endothelial nitric oxide synthase (eNOS) positive reactions

Objective	Group		
	Control	PE	GS
eNOS	25,599±1,182	18,250±9,816	488,400±10,789*

Values are mean ± standard deviation (n = 6). Image analysis for 100,000,000 pixel cells. PE, phenylephrine 10-6 M; GS, PE 10-6 M + Gami-Shinkiwhan (GS) extract 3mg/ml; *p < 0.05 compared with PE.

3.6 Changes in PDE5 Distribution in Corpus Cavernosum

Histochemical analysis revealed that PDE5 was positive in the smooth muscles around the central part of the corpus cavernosum. Image analysis revealed that positive

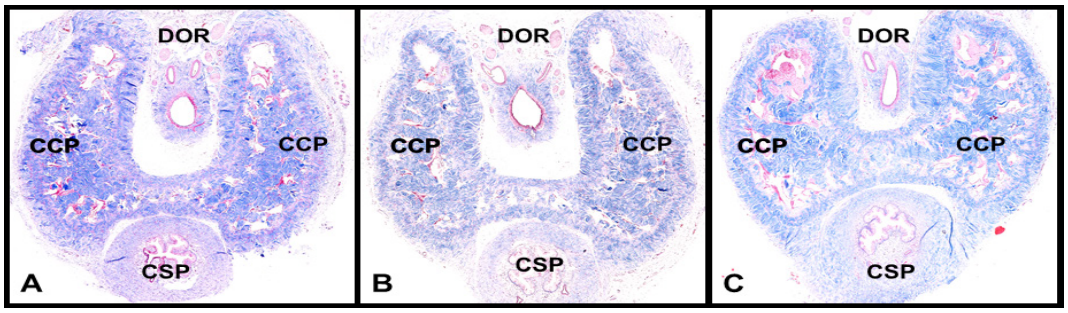

Figure 4. Results of Masson's trichrome stain in rat penises (×40). A, Control group; B, phenylephrine (PE) group; C, Gami-Shinkiwhan (GS) group. CCP, corpus cavernosum penis; CSP, corpus spongiosum penis; DOR, dorsal region of penis.

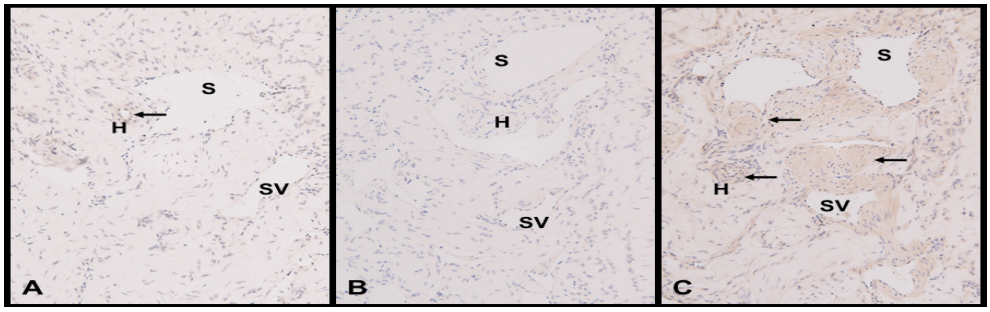

Figure 5. Immunohistochemistry of endothelial nitric oxide synthase (eNOS) in rat penises (×200). A, Control group; B, phenylephrine (PE) group; C, Gami-Shinkiwhan (GS) group; H, helicine artery in corpus cavernosum penis (CCP); S, Sinus in CCP; SV, small vein. Arrows indicate positive eNOS reactions.

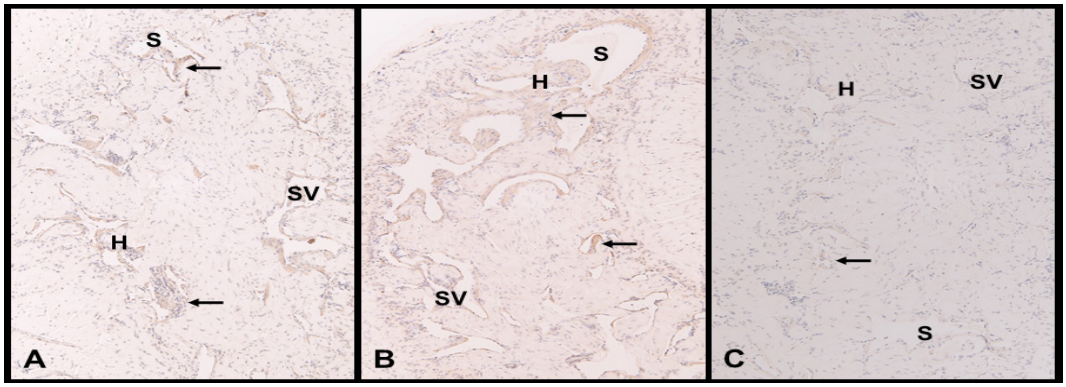

Figure 6. Immunohistochemistry of phosphodiesterase type 5 (PDE5) in rat penises (×100). A, Control group; B, phenylephrine (PE) group; C, Gami-Shinkiwhan (GS) group. Arrows indicate positive PDE5 reactions.

significantly decreased in GS group compared to PE group in Table 6, Figure 6.

Table 6. Analysis of phosphodiesterase type 5 (PDE5) positive reactions

Objective	Group		
	Control	PE	GS
PDE5	352,232±11,743	419,428±17,787	67,139±2,309*

Values are mean ± standard deviation (n = 6). Image analysis for 100,000,000 pixel cells. PE, penylephrine 10-6 M; GS, PE 10-6 M + Gami-Shinkiwhan (GS) extract 3mg/ml; *p < 0.05 compared with PE.

3.7 Measurement of GS Cytotoxicity

Cell viability was measured in HUVECs by adding different concentrations of GS. No differences in cell viabilities were observed with the addition of different concentrations of GS (100, 300, 500 and 1000 μg/ml) as shown in Figure 7.

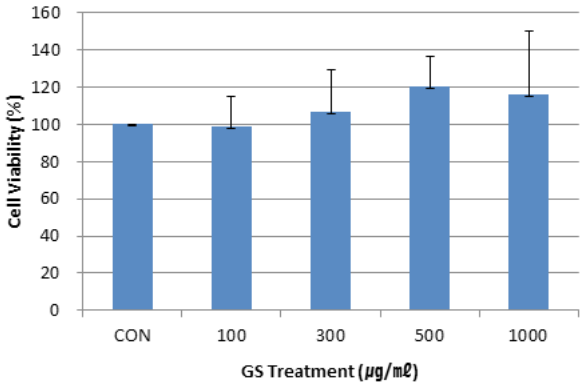

Figure 7. Effects of Gami-Shinkiwhan (GS) extract on the viability of human umbilical vein endothelial cells (HUVECs). Representative bars indicate the cell viability of HUVECs treated with GS extract for 24hr at 37°C .

3.8 Effects of GS on NO Synthesis

The amount of NO was measured after treating HUVECs with different concentrations of GS. A significant increase in NO was observed when 1000 μg/ml of GS solution was added compared to the control group as shown in Figure 8.

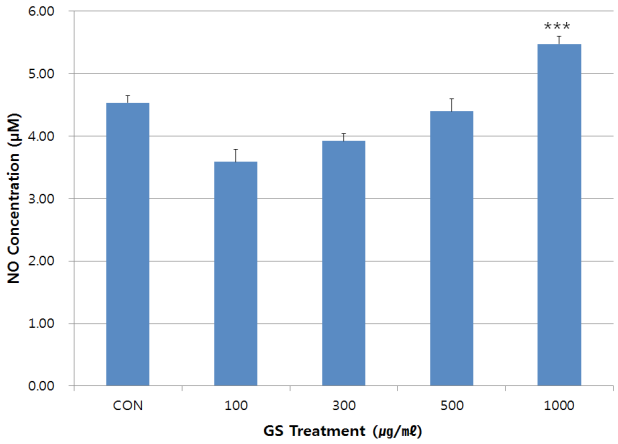

Figure 8. Effects of Gami-Shinkiwhan (GS) extract on nitric oxide (NO) concentration in human umbilical vein endothelial cells (HUVECs). Representative bars show the NO concentration in HUVECs treated with GS extract for 24hr at 37°C .***p< 0.001 compared with the control.

4. Discussion

An erection is defined as the relaxation of the corpus cavernosum caused by the influx of blood to the erectile tissues, which is stimulated by the mechanical excitation of the glans or by psychological, visual, olfactory, or auditory stimulations, followed by a blood flow block caused by venous sinus tension[20,21].

NO, an endothelium-derived relaxing factor secreted by the cavernosum endothelial cells, plays an important role in the relaxation of corpus cavernosum[22]. It is synthesized from $_{L}$-arginine by eNOSs, and then spreads to the smooth muscle cells and activates guanylate cyclase (GC). The activated GC then converts guanosine triphosphate (GTP) into cyclic monophosphate (cGMP). NO increases the amount of cGMP, which in turn activates protein kinase G (PKG). The activated PKG then inhibits the influx of extracellular calcium. This decrease in intracellular calcium cases an erection by relaxing and expanding the smooth muscles and arterioles within the corpus cavernosum[23-28].

In this study, the effects of GS on the relaxation of the smooth muscles in corpus cavernosum were investigated using organ bath studies, histochemical staining, and cell viability experiments. In addition, the relaxing mechanism of GS in corpus cavernosum smooth muscles was investigated by measuring NO production.

Results of the organ bath study revealed that 0.3, 1, and 3 mg/ml GS solutions caused significant relaxation of the corpus cavernosum fragments from rabbits, which were contracted using PE. These results indicate that GS effectively relaxes corpus cavernosum smooth muscles.

Histochemical experiments showed that the increase in smooth muscle distribution was larger in the GS group than in the PE group, which coincides with the results of the organ bath studies. These results prove that the erectile vessels and smooth muscles were expanded due to the relaxation of the corpus cavernosum smooth muscles induced by GS.

The organ bath study showed that the relaxation of the corpus cavernosum smooth muscles were more significantly inhibited when the samples were pretreated with $_{L}$-NNA before adding GS.

$_{L}$-NNA inhibits eNOS in corpus cavernosum endothelial cells, which in turn inhibits the conversion of $_{L}$-arginine into NO[25]. Therefore, the inhibition of the relaxing effects of GS on corpus cavernosum, induced by $_{L}$-NNA pretreatment, indicates that the relaxing effects of GS are related to NO production through eNOS.

The organ bath study in Ca^{2+}-free solutions showed that Ca^{2+} induced contractions, as they were inhibited when the samples were treated with GS.

Contraction of the smooth muscles are caused by the activation of the myosin light chain kinase caused by Ca^{2+}-calmodulin complexes that were formed by extracellular and intracellular Ca^{2+} ions[29].

The inhibition of Ca^{2+} further promoted the contractions induced by GS, indicating that GS prevents the influx of extracellular Ca^{2+} ions into the cell, which prevents the formation of Ca^{2+}-calmodulin complexes and in turn inhibits contractions.

Therefore, GS activates GC by introducing NO into the smooth muscles. The activated GC converts GTP into cGMP. Corpus cavernosum smooth muscles are relaxed as cGMP inhibits the influx of extracellular Ca^{2+} into the cell.

Histochemical analysis revealed that positive PDE5 was significantly lower in the GS group compared to the PE group.

PDE5 inhibits erections by breaking down cGMP and disrupting NO from converting GTP into cGMP. The smooth muscles are contracted as a result of an unsuccessful inhibition of extracellular Ca^{2+} influx into the cell[30,31].

GS lowers the activity of PDE5 and in turn promotes the conversion of GTP into cGMP. cGMP inhibits the influx of extracellular Ca^{2+} into the cell, which in turn causes the relaxation of corpus cavernosum smooth muscles.

These results coincide with the results of the organ bath study, which showed that GS inhibits the influx of extracellular Ca^{2+} and therefore relaxes the corpus cavernosum smooth muscles.

The results of this study show that GS has the potential to be used as an erectile dysfunction treatment since it relaxed the contracted corpus cavernosum smooth muscles in rabbits. In addition, the relaxing mechanism of GS operates by increasing the activity of eNOS in corpus cavernosum endothelial cells, which in turn increases the production of NO. The synthesized NO converts GTP into cGMP in the corpus cavernosum. Along with the inhibition of PDE5 by GS, cGMP inhibits the influx of extracellular Ca^{2+} into the cell and therefore improves erections.

5. Conclusion

As part of the development of erectile dysfunction treatment, animal corpus cavernosum fragments were used in organ bath studies and histochemical/immunohistochemical experiments to investigate the effects and mechanism of GS on erections. The following results were obtained:

- 0.3, 1, and 3 mg/ml GS solutions caused significant relaxing effects on corpus cavernosum smooth muscles that were contracted using PE.
- The relaxing effects of GS on corpus cavernosum smooth muscles were significantly inhibited by $_{L}$-NNA pretreatment.
- GS significantly inhibited Ca^{2+}-induced contractions in a Ca^{2+}-free solution.
- The proportion of the smooth muscles and positive eNOS findings were larger in the GS group compared to the PE group. Positive PDE5 findings were lower in the GS group.
- GS induced NO synthesis in HUVECs, which was significantly larger than in the control group.

The results suggest an increased production of NO through eNOS activation in the corpus cavernosum tissues and that the inhibition of PDE5 activates cGMP. The activated cGMP inhibits the influx of extracellular calcium into the cell, which in turn causes relaxation of corpus cavernosum smooth muscles.

6. Acknowledgements

This study has been sponsored by the Korea Health Industry Development Institute, with funding from the Ministry of Health and Welfare (HI16C-0469-010016).

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