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NON MAJOR GINSENOSIDES CONTRIBUTE TO THE RELAXATION OF *PANAX GINSENG* IN RABBIT CORPUS CAVERNOSUM

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The corporal relaxant effects of total ginsenosides and nine subfractions (F1~F9) separated from it were evaluated in the rabbit corpus cavernosal strips *in vitro*. In the tissues precontracted by phenylephrine (PE, 3×10^{-6} M), ginsenosides induced a dose-dependent (from 1 to 20 mg/ml) relaxing effect on rabbit corpus cavernosal strips with an EC_{50} of 3.65±0.27 mg/ml. Three pure ginsenoside — Rg_1, Rb_1, and Re, at optimal dosages, do not affect the vascular tone at al in PE-precontracted corpus cavernosum. Total ginsenosides was further subfractionated by column chromatography and obtained nine fractions (F1~F9). Among them, F2 (0.1~5 mg/ml) displayed the most potent relaxing activity and turned out to be more effective (EC_{50}=0.49±0.03 mg/ml) than total ginsenosides. Additionally, TLC analysis revealed that F2 contained some other components different from Rg_1, Rb_1 and Re. These findings suggest none of Rg_1, Rb_1 and Re contributes to the beneficial effect of total ginsenosides. In summary, F2 may possibly be used as a drug for intracavernosal injection therapy of impotence. However, further separation and identification the components of F2 and in vivo study are needed before clinical use in an oral form.

Key Words: *Panax ginseng*, Ginsenosides, Corpus cavernosum.

INTRODUCTION

Panax ginseng is a medicinal plant occupying an esteemed position among the tonic medicines. It has been widely used in traditional medicine for the past 20 centuries to enhance stamina and to recuperate from physical stress, particularly when the physical capacity is compromised.[1] Ginseng has also been regarded as an aphrodisiac in the Orient from the ancient past. There has been a clinical study on the effects of the consumption of ginseng on patients with erectile dysfunction.[2] Recent studies also revealed that crude extract of Korean red ginseng (KRG) and/or total ginsenosides, exerts a direct relaxing effect on rabbit corpus cavernosal tissue in a dose-dependent manner.[3-4] Ginsenosides, however, are a mixture of triterpene

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glycosides.[5] The major form of glycosides belong either to the protopanaxadiol group (Rb_1 and Rc are the major components) or to the protopanaxatriol group (Rg_1 and Re are the major components.[6-7] Ginsenosides is composed of a mixture of chemical fractions that may have different or even counteracting effects. Thus, the effect of ginseng extract may be caused by a single active ingredient or by the combined action of many active agents existed in the extract. The purpose of the present investigation was to clarify the corporal active component(s) presented in total ginsenosides.

MATERIALS AND METHODS

Plant material

Panax ginseng (Korea red ginseng) was obtained in a market in Taipei and identified by Mr. C. J. Chou (principal investigator in pharmacognosy, National Research Institute of Chinese Medicine). A voucher specimen is maintained in the herbarium of our institute. Ginsenoside standards were obtained from Extra Synthese Genay France.

Extraction and isolation

5845 gm of pulverized ginseng was extracted by refluxing with 95% ethanol (20 ml × 4) for 15 min at 60 °C. After removal of the solvent by evaporation *in vacuo*, the extract (2004 gm) was dissolved in water. This solution was partitioned into ether and aqueous layer, and the aqueous layer was then extracted with *n*-butanol (*n*-BuOH) saturated with water. The *n*-BuOH layer was concentrated *in vacuo* and lyophilized to afford total ginsenosides (57.3 gm).[8] Fractionation of crude ginseng saponins was done by column chromatography on silica gel (Diaion HP20, 70-230 mesh, Nacalai Chemical Company) and eluted by a gradient of CH_2Cl_2/MeOH (from 10:0 to 1:9) mixture into 9 fractions (F1-F9). Each fraction was dried under reduced pressure and the corporal relaxant activity was further evaluated.

Thin-layer chromatography (TLC) conditions for identification

A chromatographic analysis was performed by TLC on silica gel using a mixture of n-BuOH/EtOAc/H_2O (4:1:1, v/v) as a developing agent and sprayed with H_2SO_4 to be colored. Rf value was defined as spread distance of sample over total gel distance.

Tissue procurement

Male New Zealand white rabbits (3-4 kg) were anaesthetized with sodium pentobarbital (30 mg/kg, i.p.) and exsanguinated. Rabbit penises were surgically removed *en bloc*, with care being taken to keep the tunica albuginea intact. The corpus spongiosum and urethra were excised. The corpus cavernosum tissue was carefully dissected free from the surrounding tunica albuginea and mounted in organ baths (see below).[9-10]

Organ bath experiments

Strips of rabbit corpus cavernosum were mounted with surgical suture to a fixed metal loop from below and a metal wire from above, connected to a force transducer (Model FT03; Grass Instruments, Quincy, MA). The preparation was then immersed in 12-ml baths maintained at 37 °C and containing Krebs' solution (aerated with 5 % CO_2, 95 % O_2 to attain pH 7.4). Optimal isometric tension was achieved by gradual, incremental stretching. The tissue was periodically tested by contracting with 3 μM phenylephrine (PE). Tissues were considered to have reached optimal isometric tension when two successive contractions were within 10 % of each other. After this determination, tissues were relaxed maximally with 1 μM acetylcholine (ACh) to determine endothelium function. After thorough washout, the tissues were contracted with PE and subjected to cumulative additions of ginsenosides or any of nine subfractions. The relaxant responses were calculated as % relaxations of active muscle tone induced by PE (running from 0 to 100 %).

Statistical analysis

All values are expressed as mean ± s.e.m. The EC_{50} (the concentration required to cause half-maximal relaxation) was determined by liner interpolation for each concentration-response curve. Significant differences between groups were assessed with Student's *t* test. A *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

Effect of total ginsenosides on corpus cavernosal strips

On the phenylephrine (PE)-precontracted cavernosal strips, total ginsenosides began to exert a relaxing effect at the concentration of 0.05 mg/ml and the cavernosal strips reached 97.2 ± 5.6 % relaxation at the concentration of 20 mg/ml (EC_{50} = 3.65 ± 0.27 mg/ml) (Fig. 1a and Fig. 2, filled circles). In the tissue precontracted by PE, corpus cavernosum also showed relaxation in response to subfractions (F1 ~ F9) of ginsenosides in a concentration-dependent manner, of which F2 showed the most potent relaxing effect (EC_{50} = 0.49 ± 0.03 mg/ml) (Tab. 1). The corporal relaxant potency of F2 was more pronounced than total ginsenosides (Fig. 2, filled squares).

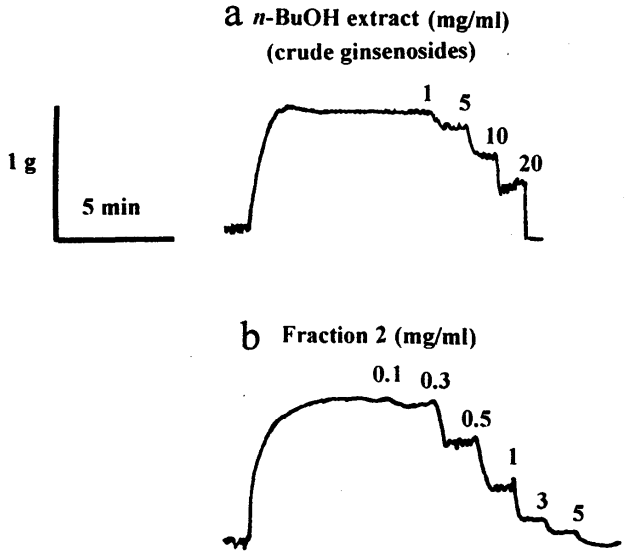

Figure 1. Representative traces showing the dose-response evoked by (a) *n*-BuOH extract (crude ginsenosides) and (b) F2 subfraction in rabbit corpus cavernosal strips precontracted with phenylephrine (3×10^{-6} M).

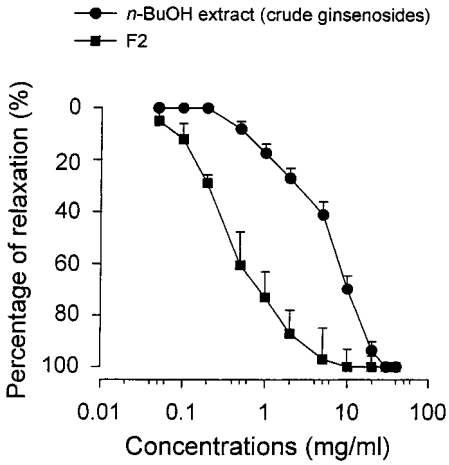

Figure 2. Relaxation effects of n-BuOH extract (filled circles) and F2 subfraction (filled squares) on phenylephrine-precontracted rabbit corpus cavernosal strips. Results are expressed as percent relaxation according to the concentration in logarithmic scale and given as mean±s.e.m. (n=8-11).

Table 1. The EC_{50} value of nine subfractions from crude ginseng saponins in phenylephrine-precontracted avernosal strips[a]

Subfractions	No. of test	EC_{50} (mg/ml)
F1	6	0.73 ± 0.13
F2	7	0.49 ± 0.03
F3	9	1.02 ± 0.71
F4	8	1.20 ± 0.13
F5	5	3.41 ± 0.52
F6	4	19.09 ± 1.28
F7	6	2.47 ± 0.31
F8	4	19.79 ± 1.26
F9	4	2.53 ± 0.52

[a] The data are expressed as mean ± s.e.m.

There are several drug types that through enhancing the NO-cyclic GMP signal pathway, may prove beneficial in treating erectile dysfunction. One such class of drugs is the phosphodiesterase (PDE) inhibitors that prevent the hydrolysis of cyclic GMP, thereby evaluating level of cyclic nucleotide. Thus, a non-specific PDE inhibitor papaverine was introduced into this experiment. Results showed that papaverine added to the bath (10^{-7} ~ 10^{-4} M) while the muscle was precontracted by PE, evoked significant corporal relaxation in a concentration-dependent manner (corporal relaxant percentages were 0, 7.0 ± 1.1, 19.2 ± 2.1, 42.8 ± 7.4, 88.7 ± 6.0, 97.0 ± 1.9, and 100 %, respectively)

Effects of Rg_1, Rb_1 and Re on the corpus cavernosal strips

As shown in Fig. 3, single application to the corpus cavernosal strips, none of Rg_1, Rb_1 and Re evoked detectable relaxation at concentration ranged from 0.1 to 1.0 mg/ml. To elucidate whether synergistic effect could be observed,

three pure ginsenosides were applied simultaneously to the tissue bath. Results showed that even combined

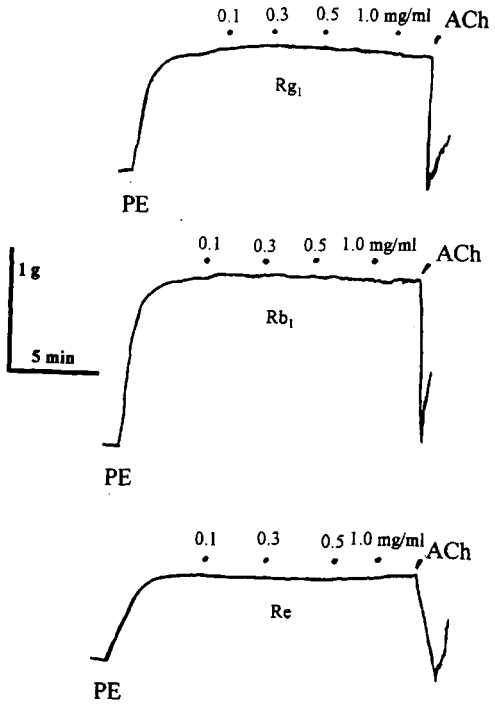

Figure 3. Representative traces showing the sole effect of purified ginsenosides (0.1~1.0 mg/ml) on rabbit corpus cavernosal strips precontracted with phenylephrine ($3x10^{-6}$ M).

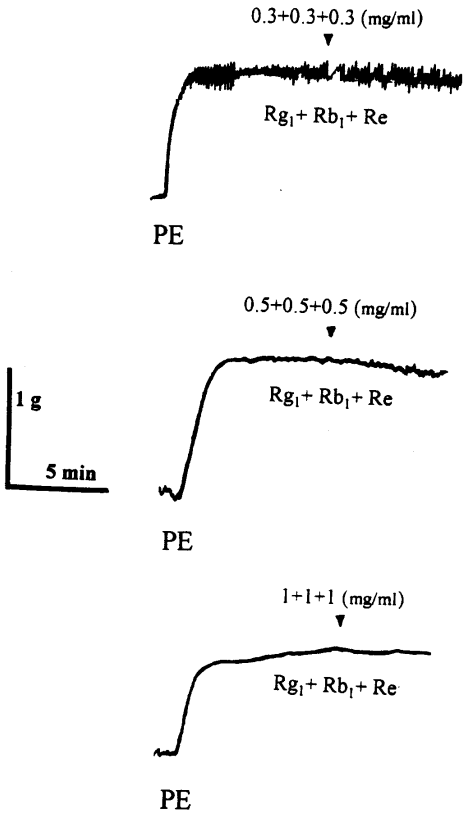

Figure 4. Representative traces showing the combined effect of purified ginsenosides (0.3 ~ 1.0 mg/ml) on rabbit corpus cavernosal strips precontracted with phenylephrine (3×10^{-6} M).

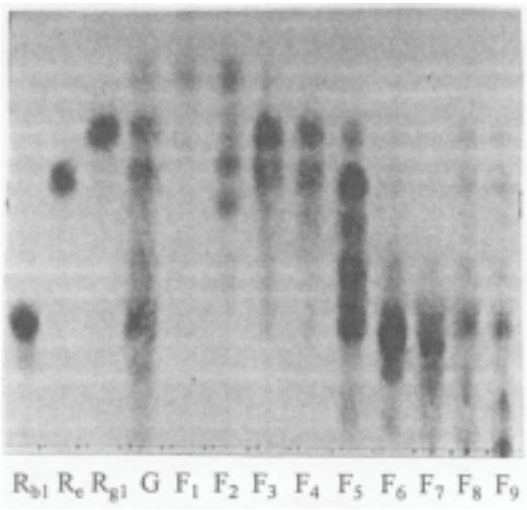

Figure 5. TLC separation of crude ginsenosides (G) and nine subfractions (F1~F9).

administration of Rg_1, Rb_1 and Re still failed to affect the cavernosal muscle tone of the PE-precontracted cavernosal strips (Fig. 4).

Analysis of subfractions of total ginsenosides by TLC

By means of TLC, the contents of nine subfractions (F1~F9) were determined and compared with the standards. As shown in Fig. 5, ginsenoside-Rb_1, Re and Rg_1were separated from each other with different Rf values of 0.27, 0.62 and 0.73, respectively. Total ginsenoside crude extract (G) contained ginsenoside-Rb_1, Re, Rg_1 as well as the other constituents. On the other hand, TLC analysis revealed that F2 contained three types of H_2SO_4 positive spots with Rf values of 0.53, 0.64, and 0.86, respectively. These three Rf values of F2 were distinguished from Rg_1, Rb_1, and Re. This experiment was repeated for 3 time and obtained the same result.

DISCUSSION

Even though studies have been carried out previously to examine the corporal relaxant effect of ginsenosides on rabbit corpus cavernosum,[4] total ginsenosides extracted from *Panax ginseng* are a mixture of several ginsenosides. The purpose of the present investigation was to clarify the vasoactive component(s) presented in the ginsenoside mixture. Soldati and Sticher (1980) pointed out that content of Rg_1, Rb_1 and Re in the root of *Panax ginseng* were 0.38, 0.38 and 0.15 %, respectively.[11-12] Based on this investigation, we can calculate approximately the amount of Rg_1, Rb_1 and Re in total ginsenosides. Qualitative identification revealed that 10 mg/ml of total ginsenosides contained about 1.25, 1.25 and 0.50 mg/ml of Rg_1, Rb_1 and Re, respectively. Comparison study showed that 10 mg/ml of total ginsenoside induced 67.5 ± 2.4% relaxation, but Rg_1, Rb_1 and Re at concentrations ranged from 0.1 to 1.0 mg/ml all did not evoke detectable relaxation. Consideration of the possibility of synergistic effect, a mixture of equal amount of Rg_1, Rb_1 and Re (1:1:1) was applied to the PE-precontracted cavernosal strips. Result showed that the mixture at concentrations ranged from 0.9 to 3.0 mg/ml still failed to evoke significant corporal relaxation. Result obtained here indicated that crude ginseng saponin relaxed corpus cavernosum, but ginsenoside — Rg_1, Rb_1 and Re turned out to be effectiveless. These findings imply that Rg_1, Rb_1 and Re cannot account for the corporal relaxant effect to total ginsenosides. Thus, an identification of more potent active components was progressed by column chromatography.

Nine fractions isolated from ginsenosides showed corporal relaxant effect with various efficacy. Among them, F2 was the most potent one and was 10 times potent than total ginsenosides.

There are many potentially active constituents in F2 that could influence its final action. Therefore, further studies are required in this area to clarify the individual mechanism of each component of F2 and purify and select the proper components for achieving better relaxation of the corporal smooth muscle. Studies are currently under way to isolate and identify the active components in F2, as well as to determine its profile of action in vivo.

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人參皂　中的非主要皂　成份引發離體大白兔陰莖海棉組織產生舒張反應

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本實驗評估人參總皂　(crude ginseng saponin)、總皂　次萃取物(subfractions, F1～F9)、及皂　標準品(Rg$_1$, Rb$_1$, Re)在離體大白兔陰莖海綿組織的舒張活性。結果顯示人參總皂　可以有效舒張 phenylephrine(3×10^{-6} M)預收縮的陰莖海綿體，一半有效濃度(EC$_{50}$)為 3.65 ± 0.27 mg/ml；相反地，皂　標準品(Rg$_1$, Rb$_1$, Re)在相當劑量範圍下，則完全不影響海綿體張力，此結果暗示人參總皂　中的三個主要皂　(Rg$_1$, Rb$_1$, Re)並非是舒張海綿體的主要活性成份。繼續以管柱分離總皂　得到 9 個部份(F1～F9)，分別呈現不同程度的海綿體舒張活性，其中以 F2 的作用最強(EC$_{50}$ = 0.49 ± 0.03 mg/ml)、且顯著強於人參總皂　。以上結果暗示 F2 可能有機會作為治療陽痿的陰莖內注射用藥，但在臨床使用前必須再評估其組成、在活體動物的實驗結果以及是否可作為口服投藥.

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