Original Article
Inhibitory effects of salviae miltiorrhizae radix (danshen) and puerariae lobatae radix (gegen) in carbachol-induced rat detrusor smooth muscle contractility

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Abstract: Both danshen (D) and gegen (G) have proven relaxant effects on vascular smooth muscle, thus their potential bladder inhibitory effects have impending interests in urology. The aim of this study was to demonstrate the novel effects of D and G on detrusor smooth muscle contractility. Urothelium-intact (+UE) and urothelium-denuded (−UE) detrusor strips were isolated from the rat. Isometric tension was measured using a myograph system. Carbachol (CCh) was used to pre-contract the detrusor strips prior to stepwise relaxation by adding extracts of D, G, and a DG (7:3) formulation. Tonic relaxation level and phasic contractile activity under the herbal treatments were analyzed. There was no difference in the herbal effects between +UE and −UE strips. D alone induced a much smaller relaxation than G alone or DG. G alone also suppressed phasic amplitude but not phasic frequency while DG suppressed both parameters. D and G acted synergistically to yield the observed effects on detrusor smooth muscle. The findings showed that the DG formulation were able to relax the detrusor as well as suppress phasic contractions, both actions important in maintaining normal bladder filling and urine storage processes. Hence DG may have new application in the management of bladder disorders.

Keywords: Detrusor smooth muscle, contractility, danshen, gegen, Chinese medicine

Introduction

Urine storage and release are two principal functions of the bladder. To fill the bladder with urine, bladder smooth muscle undergoes extensive relaxation to increase intravesical volume [1]. Meanwhile, phasic contractions of individual smooth muscle bundles help maintain a bladder shape that facilitates voiding when necessary [2]. When phasic contractions become too vigorous and frequent, however, urine spillage may occur, as is observed in patients with overactive bladders [3]. Thus, the ability to relax properly and to prevent excessive phasic contractions is pivotal to normal bladder function. Currently M_3-antimuscarinics are the most commonly used drugs for detrusor overactivity (DO), which by definition, refers to involuntary detrusor contractions during bladder filling [3, 4]. Other clinically used medications include β_3-adrenoceptor agonists and phosphodiesterase inhibitors [4]. These western medicines possess varying degrees of adverse effects one way or another [4]. As such, traditional Chinese herbal medicines become a hotbed to search for drugs with efficacy toward DO but with fewer undesirable side effects. Despite the long history of using Chinese herbs to treat various ailments, documentation on the management of bladder-related urological disorders has been very scarce. Without any lead on possible testable Chinese herbs on bladder smooth muscle, attention was turned to look for herbs that act on other smooth muscle types, such as vascular smooth muscle. In the vascular system, a Salviae Miltiorrhizae Radix (Danshen, D) and Puer-

ariae Lobatae Radix (Gegen, G) formulation has proven vasorelaxing effects [5, 6]. The same formulation, composed of 7-part D and 3-part G, was used in the present study, where the effects of bladder relaxation were determined. In the vascular studies mentioned above, DG-induced vasorelaxation was independent of the endothelium, which is known to secrete vasoactive substances [5]. Since the urothelium (UE) has also been receiving much interest lately due to its bladder-modulatory effects [7-10], we also compared whether DG elicited its actions differently in UE-intact (+UE) and UE-denuded (−UE) detrusor strips.

Materials and methods

Tissue preparation

All procedures were performed according to rules outlined by the Institutional Animal Care and Use Committee at Nanyang Technological University, Singapore (Project approval No.: ARF SBS/NIE-A 003). Six- to seven-week-old male Sprague-Dawley rats were killed by CO_2 asphyxiation. The whole bladder was harvested as previously described and immediately placed in carbogen (95% O_2 and 5% CO_2)-aerated ice-cold Krebs' solution [11]. The bladder base, which made up about one third of the bladder, was discarded. Only tissues isolated from the bladder dome (detrusor) were used. The bladder dome was cut open along the lateral sides and the UE was exposed. Fine pins were used to fix the tissue on a Sylgard®-coated petri dish. Using a razor blade, two transverse strips measuring 5 mm by 1 mm each were dissected from the detrusor [11]. Both urothelium-intact (+UE) and urothelium-denuded (−UE) strips were used. To obtain −UE strips, the UE was carefully excised with fine dissecting scissors as described elsewhere [7-10]. All strips were mounted on a tissue myograph system (Danish Myo Technology Model 800MS, Denmark) containing carbogen-aerated Krebs' solution at 37°C. Isometric tension was monitored in both transverse and longitudinal directions and recorded using a Powerlab interface and the Lab-Chart software (ADInstruments, Australia). The detrusor strips were allowed to equilibrate for 30 min with multiple washouts. During the equilibration and washout periods, baseline tension was consistently adjusted to 2 ± 0.1 g prior to agonist stimulation [9, 10]. Viability of the strips was tested using carbogen-aerated K^+

-Krebs' solution. Experiments were commenced after an additional 30 min of continuous washout.

Experimental protocol

The detrusor strips were pre-contracted with the muscarinic agonist carbachol (CCh, 10 µM) for 20 min. By then, the contraction level would have reached a steady state. The herbal extracts (from 0.1 to 3 mg/mL in half-log increments) were added at 5-min intervals. The selected concentration range of the herbs was based on a study showing relaxant effects in vascular smooth muscle [5]. The average tension over the last 2 min of each herb addition was taken to determine the relaxant response. All relaxant responses were compiled to plot concentration-response curves (CRCs) for each herbal preparation (see Preparation of herbal extracts). To allow comparison of effects between equivalent herbal concentrations, DG concentrations on the graphs had been converted to represent the G concentration in the formulation. For example, when 1 mg/mL DG was indicated, it represented a formulation containing 1 mg/mL G. Considering the 7-part D to 3-part G constitution, the corresponding D concentration would be 2.33 mg/mL in the said example. The reason behind using G as a reference was due to its greater relaxant activity demonstrated. Each phasic contractile event was identified manually, with the cut-off peak amplitude fixed at 5 % of the CCh response at steady state. Two parameters of phasic contractile activity under herbal treatment, phasic amplitude and phasic frequency, were analyzed using the LabChart software (ADInstruments, Australia) [10]. Control phasic contractile activity was determined from the 2-min period prior to addition of herbs. Phasic amplitude (and frequency) under each herbal treatment was expressed as percentage change from control.

Preparation of herbal extracts

Raw herbs of danshen (D) and gegen (G) were purchased, respectively, from shops in Sichuan and Guangdong in China. Morphological and chemical authentication was done in accordance to the Pharmacopoeia of the People's Republic of China 2010. Crude extracts were obtained as stated elsewhere [5] and summarized as follows. Three preparations of raw herbs, D, G and DG (i.e. 7-part D and 3-part G by

weight) were soaked in water (10-fold v/w) for 90 min and extracted at 100°C for 60 min twice and once for 30 min. Products from the three extractions were combined and dried at 65°C at -70 kPa. The extracts were stored in a dessicator prior to use.

Drugs and chemicals

The composition of Krebs' solution was as follows [in mM]: NaCl [119], $MgCl_2$ [1.2], NaH_2PO_4 [1.2], $NaHCO_3$ [15], KCl [4.6], $CaCl_2$ [1.5], *D-*glucose [11]. For K^+-Krebs' solution, no NaCl was added but 124 mM KCl was used instead. All constituents remained the same otherwise. All chemicals and drugs used in this study were purchased from Sigma-Aldrich Co. (Singapore). All drugs were dissolved in Ca^{2+}-free Krebs' solution.

Statistical analysis

Raw values of relaxant responses and phasic amplitude readings were normalized to dry tissue weights, measured after the experiments, so that weight-insensitive data could be obtained. Calculations and statistical analysis were performed using the Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). All data shown in graphs were mean values ± S.E.M. Two-way ANOVA and Bonferroni post-hoc test were used to detect differences in relaxations attained at each herb concentration in the CRC experiments. One-sample Student's t-test was used to detect changes from control phasic amplitude and frequency. P values of less than 0.05 ($P < 0.05$) were considered to be statistically different. Analysis of herb-herb (i.e. D-G) synergism was done using the Calcusyn 2.1 software (Biosoft, Cambridge, UK), where isobologram data and combination indices (CIs) were computed. Isobolograms were generated by first defining the x- and y-axes as concentration of each of the 2 herbs in the formulation [12]. In our isolobograms, D concentrations were expressed on the x-axis, while the y-axis represented G concentrations. A straight line was subsequently drawn, connecting the individual D and G concentrations that elicits a given response level (e.g. 50 %) [12]. This line would intersect both x and y axes. The next step was locating the x,y coordinates of the DG concentration (in the formulation) that achieved the same response level. According to Tallarida [12], a point representing the concentration of the herbal formulation that lies to the left of the

straight line drawn previously would indicate herb-herb synergism. CIs were determined to provide additional evidence of the synergistic effects between D and G [13].

Results

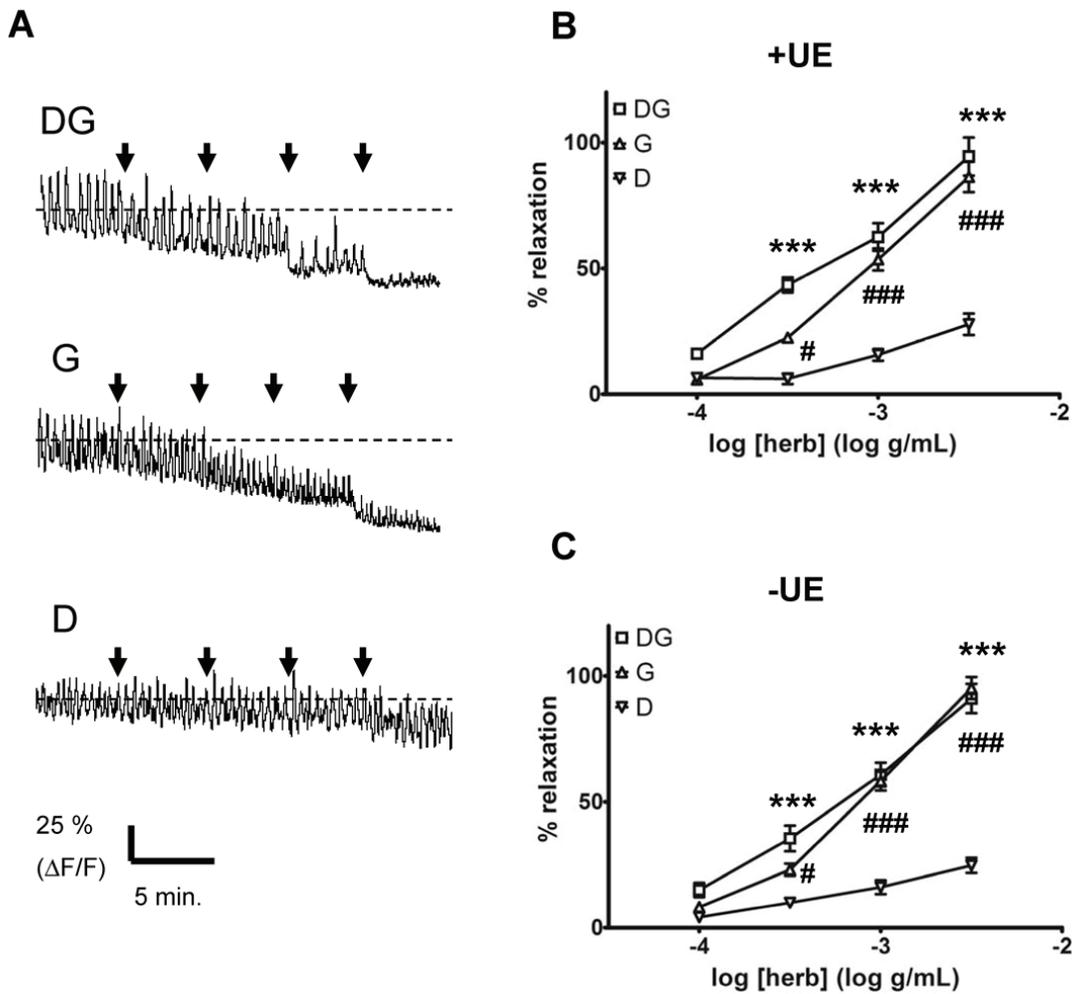*Detrusor relaxation induced by danshen (D), gegen (G) and the DG formulation*

Addition of all three herbal preparations to CCh-precontracted detrusor strips resulted in relaxation of the detrusor strips. **Figure 1A** shows representative traces of the herbal effects on +UE detrusor strip contractility. Both DG (n = 7) and G (n = 7) resulted in marked relaxation, as indicated in **Figure 1B** also. Much smaller relaxation was seen when D (n = 8) was added instead. The relaxant effect of D was significantly smaller than that induced by DG or G. There was no statistical difference between the effects of G and DG, but the latter seemed to induce slightly greater relaxation especially at around half-maximal response level. **Figure 1C** shows the relaxation concentration-response curves obtained in –UE strips (n = 7 to 8 for each herb). The results were similar to those in +UE strips, suggesting the herbs eliciting their effects via an UE-independent manner.

Synergistic effects between D and G in inducing detrusor relaxation

The DG preparation was composed of a 7:3 formulation, where D made up the bulk portion. Although being the minor component in the formulation, it is evident from **Figure 1** that G was more efficacious in inducing relaxation. The synergy between D and G was thus investigated. In **Figure 2**, isobolograms were constructed using herbal concentrations at the half-maximal response level. In both +UE and –UE strips, the DG data points were located to the lower left of the intersecting lines. In addition, the computed CIs were 0.16655 (for +UE, **Figure 2A**) and 0.56981 (for –UE, **Figure 2B**). According to Chou [13], a CI smaller than 1 suggests synergism. Thus, both graphical and mathematical data suggest synergistic effects between D and G in inducing detrusor relaxation.

Suppressed phasic contractile activity under G and DG treatments

Phasic amplitude and frequency were the two parameters of phasic contractions examined.
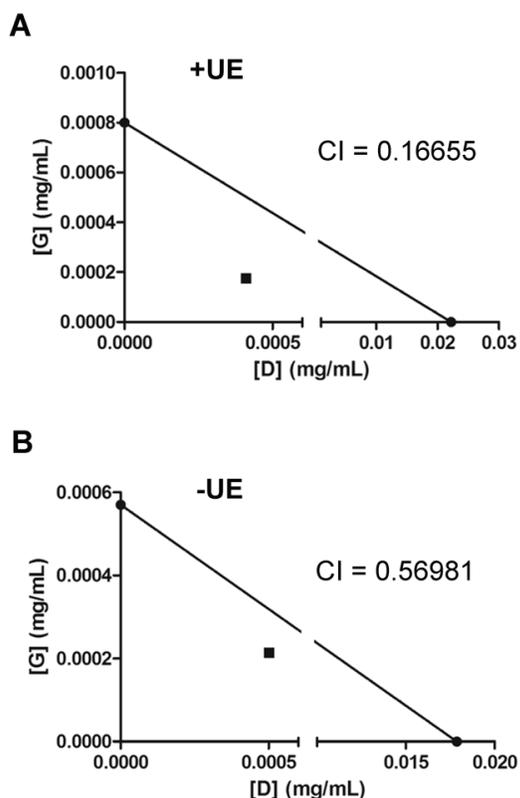
Figure 1. Relaxant effects of danshen and gegen in rat detrusor strips. (A) Raw traces showing detrusor relaxations (in urothelium-intact (+UE) strips) induced by danshen-gegen (7D:3G) formulation, gegen (G) alone and danshen (D) alone. Increasing concentrations of the various herbs were added cumulatively (indicated by arrows, in this order: 0.1, 0.3, 1, 3 mg/mL) after carbachol-induced contraction has reached a steady state. ΔF/F indicates net change in contractile force. Dashed line indicates the control tension level (i.e. before addition of the herbs). (B and C) Concentration-response curves of the herb-induced detrusor relaxations in +UE and urothelium-denuded (-UE) strips. Relaxant responses under both DG and G treatments were significantly larger than D alone. Each data point represents the mean from n= 7 to 8 rats. ***P < 0.001 between DG and D. #P < 0.05 between G and D. ###P < 0.001 between G and D.

There was no notable difference between the phasic activity in +UE (**Figure 3A**) and –UE (**Figure 3B**) detrusor strips, same as in the tonic relaxant responses shown in **Figure 1**. When compared with control, D (n = 8 for both +UE and –UE) failed to alter either phasic amplitude (**Figures 3Aaiii** and **3Baiii**) or frequency (**Figures 3Abiii** and **3Bbiii**). The raw traces in **Figures 3Aciii** and **3Bciii** also showed the absence of any D effects in the phasic contractions. For G and DG, significant changes from control, i.e.

where percent change from control significantly deviated from zero, if any, were detected only at the highest herbal concentration (i.e. 3 mg/mL). The only exception was the smaller phasic amplitude under 1 mg/mL G treatment in –UE strips (**Figure 3Baii**). Nevertheless, at the maximal concentration of 3 mg/mL, G (n = 7 for both +UE and –UE) decreased phasic amplitude (**Figures 3Aaii** and **3Baii**) but not phasic frequency (**Figures 3Abii** and **3Bbii**) in both +UE and –UE strips. The raw traces in **Figures 3Acii**

A

+UE

CI = 0.16655

B

-UE

CI = 0.56981

Figure 2. Synergistic effects between danshen and gegen in relaxing (A) +UE and (B) −UE detrusor strips. At half-maximal relaxation, the DG concentration (filled square) is located to the left of the line intersecting the two axes on the isobologram. The computed combination indices (CIs), both < 1, also indicate danshen-gegen synergy (see Materials and Methods for details on isobolograms and CIs).

and **3Bcii** showed that the presence of G largely reduced phasic amplitudes while preserving small rhythmic fluctuations distinct from the baseline. In contrast, DG suppressed not only phasic amplitude but also frequency, as evident from the lack of distinct phasic activity shown in **Figures 3Aci** and **3Bci**. The summarized data of DG (n = 7 for +UE; n = 8 for −UE) were shown in **Figures 3Aai, 3Abi, 3Bai** and **3Bbi**, where both phasic amplitude and frequency was significantly decreased.

Synergistic effects between D and G in suppressing phasic contraction

Similar to the analysis of synergism between D and G in the relaxant response, the same analysis was done concerning phasic amplitude and frequency in **Figure 4**. **Figure 4A** shows the

isobolograms and CIs determined from the herbal effects on phasic amplitude in +UE (**Figure 4Aa**) and −UE (**Figure 4Ab**) strips. In both cases, the DG data points were located to the lower left of the intersecting lines. The CIs of DG in +UE and −UE strips were, respectively, 0.40360 and 0.69177, both smaller than 1. The same observation was made with the DG data points in the phasic frequency isobolograms (**Figures 4Ba** and **4Bb**), with CIs of 0.01018 (for +UE) and 0.07555 (for −UE). Collectively, D and G acted synergistically in suppressing phasic contractions in the detrusor.

Discussion

Due to the ever increasing incidence of urological disorders including detrusor overactivity (DO) in the aging population [14], much effort has been devoted to the search for medical interventions to better manage various bladder-related problems. In western medicine, the current mainstay treatment is M_3-antimuscarinics [4]. Newer, more bladder-selective antimuscarinics devoid of the typical side effects have been under development. Another promising drug class is β_3-adrenoceptor agonists, where several test compounds are under trial as well [15, 16]. Examples of other less common but nevertheless clinically proven drugs targeting DO include phosphodiesterase inhibitors and Ca^{2+}-channel blockers [4]. Unlike the above examples of western medicines that have been extensively documented in the literature, information on the potential use of traditional Chinese herbal medicines in DO management has been extremely scant. Indeed, the effects of Chinese herbs on other organ systems, such as the central nervous and cardiovascular systems, have been studied by many. This is in stark contrast to the lack of investigations in Chinese herbal effects on isolated bladder contractile function. This study represented a pilot effort to systematically examine the effects of crude Chinese herbal extracts on bladder smooth muscle contractility.

Contractility of the urinary bladder is governed by the detrusor smooth muscle with urothelial inputs [14, 17]. Blood vessel contractility, like other hollow organs such as the bladder, is also under the regulation of vascular smooth muscle. The shared importance of smooth muscle in bladder and vascular functions resulted in the choice of Salviae Miltiorrhizae Radix (Danshen,
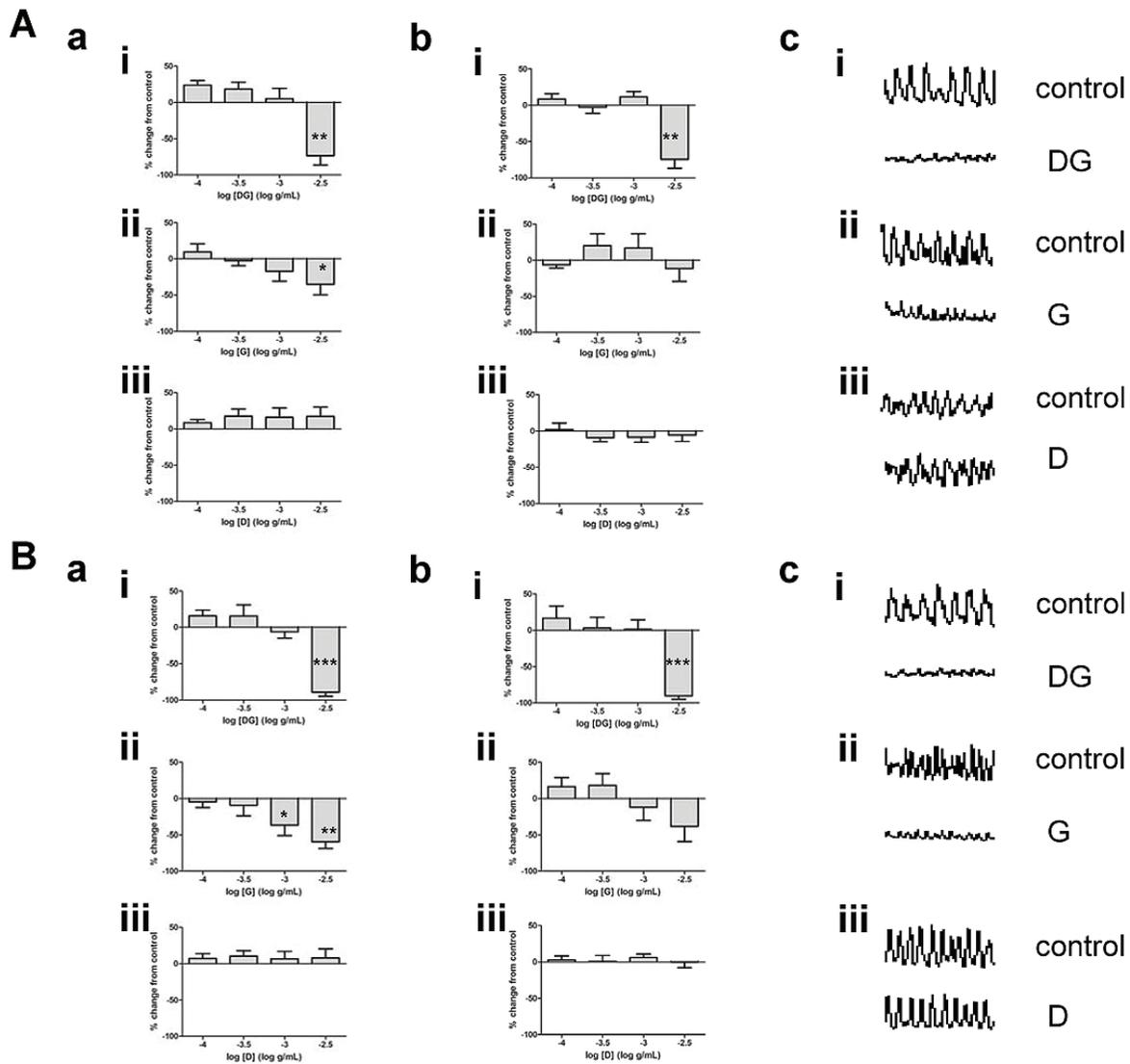
Figure 3. Phasic contractile activity of (A) +UE and (B) –UE detrusor strips under the various herbal treatments. Two parameters, (a) phasic amplitude and (b) phasic frequency, were examined in detrusor strips treated with (i) DG, (ii) G alone and (iii) D alone. The percentage changes of each phasic contractile parameter before and after the herbal treatment were summarized in the bar graphs. In both +UE and –UE strips under maximal DG treatment, both phasic amplitude and frequency were significantly suppressed, i.e. % change from control was significantly smaller than zero. At the equivalent G concentration, only phasic amplitude was significantly smaller than control. Each bar represents the mean from n = 7 to 8 rats. *P < 0.05 vs. zero. **P < 0.01 vs. zero. ***P < 0.001 vs. zero. (C) Sample traces of the phasic contractions seen before (i.e. control) and after the addition of a maximal concentration (i.e. 3 mg/mL) of (i) DG, (ii) G alone and (iii) D alone.

D) and Puerariae Lobatae Radix (Gegen, G) in this study. Both D and G (or their constitutents) have been shown to induce vasorelaxation individually [18, 19]. The DG formulation adopted in this study was modified from literature where Chinese herbal treatment of cardiovascular conditions was described [20]. Thus the potential relaxant and suppressant effects of D and G on detrusor smooth muscle were investigated in this study.

The inhibitory effects of D and G on detrusor contractility were reported here for the first time. Detrusor contractility was inhibited in two

A

a

B

a

b

b
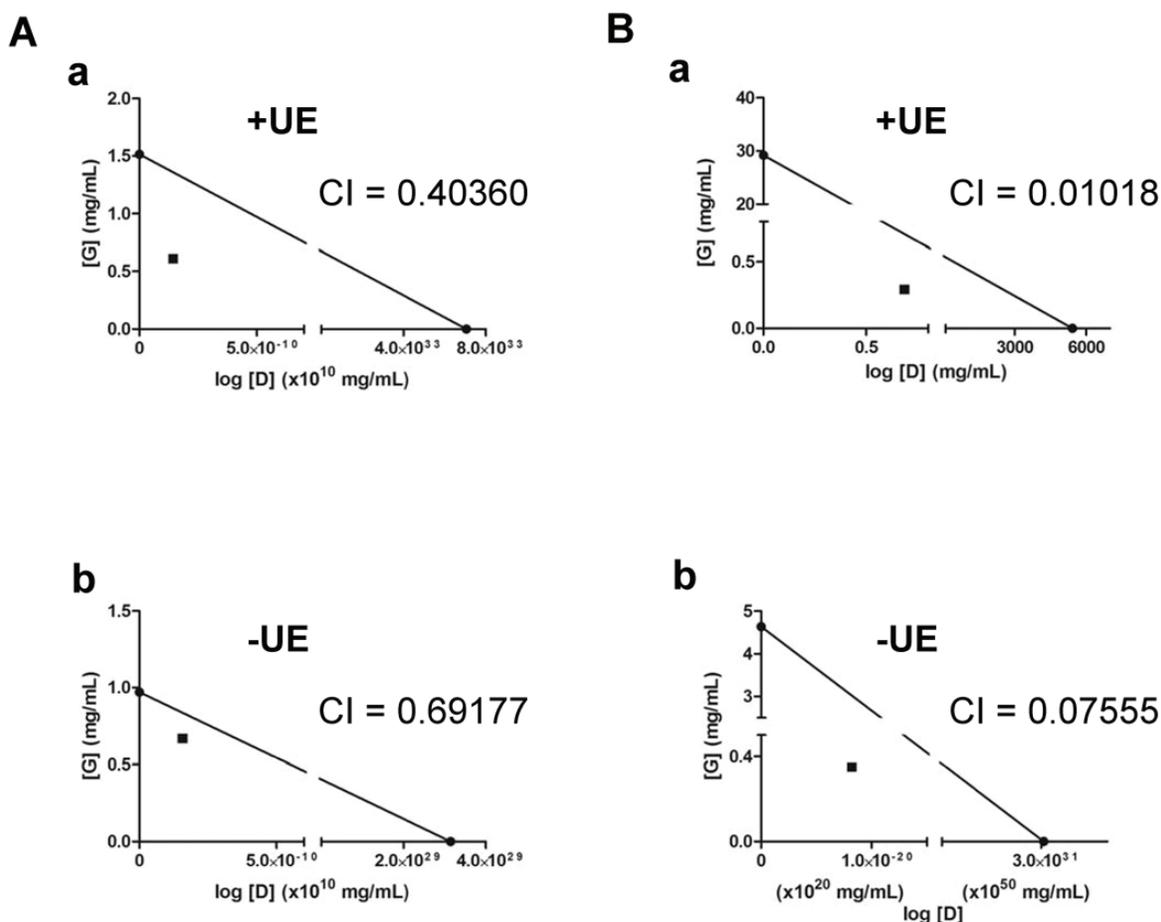
Figure 4. Synergistic effects between danshen and gegen in suppressing (A) phasic amplitude and (B) phasic frequency in (a) +UE and (b) -UE detrusor strips. At half-maximal responses, the DG concentration (filled square) is located to the left of the line intersecting the two axes on the isobologram. The computed combination indices (CI), both < 1, also indicate danshen-gegen synergy.

different aspects, namely tonic and phasic contractions, depending on the herbal preparation. The herbal effects were on the detrusor smooth muscle directly, devoid of urothelial influences. D alone was able to relax the detrusor modestly without any effect on phasic contractile activity. G alone induced full relaxation while suppressing phasic amplitude only. When combined, D and G together induced equi-effective relaxation as G alone, but also inhibited both phasic amplitude and frequency. The isobologram and CI data suggested D and G acted synergistically in eliciting the bladder responses; this interpretation was based on the criteria listed in Chou [13]. The combined use of D and G yielding better responsiveness in the detrusor may strengthen the basis of using herbal formula-

tions instead of single herbs. Furthermore, active ingredients in D and G that possess smooth muscle relaxant properties may be identified using bioassay-guided fractionation of the crude extracts. Similar biological activity-based chemical separation of herbal extracts has been conducted by others [21]. Toward developing DG into clinical applications, the oral availability of D and G, as well as any active by-products in the bladder lumen after hepatic metabolism, may be determined. The pharmacokinetic properties of D have been described in the literature [22]. The same information about G, being the major relaxant component of the DG formulation in detrusor smooth muscle, should also be determined in the future. In the long run, the use of DG, currently in cardiovascular conditions, may

possibly be expanded to manage bladder disorders as well.

In bladder-related disorders, involuntary detrusor contractions are implicated in DO [3, 14]. Excessive phasic contractions may contribute to global detrusor contractions resulting in urine spillage, as observed in patients with overactive bladders [23]. Suppressing the excessive phasic contractile activity may facilitate bladder filling without disturbance. Bladder filling also depends on the ability of the detrusor smooth muscle to relax [1, 14]. The DG formulation in this study demonstrated efficacy in both relaxing the detrusor and in suppressing phasic contractile activity. These DG actions may be extrapolated in an intact organism as improving bladder filling and urine storage. A handful of studies on unrelated herbal medicines reported improvement in bladder function without confirming whether the observed effect was of central or peripheral origin [24, 25]. Nor did the mentioned studies examine tonic and phasic contractions separately, given the importance of both in proper bladder function. In contrast, we previously reported enhanced phasic contractile activity and diminished relaxant ability in Gastrodiae Rhizoma-treated rat bladders, suggesting potential adverse effects of these herbs [26]. In this study, we demonstrated the direct inhibitory actions of DG on the bladder. The findings here may serve as a strong basis for explorations in the long-term treatment effects of DG on bladder function *in vivo*. Moreover, the underlying relaxant and inhibitory mechanisms of DG may be elucidated, with established pharmacological tools such as endogenous enzyme inhibitors, channel modulators and various receptor agonists and antagonists [2, 9, 10, 27-30].

In summary, we showed that both D and G possess inhibitory effects on detrusor smooth muscle contractility. The combined herbal formulation of DG was most efficacious both in relaxing detrusor smooth muscle and suppressing phasic contractile activity. The synergy between D and G effects may translate to the clinical application of these herbs in the management of bladder disorders such as DO.

Acknowledgements

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