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Effects of Gegen (*Puerariae lobatae* Radix) water extract on improving detrusor overactivity in spontaneously hypertensive rats

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ABSTRACT

Aim: *Ex vivo* experiments showed that the water extract of *Puerariae lobatae* Radix (named Gegen in Chinese) induced detrusor relaxation. The aim of this study was to prove the *in vivo* efficacy of Gegen on improving detrusor overactivity and its possible synergism with darifenacin (a first-line muscarinic receptor-3 inhibitor) in spontaneously hypertensive rats (SHR), a rat model exhibiting symptoms of detrusor overactivity.

Method: After daily oral administration of Gegen 30 (Gegen, 30 mg/kg); Gegen 300 (Gegen, 300 mg/kg); Low_Dar (darifenacin, 3 mg/kg); High_Dar (darifenacin, 30 mg/kg) Low_Dar+Gegen 30 or High_Dar+Gegen 30 for 3 weeks, bladder detrusor strips of the rats were isolated and assessed with different stimulators for the measurement of tonic and phasic contractile activities (including phasic amplitude and frequency). Modes of stimulation included the use of carbachol, isoprenaline and electrical field stimulation (EFS).

Results: All drug treatments significantly reduced carbachol-stimulated tonic contractile activities, but did not change the phasic amplitude. Meanwhile, the treatments with Gegen 300; Low_Dar; Low_Dar+Gegen 30; and High_Dar+Gegen 30 decreased carbachol-stimulated phasic frequency. Gegen 300 and Low_Dar+Gegen 30 showed stronger potency on lowering EFS-induced responses. Under isoprenaline-induced relaxation, only Gegen 300 significantly enhanced this relaxation by decreasing tonic contraction; Gegen 300; Low_Dar; Low_Dar+Gegen 30; and High_Dar+Gegen 30 increased the reduction of phasic frequency, but all treatment did not alter their phasic amplitude. Combination Index (CI) showed that the combination with Low_Dar and Gegen 30 had very strong synergism ($CI < 0.1$) on inhibiting EFS-induced contractile response.

Conclusion: Gegen improved detrusor overactivity through neurogenic and anti-muscarinic mechanisms. Gegen and darifenacin together attained synergism for detrusor overactivity treatment via the neurogenic pathway.

Introduction

Overactive bladder, being one of the causes of urge incontinence, is common among the elderly women and men with benign prostatic hypertrophy. The prevalence of overactive bladder symptom was about 16.6% in adults over 40-year of age in six European countries (Milsom et al., 2001), and similar data were reported in the USA with approximately 34 million affected people (Asimakopoulos et al., 2012). This symptom is also prevalent globally as revealed in the recent systematic review (Milsom et al., 2014). Detrusor overactivity with high contractile frequency and urgency manifests as excessive and involuntary detrusor contraction in the bladder. It is hypothesized that detrusor overactivity is caused by neurogenic problem from central nervous system, while it can also be induced by myogenic and autonomic automation derived from the periphery (Drake et al., 2006).

Abbreviations: CI, combination index; EFS, electrical field stimulation; High_Dar, darifenacin, 30 mg/kg; Low_Dar, darifenacin, 3 mg/kg; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

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Since the M_3 subtype of muscarinic receptors is mostly expressed in the bladder, M_3-selective anti-muscarinic agents (e.g. darifenacin) provide the first-line treatment for detrusor overactivity, acting via the competition with acetylcholine at the M_3 receptors in the detrusor smooth muscle to inhibit detrusors overactivity (Andersson et al., 2009). Unfortunately, these M_3-selective anti-muscarinic agents cause significant clinical adverse effects (e.g. constipation and dry mouth) in many patients; When the dosage was doubled to be effective, more than 30% of the patients had suffered from the side effects (FDA, 2004). It is therefore very much justified that new treatment strategies should be developed, which preserve the therapeutic efficacy against detrusor overactivity on one hand and minimize the adverse effects related to the effective dosages on the other.

Chinese medicinal herbs have been traditionally used for thousands of years in Asia. The safety of these herbs are well documented in ancient classics, but information about herbs for the treatment of detrusor overactivity is not available. We have made attempt to identify some Chinese herbs that might be useful for detrusor overactivity, and *Puerariae lobatae* Radix (named Gegen in Chinese) appeared to be a promising candidate (Liang et al., 2012). Gegen is the dried root of *Pueraria lobata* (Willd.) Ohwi, which is traditionally used for promoting circulation, increasing the blood flow, reducing dry mouth, and treating cardiovascular diseases such as hypertension, angina pectoris and type 2 diabetes mellitus (Zhang et al., 2013). In our previous *ex vivo* study, Gegen water extract induced detrusor relaxation in urothelium-independent manner on isolated bladder strips (Liang et al., 2012); Gegen also suppressed phasic amplitude but not phasic frequency. The *ex vivo* efficacy of Gegen on the bladder strips suggested its potential value as an adjunctive treatment for detrusor overactivity. Hence, the aim of this study was to prove the *in vivo* efficacy of Gegen on improving detrusor overactivity. A spontaneously hypertensive rat (SHR) model was used in this study, due to the fact that increased urinary frequency and urodynamic findings of filling-phase contractions have been well documented in the SHR (Steers et al., 1999). Meanwhile, Wistar Kyoto rats (WKY), a healthy animal model, were used as controls for comparison with SHR. However, in our preliminarily study using the current experimental protocol, significant difference between WKY and SHR detrusors from 12-week-old male rats was found, but not in female detrusors (Data not shown). Thus, male SHR model was used in this study. This study also investigated the possible synergistic effects of Gegen and darifenacin in the SHR model.

Methods and materials

Chemicals

Darifenacin hydrobromide, carbachol and other unspecified chemicals were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). The composition of Krebs' solution was as followed: NaCl, 119 mM; KCl, 4.6 mM; $MgCl_2$, 1.2 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 15 mM; $CaCl_2$, 1.5 mM; and D-glucose, 11 mM.

Preparation of herbal extract

The dried root of *Pueraria lobata* (Willd.) Ohwi was purchased from Guangzhou Zhixin Pharmaceutical Co. (Guangzhou, China). It was chemically authenticated by thin layer chromatography (TLC) according to Chinese Pharmacopoeia 2010. Its voucher specimen (no. 2013-3409) was kept in the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong. The raw materials (500 g) of Gegen were extracted by boiling with water (5 L) for an hour and repeated once. After filtered, the supernatant was collected and subjected to freeze-drying for the collection of dried Gegen water extract. The dried extract was stored in the desiccator before use.

HPLC profile

Gegen powder was totally dissolved in water, filtered by 0.22 μm filter, and then subjected to chemical analysis using Agilent 1100 Infinity HPLC system (Santa Clara, CA, USA) which was equipped with an online degasser, a binary-pump, an autosampler and a diode array detector. HPLC analysis was performed with an Alltima HPLC C18 column (250 mm x 4.6 mm, 5 μm) guarded by a guard column with the same stationary phase. The column was maintained at room temperature, and the flow rate was set at 1 ml/min. The mobile phase consisted of (A) 0.1% acetate acid and (B) acetonitrile with the following gradient: 10–12.5% B from 0 min to 20 min; 12.5–15% B from 20 to 30 min; 15–60% B from 30 min to 40 min; and 60–90 % B from 40 min to 45 min. The UV absorbance was detected at 254 nm, and injection volume was 10 μl.

Animals

Male spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats (9-week old) were provided by the Laboratory Animal Services Centre, The Chinese University of Hong Kong (CUHK). Animals were kept with free access to food and water under a 12:12-h light-dark cycle. All animal experiments have been approved by the CUHK Animal Experimentation Ethic Committee (AEEC No. 12/046/MIS-4) according to the guidance set by the Department of Health, Hong Kong SAR.

Drug treatment for animals

According to the recommended dosage (10–15 g) of raw herb in the Chinese Pharmacopoeia 2010 and the yield (~47%) of Gegen water extract prepared in this study, the minimum human equivalent dose (HED) of Gegen water extract was about 4.7 g. As calculated by the body surface area normalization method with its HED and the translational factor (6.16) between human and rat, the rat dose of Gegen to HED was about 483 mg/kg (=HED/60 kg x 6.16). Then, a lower dose at 300 mg/kg was selected based on this calculation. For the study of detrusor overactivity in experimental SHR rats, Gegen 300 (Gegen, 300 mg/kg) and Gegen 30 (Gegen, 30 mg/kg) were given orally by gavage. According to pharmaceutical recommendations and basing on the acute treatment results described in the previous study, Low_Dar (darifenacin, 3 mg/kg) and High_Dar (darifenacin, 30 mg/kg), as the positive control, were administrated intragastrically to SHR rats, respectively (Patra and Thorneloe, 2011). To study the combination effect, Low_Dar+Gegen 30 and High_Dar+Gegen 30 were used. All rats received different treatments continuously for 3 weeks. SHR served as the disease model, while WKY rats served as the normal control. Gegen and darifenacin were dissolved in water, and control rats received water alone.

Ex vivo *experiments*

After 3-week treatments, rats were euthanized by CO_2 asphyxiation. The whole bladder was isolated and immediately put into carbogen-bubbled ice-cold Krebs' solution. The bladder base, one–third of the bladder, was discarded. Only tissues isolated from the bladder dome (detrusor) were used. Detrusor strips were prepared along the transverse and longitudinal orientations. Detrusor strips were mounted on a myograph system (Danish Myo Technology Model 800MS, Denmark) with carbogen-aerated Krebs' solution at 37 °C. Isometric tension was recorded through the PowerLab data acquisition system with the LabChart software (ADInstruments