

**Effects of goshajinkigan (Niu-Che-Sen-Qi-Wan) for
resiniferatoxin-sensitive afferents on detrusor overactivity induced by
acetic acid in conscious rats**

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Running title: Effects of Niu-Che-Sen-Qi-Wan for detrusor overactivity in rats

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ABSTRACT: This study was performed to investigate the effects of goshajinkigan, a traditional Chinese herbal mixture, in conscious rats undergoing continuous cystometry. Systemic resiniferatoxin (RTX) pretreatment can block resiniferatoxin-sensitive (C-fiber) nerve-mediated bladder overactivity, such as that induced by intravesical administration of acetic acid. The effects of pretreatment with goshajinkigan and resiniferatoxin (RTX) alone or in combination on acetic acid-induced bladder overactivity in conscious rats were also compared. Female SD rats were divided into 4 groups. Group (1), (3) received normal food for 4 weeks, while groups (2) and (4) received goshajinkigan (0.09 g/kg/day) during the same period. Two days after bladder catheterization, groups (3) and (4) received RTX (0.3 mg/kg) injection, while groups (1) and (2) received vehicle alone. Cystometric investigations were performed on all animals 24 h after RTX or vehicle injection. The effects of intravesical instillation of acetic acid (pH=4.0) were compared with those of intravesical saline. Goshajinkigan significantly increased threshold pressure, voiding interval, micturition volume, and bladder capacity. Intravesical instillation of acetic acid induced bladder overactivity in both normal rats and in those pretreated with goshajinkigan. However, the effects of acetic acid on voiding interval and micturition volume were significantly different between rats given normal diet and those pretreated with goshajinkigan. The effect of acetic acid was not different between goshajinkigan- and RTX-pretreated rats. The results of the present study indicated that goshajinkigan increases voiding interval, micturition volume, and bladder capacity, and pretreatment partially blocks the bladder overactivity induced by intravesical administration of acetic acid in rats.

Keywords: goshajinkigan, Niu-Che-Sen-Qi-Wan, resiniferatoxin, overactive bladder, *Rheumanna glutinosa*, *Achyranthes Root*, *Cornus officinalis*

INTRODUCTION

The urinary bladder is rich in afferent nerve fibers, which can be divided into two types: myelinated A δ fibers and unmyelinated C-fibers. C-fibers can be activated by chemical irritants, such as acetic acid and heat, but they are currently considered to play no role in normal micturition (Andersson, 2003). However, in some neurological diseases, such as multiple sclerosis or spinal cord lesions, the C-fibers may become sensitive to mechanical stimulation and take part in voiding reflexes. Clinically, capsaicin and resiniferatoxin (RTX), which desensitize C-fiber afferents *via* vanilloid receptor-1 (VR1) (Caterina *et al.*, 1997), have been reported to be effective in the treatment of bladder overactivity induced by different neurological diseases (Craft *et al.*, 1995; Komiyama *et al.*, 1999).

Most drugs in current use for the treatment of incontinence are believed to act peripherally; they can be classified as drugs that affect sensory nerves and those that mainly reduce detrusor contractility. Antimuscarinic agents that reduce detrusor contractility are still the first line of therapy. However, side effects, such as dry mouth, remain the main reasons for poor patient compliance in clinical practice (Chapple *et al.*, 2002). Goshajinkigan is a traditional Chinese herbal mixture that contains ten ingredients (Table 1), and has long been used in China and Japan to alleviate the subjective symptoms of diabetic neuropathy (Tawata *et al.*, 1994). Due to this clinical effect, we felt that this drug might be useful in improving bladder overactivity.

In the present study, we investigated the effects of goshajinkigan, a traditional Chinese herbal mixture, in conscious rats undergoing continuous cystometry. Systemic RTX pretreatment can block the bladder overactivity mediated by RTX-sensitive (C-fiber) nerves, such as that induced by intravesical administration of acetic acid. We also compared the effects of pretreatment with goshajinkigan and RTX, alone or in combination, on acetic acid-induced bladder overactivity in conscious rats.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats from 8 to 12 weeks old were used in this study, which was conducted in accordance with the guidelines approved by the Animal Ethics Committee, Shinshu University School of Medicine. All the rats were group-housed in cages for at least one week before the experiment and were fed laboratory chow and water *ad libitum*. The temperature of the animal house was maintained at $23\pm 1^{\circ}\text{C}$ with a 12-h light/dark cycle (lights on at 09:00).

Goshajinkigan pretreatment

Four weeks before bladder catheter implantation, the animals were assigned at random to 4 groups, each of which consisted of 6 animals. Groups (1) and (3) received normal food, while groups (2) and (4) received special food containing goshajinkigan (0.09 g/kg/day) for 4 weeks. The herbal extract of goshajinkigan was kindly provided by Tsumura Co. (Tokyo, Japan). The extract was composed of 10 medicinal plants, as shown in Table 1, extracted with hot water, filtered, and lyophilized. The dosage used in the experiments was the extracted weight. A dose of 7.5 g/day is used in humans. The animal food in the present study contained 1.08% extract of goshajinkigan, representing a dose approximately 10-fold higher than that used in humans based on previous reports (Tawata *et al.*, 1994; Hu *et al.*, 2003). The animals took about 15 g/day of this special diet.

Bladder catheter implantation

Rats were anesthetized by i.m. injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). The bladder was exposed through an abdominal midline incision and a small incision was made at the bladder dome. A polyethylene catheter (PE50; Nippon Becton Dickinson, Tokyo, Japan) was implanted into the bladder as described previously (Malmgren *et al.*, 1987). The free end of the catheter was tunneled subcutaneously and exteriorized at the back of the neck. All the rats were maintained individually in cages under a 12-h alternating light-dark cycle, with food and water available *ad libitum*.

RTX pretreatment

Two days after bladder catheter implantation. Animals in groups (3) and (4) received

RTX (0.3 mg/kg, 0.75 mg/ml, s.c.) by subcutaneous injection, while those in groups (1) and (2) received vehicle alone.

Cystometric investigation

Cystometric investigation was performed without anesthesia 3 days after bladder catheter implantation. The bladder was connected *via* a T-tube to a pressure transducer (P23 DC; Statham, Oxnard, CA, USA) and a microinjection pump (Model 200; Muromachi-Kikai, Tokyo, Japan). The conscious rats were placed without any restraint in a metabolic cage (Nalgen, 650-0100, Nalge Nunc, Rochester, NY), which also enabled measurement of micturition volume by means of a fluid collector connected to a force displacement transducer (Type 45196; NEC San-ei Instruments, Tokyo, Japan). Saline maintained at room temperature was instilled into the bladder at a rate of 10 ml/h. Intravesical pressure and micturition volumes were recorded continuously on a pen oscillograph (Recti-Horiz-8K; NEC San-ei Instruments; recording speed, 10 mm/min). Three reproducible micturition cycles were recorded and used as baseline values. When the baseline cystometric investigations were finished, acetic acid solution (pH 4.0) was instilled intravesically by changing the syringe of the infusion pump, and micturition cycles were recorded continuously for a further 60 minutes. The drugs actually reached the bladder approximately 8 min after the syringe change because of dead space in the connecting tube between the syringe and the bladder. Following acetic acid administration, two micturition cycles showing the most pronounced changes were analyzed. The following cystometric parameters were investigated: basal pressure, threshold pressure (bladder pressure immediately prior to micturition), micturition pressure, voiding interval, micturition volume, residual volume, and bladder capacity (Malmgren *et al.*, 1987). Six animals were used for intravesical administration of each drug.

Administration of drugs

Resiniferatoxin (Sigma, St. Louis, MO) was dissolved in absolute ethanol as a 1 mM stock solution and stored at -70°C . The stock solution was diluted to appropriate final concentrations in saline just before use. Acetic acid (Wako, Osaka, Japan) was diluted with saline and pH was adjusted to 4.0 (about 200 μl of 99% acetic acid per 1000 ml saline) using a pH/ion meter (F23, Horiba, Kyoto, Japan).

Statistical analysis

Results are given as means \pm SD. Student's two-tailed *t*-test was used for comparison of baseline values between the control group, goshajinkigan-pretreated group, and RTX-pretreated group, and was also used for comparisons of paired samples before and after acetic acid administration. One-way factorial ANOVA followed by Scheffe's F-test was used for comparisons of body weight and drug effects between different animal groups, with $p < 0.05$ accepted as significant.

RESULTS

Body weight of the rats

The rats weighed (1) 227 ± 7.5 g, (2) 231 ± 6.8 g, (3) 230 ± 5.8 g, and (4) 225 ± 6.3 g just before cystometric investigation. There were no significant differences in body weight among the groups.

Effects of goshajinkigan pretreatment

Four weeks of goshajinkigan pretreatment induced significant increases in threshold pressure ($p<0.05$), voiding interval ($p<0.05$), micturition volume ($p<0.05$), and bladder capacity ($p<0.05$, Table 2).

Effects of RTX pretreatment

Systemic RTX pretreatment induced a significant decrease in basal pressure, and significant increases in threshold pressure, voiding interval, micturition volume, and bladder capacity ($p<0.001$, Table 2).

Effects of intravesical acetic acid in vehicle-pretreated rats

Intravesical instillation of acetic acid (pH 4.0) induced statistically significant increases in basal pressure ($p<0.05$) and micturition pressure ($p<0.05$), and significant decreases in voiding interval ($p<0.001$), micturition volume ($p<0.001$), and bladder capacity ($p<0.01$) (Fig. 1, Table 2).

Effects of intravesical acetic acid in goshajinkigan-pretreated rats

In rats pretreated with goshajinkigan, intravesical instillation of acetic acid (pH 4.0) induced a statistically significant increase in basal pressure ($p<0.05$), and significant decreases in threshold pressure ($p<0.05$), voiding interval ($p<0.05$), micturition volume ($p<0.05$), and bladder capacity ($p<0.05$). There were significant differences in voiding interval ($p<0.05$) and micturition volume ($p<0.05$) between the normal food group and goshajinkigan-pretreated group (Fig. 2, Table 2).

Effects of intravesical acetic acid in RTX-pretreated rats

In rats pretreated with RTX (0.3 mg/kg), intravesical instillation of acetic acid (pH 4.0) induced significant decreases in threshold pressure ($p<0.05$). There were significant differences in basal pressure ($p<0.05$), micturition pressure ($p<0.05$), voiding interval ($p<0.01$), micturition volume ($p<0.01$), and bladder capacity ($p<0.05$) between the vehicle-pretreated group and RTX-pretreated group (Table 2).

Effects of intravesical acetic acid in both goshajinkigan- and RTX-pretreated rats

In rats pretreated with both goshajinkigan and RTX, intravesical instillation of acetic acid (pH 4.0) did not induce any statistically significant changes in the voiding parameters (Table 2).

DISCUSSION

In the present study, injection of RTX (0.3 mg/kg, s.c.) 24 h before cystometric investigation induced significant increases in threshold pressure, voiding interval, micturition volume, and bladder capacity as compared with the same parameters in the vehicle-pretreated controls. In humans, RTX-sensitive nerves are considered to play no role in micturition under normal conditions (Andersson, 2002). However, in rats, this afferent nerve may play a role in the modulation of micturition (Zhang *et al.*, 2003).

Goshajinkigan has long been used in China and Japan, mainly to relieve the subjective symptoms of diabetic neuropathy (Tawata *et al.*, 1994). Due to this clinical effect, we felt that this drug might be useful to improve bladder overactivity with fewer of the side effects, such as dry mouth and constipation, associated with conventional therapeutic agents. However, the possible mechanisms of the effectiveness of this drug for overactive bladder were not confirmed sufficiently from the viewpoint of both basic and clinical studies. The present study using continuous cystometry in conscious animals indicated that 4-week administration of this drug significantly increased micturition volume, bladder capacity, and micturition interval. As the extract was administered orally, it was not easy to determine the site of action of this drug in the micturition cycle of the rats. To clarify one of the mechanisms of action, we focused on the RTX-sensitive neurons, which are considered to induce bladder overactivity (Craft *et al.*, 1995; Komiyama *et al.*, 1999). However, further studies are needed to clarify the other mechanisms. In the present study, acetic acid was shown to induce bladder overactivity, but this overactivity did not appear in RTX-pretreated animals. This observation implied that this overactivity was mediated by RTX-sensitive afferent neurons in the bladder. Acetic acid caused bladder overactivity in both control and goshajinkigan-pretreated rats. However, there were significant differences in voiding interval and micturition volume between these two groups. Our results suggested that goshajinkigan pretreatment has a blocking effect on intravesical acetic acid-induced bladder overactivity, possibly *via* inhibition of bladder RTX-sensitive afferent nerves, but the effect was relatively mild as compared with that of RTX pretreatment.

The present study showed that goshajinkigan increased voiding interval,

micturition volume, and bladder capacity, and that pretreatment partly blocked the bladder overactivity induced by intravesical acetic acid *via* RTX-sensitive afferent neurons.

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LEGENDS TO TABLES AND FIGURES

Table 1

Composition of Goshajinkigan.

Samples of 4.5 g of extract of goshajinkigan were composed of these plants.

Table 2

Effects of intravesical administration of acetic acid (pH 4.0) on cystometric parameters in vehicle-, RTX-, and goshajinkigan-pretreated rats.

Figure 1

Effects of intravesical instillation of acetic acid (pH 4.0) in a normal rat. BP, bladder pressure; MV, micturition volume; the asterisk (*) indicates adjustment to baseline position.

Figure 2

Effects of intravesical instillation of acetic acid (pH 4.0) in a goshajinkigan-pretreated rat. BP, bladder pressure; MV, micturition volume; the asterisk (*) indicates adjustment to baseline position.

Fig. 1 Effects of intravesical instillation of acetic acid (pH=4.0) in a normal rat.
BP=bladder pressure; MV=micturition volume; asterisk () indicates adjustment to baseline position.

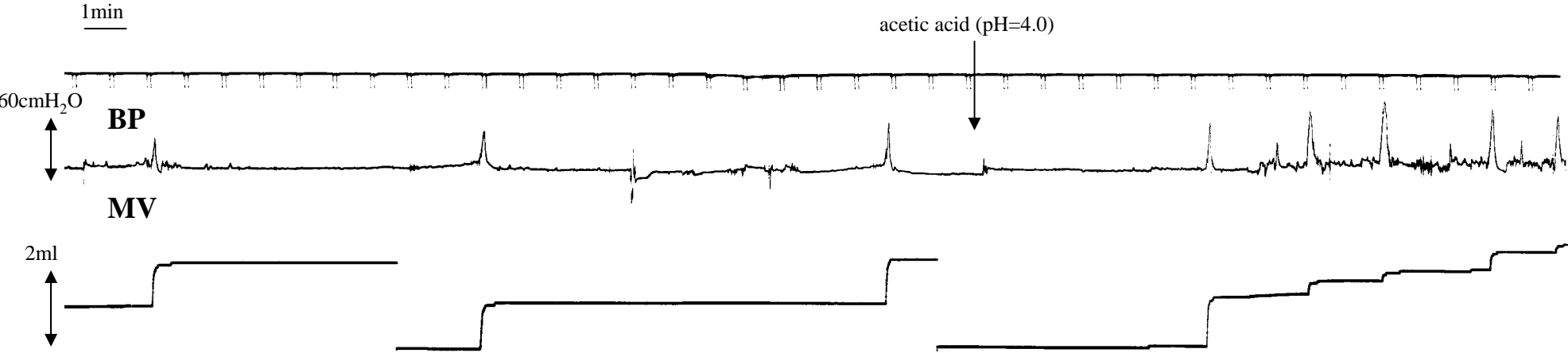


Fig. 2 Effects of intravesical instillation of acetic acid (pH=4.0) in a goshajinkigan-pretreated rat.
BP=bladder pressure; MV=micturition volume; asterisk () indicates adjustment to baseline position.

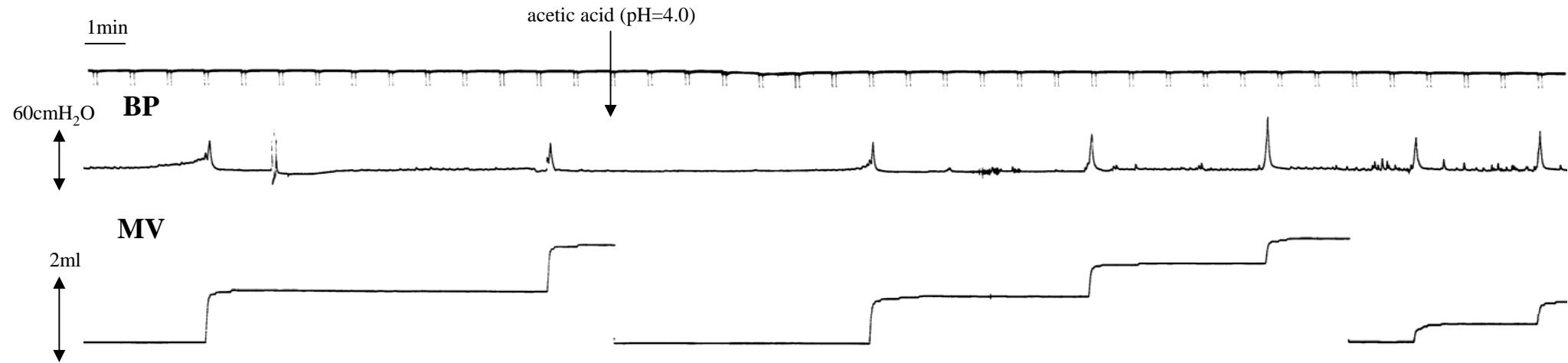


Table 1 Composition of Goshajinkigan.

Samples of 4.5 g of extract of goshajinkigan were composed of these plants.

Plant name	(g)
<i>Rehmannia glutinosa</i>	5
<i>Achyranthes Root</i>	3
<i>Cornus officinalis</i>	3
<i>Dioscorea Rhizome</i>	3
<i>Plantago seed</i>	3
<i>Alisma orientale</i>	3
<i>Porica cocos</i>	3
<i>Moutan Cortex</i>	3
<i>Cinnamomum Cassia</i>	1
<i>Aconite Tuber</i>	1

Table 2 Effects of intravesical administration of acetic acid (pH 4.0) on cystometric parameters in vehicle-, RTX-, and goshajinkigan-pretreated rats.

		BP (cmH ₂ O)	TP (cmH ₂ O)	MP (cmH ₂ O)	VI (min)	MV (ml)	RV (ml)	BC
vehicle-pretreated								
normal food (n=6)	before	8.4±2.0	16.0±3.4	49.1±8.5	6.48±1.08	1.07±0.18	0.03±0.02	1.1
	after	11.2±1.0*	14.0±1.6	67.6±7.8*	2.66±0.89***	0.43±0.15***	0.04±0.02	0.4
goshajinkigan (n=6)	before	8.4±1.9	19.6±1.6	51.4±10.9	8.14±1.14	1.34±0.19	0.04±0.01	1.3
	after	9.6±2.1*	17.3±1.9*	60.1±9.6	6.11±1.04* ⁺	0.96±0.19* ⁺	0.05±0.03	1.0
RTX-pretreated								
normal food (n=6)	before	5.9±1.8	28.0±3.7	49.6±13.0	11.88±1.76	1.94±0.29	0.08±0.06	2.0
	after	5.8±0.9 [#]	25.3±3.0*	50.0±12.2 [#]	10.91±1.47 ^{##}	1.78±0.24 ^{##}	0.08±0.02	1.8
goshajinkigan (n=6)	before	5.5±1.4	22.7±3.0	57.3±11.1	11.31±2.50	1.78±0.46	0.09±0.02	1.8
	after	6.2±2.0	20.8±3.3	57.2±10.8	10.76±1.49	1.71±0.30	0.10±0.03	1.8

BP basal pressure, TP threshold pressure, MP micturition pressure, VI voiding interval, MV micturition volume, RV residual volume

*p<0.05, **p<0.01, ***p<0.001 (effect of acetic acid in each group, Student's paired two-tailed t-test)

+p<0.05, +p<0.01, ++p<0.001 (comparison of acetic acid effect between groups: control vs goshajinkigan, ANOVA followed by Scheffe's F-test)

[#]p<0.05, ^{##}p<0.01 (comparison of acetic acid effect between groups: control vs RTX, ANOVA followed by Scheffe's F-test)