

*Full Paper***Beneficial Effects of Gosha-jinki-gan and Green Tea Extract in Rats With Chemical Cystitis**Sweety Nasrin<sup>1</sup>, Ayaka Osano<sup>1</sup>, Yoshihiko Ito<sup>1</sup>, and Shizuo Yamada<sup>1,\*</sup><sup>1</sup>Department of Pharmacokinetics and Pharmacodynamics, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

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**Abstract.** The aim of this study was to characterize pharmacological effects of gosha-jinki-gan (GJG) and green tea extract (GTE), on urodynamic parameters, bladder receptors, and urinary cytokines in rats with cyclophosphamide (CYP)-induced cystitis. Urodynamic parameters in CYP-treated rats were measured using the cystometric method. Muscarinic and purinergic receptors in rat tissues were measured by radioreceptor assays. Urinary cytokine levels were measured with ELISA kits. GJG and GTE were orally administered to rats once a day for 7 days. The GJG treatment significantly ameliorated changes in urodynamic parameters in CYP-treated rats. Similar treatment with GTE slightly attenuated changes in urodynamic parameters. The maximal number of binding sites for [<sup>3</sup>H]NMS and [<sup>3</sup>H] $\alpha\beta$ -MeATP in the bladder was significantly lower in CYP-treated rats than in sham rats. Such a reduction in receptor density was significantly attenuated by the GJG treatment. GTE treatment also significantly attenuated the down-regulation of muscarinic receptors, but not P2X receptors in bladders of rats with CYP-induced cystitis. The elevation in urinary cytokine levels in CYP-treated rats was effectively attenuated by GJG treatment. The elevation in cytokine levels in CYP-treated rats was alleviated by GTE treatment. In conclusion, GJG may be a pharmacologically useful plant extract for cystitis.

**Keywords:** plant extract, muscarinic and purinergic receptor, cyclophosphamide, cystitis, urinary cytokine

**Introduction**

Interstitial cystitis (IC) is a chronic inflammatory disease of the bladder. Major characteristics are urinary frequency, urgency, and suprapubic pain. Patients may also exhibit nocturia, tension, pain during sexual intercourse, discomfort, and difficulty in driving, travelling, or working. The antineoplastic agent, cyclophosphamide (CYP), is widely used as a therapy for various diseases (malignancy, bone marrow transplantation, and multiple sclerosis); one of the most common and severe side effects of CYP therapy is hemorrhagic cystitis (1). Therefore, CYP can induce hemorrhagic cystitis, urgency, increased frequency, and pain through its toxic metabolite acrolein (2, 3). Accordingly, an intraperitoneal

injection of CYP was shown to induce reproducible dose-dependent chemical cystitis in rats; therefore, it has been used as an experimental model of hemorrhagic cystitis (2).

Inflammation is mediated by various soluble factors, including a group of secreted polypeptides known as cytokines. Previous studies have implied that urinary cytokines or chemokines are involved in CYP-induced cystitis (4) and in patients with cystitis (5). We recently showed that treatment with CYP or hydrochloric acid caused a decrease in the number of muscarinic and purinergic receptors in the rat bladder (6, 7). Therefore, urinary cytokines and pharmacologically relevant bladder receptors may serve as direct therapeutic targets or potential biomarkers for the development of targeted therapy designed to prevent chronic bladder inflammatory conditions such as IC.

Many reports have described the efficacy and safety of phytotherapeutic natural products for urinary dysfunc-

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tion. However, the use of these agents in the treatment of lower urinary tract symptoms due to IC has been limited by a lack of scientific knowledge underlying the possible mechanism of pharmacological action. Gosha-jinki-gan (GJG) is a traditional Japanese medicine used for urinary disorders, and is composed of 10 crude drugs in fixed proportions: Rehmanniae radix (5.0 g), Achyranthis radix (3.0 g), Corti fructus (3.0 g), Moutan cortex (3.0 g), Alismatis rhizome (3.0 g), Dioscoreae rhizome (3.0 g), Plantaginis semen (3.0 g), Hoelen (3.0 g), processed Aconiti tuber (1.0 g), and Cinnamomi cortex (1.0 g). GJG has been shown to decrease the frequency of urination in patients with urinary disturbance (8). Although the mechanism of pharmacological action of GJG is not yet clear, its vasodilatory effects (9), antioxidant effects (10), antinociceptive effects (11), and aldose-reductase inhibitory effects (12) have been demonstrated. In addition, GJG was shown to inhibit bladder contraction induced by electrical stimulation of the pelvic nerve or the infusion of acetylcholine (13). These results suggest that GJG has anti-cholinergic activity similar to that of atropine. An increase in bladder capacity related to the above action plays an important role in the treatment of urinary frequency and may be effective in treating bladder cystitis. The medicinal value of green tea is well known and reportedly contains the highest concentration of powerful antioxidants called polyphenols, also known as green tea catechins (14). The catechins of green tea are (–)-epicatechin (EC), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epigallocatechin-3-gallate (EGCG). Over the last few decades, green tea has been subjected to many scientific and medical studies to determine the extent of its long-purported health benefits, with some evidence suggesting that green tea polyphenolic compounds present in the green tea extract (GTE) have antioxidant, anticarcinogenic, antiinflammatory, and antimicrobial properties in human, animal, and in vitro studies (14–16). The polyphenols in green tea can neutralize free radicals and may reduce or even help to prevent some of the damage caused by reactive oxygen species (ROS) (17). The aim of the present study was to examine the pharmacological usefulness of two different types of plant extracts, GJG and GTE, as the phytotherapeutic agents of IC by characterizing their effects on urodynamic parameters, bladder muscarinic and purinergic receptors, and urinary cytokines in rats with CYP-induced cystitis.

## Materials and Methods

### Materials

[N-Methyl- $^3\text{H}$ ] scopolamine chloride [ $^3\text{H}$ ]NMS, 3.03 TBq/mmol) and  $\alpha\beta$ -methylene-ATP [2-8- $^3\text{H}$ ]tetrasodium

salt [ $^3\text{H}$ ] $\alpha\beta$ -MeATP, 555.0 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). Gosha-ginki-gan (GJG) was donated by Tsumura (Saitama). As green tea extract (GTE), Sunphenon BG3<sup>TM</sup> was provided by Taiyo Kagaku (Yokkaichi), and contained EC, ECG, EGC, and EGCG of 9.7%, 1.7%, 24.8%, and 43.4%, respectively, with a total catechin amount of 86.5% (w/w). ELISA kits for the measurement of IL-1 $\beta$ , IL-6, and IL-17 were purchased from Signosis, Inc. (Sunnyvale, CA, USA) and USCN Life Science, Inc. (Wuhan, China). All other chemicals were purchased from commercial sources.

### Animals

Female Sprague-Dawley (SD) rats (aged 9-week-old) were purchased from Japan SLC, Inc. (Shizuoka). They were housed in the laboratory with free access to food and water and maintained on a 12-h dark/light cycle in a room with controlled temperature ( $23^\circ\text{C} \pm 1^\circ\text{C}$ ) and humidity ( $55\% \pm 5\%$ ). The animals were divided into a sham group, a CYP-treated group, and a CYP + GJG- or CYP + GTE-treated group. Animal care and experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of University of Shizuoka.

### Induction of chemical cystitis and administration of plant extracts

Rats received an injection of CYP (150 mg/kg, i.p.) for acute cystitis as previously described (4, 18), and were used for the experiment three days later. Sham rats received a volume-matched injection of saline (5 mL/kg, i.p.). CYP + GJG- and CYP + GTE-treated animals were orally administered GJG ( $1000 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) and GTE ( $400 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) prepared in 0.5% methylcellulose and distilled water, respectively, for three days before and after the CYP treatment (seven consecutive days). Vehicle was administered orally to sham and CYP-treated rats. Cystometry and measurements of receptor binding and urinary cytokines were performed seven days after the first administration of GJG or GTE.

### Cystometry

Rats were anesthetized by intraperitoneal (0.8 g/kg) and subcutaneous (0.4 g/kg) injections of urethane. The bladder was exposed through a short midline incision. Polyethylene tubing (SP-45; Natsume, Tokyo) was inserted into the dome of the bladder and ligated. The bladder catheter was connected via a T connector to a pressure transducer and an infusion pump. Then, 0.9% saline maintained at  $37^\circ\text{C}$  was instilled into the bladder at a rate of 3.0 mL/h. Intravesical pressure was recorded continuously. After stabilization for about 30 min, the

following urodynamic parameters were recorded for 30 min in each animal: micturition interval, mean micturition volume, frequency of micturition, maximum micturition pressure, basal bladder pressure, and threshold pressure. Voided urine was cumulatively collected into a urine cup placed on a microbalance. Furthermore, at the end of cystometric recording, saline infusion was stopped after confirming the first micturition, and residual urine was collected by a syringe.

#### *Tissue preparation and radioreceptor binding assay*

Rats were exsanguinated from the descending aorta under anesthesia with pentobarbital (50 mg/kg, i.p.). The bladder and submaxillary gland were dissected, washed with cold saline, and minced with scissors. For the [ $^3\text{H}$ ]NMS binding assay, the tissues were homogenized with a Kinematica Polytron homogenizer in 19 volumes of ice-cold 30 mM  $\text{Na}^+$ /HEPES buffer (pH 7.5). The homogenate was centrifuged at  $40,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The resulting pellet was finally resuspended in the same buffer for the binding assay. The bladder was homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) for the [ $^3\text{H}$ ] $\alpha\beta$ -MeATP binding assay. The homogenate was centrifuged at  $2,000 \times g$  for 10 min, and the supernatant was reserved. The resulting pellet was resuspended in 10 volumes of the same buffer and centrifuged again under the same conditions. The second supernatant was added to the supernatant from the first centrifugation. The supernatant was incubated at  $37^\circ\text{C}$  for 20 min and recentrifuged at  $40,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The resulting pellet was finally resuspended in the buffer for the binding assay. All steps were performed at  $4^\circ\text{C}$ .

The radioligand-binding assay for muscarinic and P2X receptors was performed using [ $^3\text{H}$ ]NMS and [ $^3\text{H}$ ] $\alpha\beta$ -MeATP as described previously (6, 7). In the [ $^3\text{H}$ ]NMS binding assay, tissue homogenate (250  $\mu\text{g}$  protein/assay) was incubated with different concentrations (0.06 – 1.0 nM) of [ $^3\text{H}$ ]NMS in 30 mM  $\text{Na}^+$ /HEPES buffer (pH 7.5). Incubation was performed for 60 min at  $25^\circ\text{C}$ . In the [ $^3\text{H}$ ] $\alpha\beta$ -MeATP-binding assay, the homogenate (30  $\mu\text{g}$  protein/assay) of the bladder was incubated with different concentrations (0.3 – 10 nM) of [ $^3\text{H}$ ] $\alpha\beta$ -MeATP in 50 mM Tris-HCl buffer (pH 7.4) at  $4^\circ\text{C}$  for 60 min. The reaction was terminated by rapid filtration (Cell Harvester; Brandel Co., Gaithersburg, MD, USA) through Whatman GF/B glass filters, and the filters were then rinsed 3 times with 3 mL of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid, and radioactivity was measured with a liquid scintillation counter. The specific binding of [ $^3\text{H}$ ]NMS and [ $^3\text{H}$ ] $\alpha\beta$ -MeATP was determined experimentally from the difference between counts in

the absence and presence of 1  $\mu\text{M}$  atropine and 3  $\mu\text{M}$   $\alpha\beta$ -MeATP, respectively. Protein concentrations were measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) using bovine serum albumin as a standard.

#### *Determination of cytokines in the urine of rats*

Six hours after the last administration of GJG and GTE, urine was collected from rats kept in metabolic cages. The collected urine was stored at  $-30^\circ\text{C}$  before analysis. Cytokines in the urine were determined by ELISA with rat IL-1 $\beta$ , IL-6, and IL-17 kits according to the manufacturer's instructions. Cytokine concentrations were normalized to the creatinine concentration in urine and were expressed as the amount of cytokine in picograms excreted per milligram of creatinine. Creatinine in the urine was measured by Lab Assay Creatinine (Wako, Osaka).

#### *Data analyses*

The binding data for [ $^3\text{H}$ ]NMS and [ $^3\text{H}$ ] $\alpha\beta$ -MeATP were subjected to a nonlinear regression analysis using Graphpad Prism 5 (Graphpad Software, Inc., San Diego, CA, USA). The apparent dissociation constant ( $K_d$ ) and maximal number of binding sites ( $B_{\text{max}}$ ) for [ $^3\text{H}$ ]NMS and [ $^3\text{H}$ ] $\alpha\beta$ -MeATP were estimated. Statistical analyses of the data from radioreceptor binding assays, cystometry, and cytokine measurements were performed with the Student's *t*-test and one-way analysis of variance, followed by the Bonferroni test for multiple comparisons. All data are expressed as the mean  $\pm$  S.E.M. Significance was accepted at  $P < 0.05$ .

## **Results**

#### *Effects on urodynamic parameters*

Representative traces of continuous cystometrograms in urethane-anesthetized rats are shown in Fig. 1. The micturition interval and mean micturition volume were significantly (71% and 70%, respectively) lower, and the frequency of micturition and basal pressure were significantly (2.8- and 3.8-fold, respectively) higher in CYP-treated rats than in sham rats (Table 1). The CYP treatment had little effect on the maximum micturition pressure, threshold pressure, or residual urine. These changes in urodynamic parameters reflected CYP-induced detrusor overactivity.

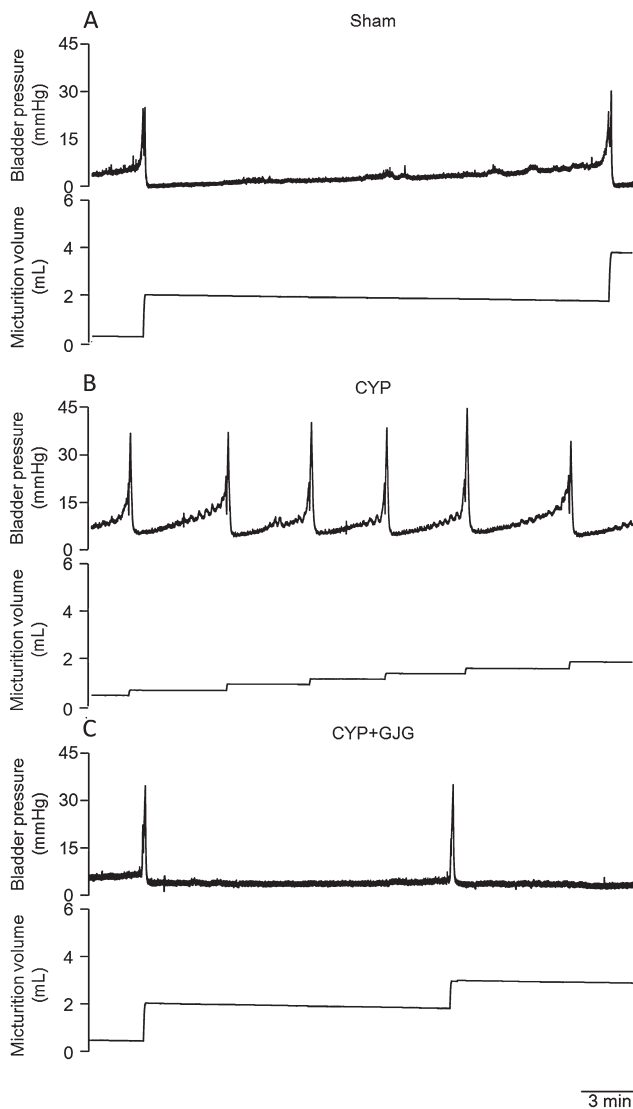
The repeated oral administration of GJG (1000  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ , 7 days) prevented the significant decrease in the micturition interval and mean micturition volume caused by the CYP treatment (Fig. 1, Table 1). The frequency of micturition and basal pressure were also significantly lower in CYP + GJG-treated rats than

**Table 1.** Urodynamic parameters in sham, CYP-treated, and CYP + GJG-treated rats

| Variable                            | Sham        | CYP          | CYP + GJG                 |
|-------------------------------------|-------------|--------------|---------------------------|
| Micturition interval (min)          | 12 ± 2.4    | 3.5 ± 0.8*   | 15 ± 2.9 <sup>††</sup>    |
| Mean micturition volume (mL)        | 0.68 ± 0.14 | 0.20 ± 0.04* | 0.94 ± 0.14 <sup>††</sup> |
| Frequency of micturition (number/h) | 6.0 ± 0.9   | 17 ± 2.9**   | 4.7 ± 0.9 <sup>†††</sup>  |
| Maximum micturition pressure (mmHg) | 41 ± 2.7    | 43 ± 6.9     | 35 ± 4.3                  |
| Basal pressure (mmHg)               | 2.6 ± 0.7   | 10 ± 2.2**   | 2.0 ± 0.5 <sup>††</sup>   |
| Threshold pressure (mmHg)           | 8.1 ± 1.1   | 15 ± 3.6     | 10 ± 0.8                  |
| Residual urine (mL)                 | 0.03 ± 0.02 | 0.06 ± 0.04  | 0.04 ± 0.04               |

Each value represents the mean ± S.E.M. (n = 5 – 6). \* $P < 0.05$ , \*\* $P < 0.01$ , significantly different from sham rats.

<sup>††</sup> $P < 0.01$ , <sup>†††</sup> $P < 0.001$ , significantly different from CYP-treated rats.



**Fig. 1.** Representative cystometric traces of bladder pressure and micturition volume for sham (A), CYP-treated (B), and CYP + GJG-treated (C) rats.

in CYP-treated rats. These urodynamic parameters in CYP-treated rats were shown to recover to control (sham) levels with the GJG treatment. No significant differences in maximum micturition pressure, threshold pressure, or residual urine volume were observed between the CYP and CYP + GJG-treated groups (Table 1). A similar treatment with GTE (400 mg·kg<sup>-1</sup>·day<sup>-1</sup>, 7 days) slightly attenuated changes in urodynamic parameters in rats with CYP-induced cystitis (Fig. 2, Table 2).

#### *Effects on bladder muscarinic and P2X receptors*

$B_{\max}$  for specific [<sup>3</sup>H]NMS binding in the rat bladder was significantly (34%) lower in CYP-treated rats than in sham rats, with little effect on  $K_d$  (Table 3). Moreover, the change in the  $K_d$  or  $B_{\max}$  for specific [<sup>3</sup>H]NMS binding in the submaxillary gland was not significant, which suggests the specific down-regulation of bladder muscarinic receptors. Similarly, the  $B_{\max}$  for specific [<sup>3</sup>H] $\alpha\beta$ -MeATP binding was significantly (51%) decreased in CYP-treated rats (Table 3). Conversely,  $K_d$  was not altered by the CYP treatment. Interestingly, the repeated oral administration of GJG (1000 mg·kg<sup>-1</sup>·day<sup>-1</sup>, 7 days) caused the significant recovery of  $B_{\max}$  for specific binding of [<sup>3</sup>H]NMS and [<sup>3</sup>H] $\alpha\beta$ -MeATP in the rat bladder, as shown by an increase of 44% and 41%, respectively (Table 3). In contrast, the GJG treatment had no significant effect on the  $K_d$  of either radioligand in the rat bladder, or on the  $K_d$  and  $B_{\max}$  for specific [<sup>3</sup>H]NMS binding in the submaxillary gland. Orally administered GTE (400 mg·kg<sup>-1</sup>·day<sup>-1</sup>, 7 days) caused the significant recovery (74%) of  $B_{\max}$  for the specific binding of [<sup>3</sup>H]NMS in the rat bladder (Table 4). However, the GTE treatment had no significant effect on the  $B_{\max}$  and  $K_d$  for specific [<sup>3</sup>H] $\alpha\beta$ -MeATP binding in the rat bladder or specific [<sup>3</sup>H]NMS binding in the submaxillary gland.

**Table 2.** Urodynamic parameters in sham, CYP-treated, and CYP + GTE-treated rats

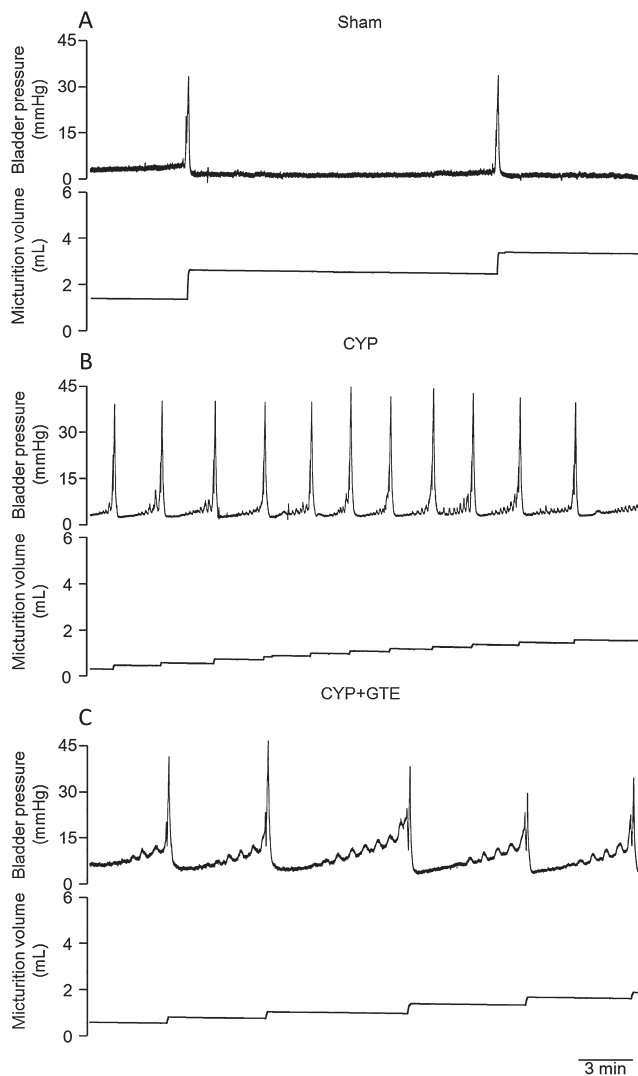
| Variable                            | Sham        | CYP          | CYP + GTE   |
|-------------------------------------|-------------|--------------|-------------|
| Micturition interval (min)          | 7.5 ± 0.51  | 3.9 ± 1.3*   | 5.9 ± 1.1   |
| Mean micturition volume (mL)        | 0.50 ± 0.09 | 0.14 ± 0.05* | 0.35 ± 0.06 |
| Frequency of micturition (number/h) | 8.2 ± 0.56  | 28 ± 10.3*   | 11.2 ± 2.2  |
| Maximum micturition pressure (mmHg) | 31 ± 4.5    | 35 ± 3.7     | 39 ± 6.1    |
| Basal pressure (mmHg)               | 2.1 ± 0.4   | 3.6 ± 1.0    | 4.1 ± 0.9   |
| Threshold pressure (mmHg)           | 9.3 ± 1.0   | 7.9 ± 0.8    | 11.7 ± 1.5  |
| Residual urine (mL)                 | 0.02 ± 0.02 | 0.12 ± 0.04  | 0.06 ± 0.02 |

Each value represents the mean ± S.E.M. (n = 5 – 6). \**P* < 0.05, significantly different from sham rats.

**Table 3.**  $K_d$  and  $B_{max}$  for specific binding of [ $^3$ H]NMS and [ $^3$ H] $\alpha\beta$ -MeATP in sham, CYP-treated, and CYP + GJG-treated rats

| Variable                               | $K_d$     | $B_{max}$                       |
|--|-----------|---------------------------------|
| [ $^3$ H]NMS binding                   | (pM)      | (fmol/mg protein)               |
| Bladder                                |           |                                 |
| Sham                                   | 265 ± 9   | 224 ± 15 (1.0)                  |
| CYP                                    | 234 ± 15  | 147 ± 8 (0.65)**                |
| CYP + GJG                              | 240 ± 10  | 211 ± 19 (0.94) <sup>†</sup>    |
| Submaxillary gland                     |           |                                 |
| Sham                                   | 190 ± 12  | 143 ± 7                         |
| CYP                                    | 182 ± 4   | 149 ± 8                         |
| CYP + GJG                              | 167 ± 7   | 132 ± 7                         |
| [ $^3$ H] $\alpha\beta$ -MeATP binding | (pM)      | (pmol/mg protein)               |
| Bladder                                |           |                                 |
| Sham                                   | 975 ± 133 | 9.3 ± 0.66 (1.0)                |
| CYP                                    | 970 ± 171 | 4.6 ± 0.38 (0.49)***            |
| CYP + GJG                              | 910 ± 108 | 6.5 ± 0.28(0.69)** <sup>†</sup> |

Each value represents the mean ± S.E.M. (n = 7 – 9). \*\**P* < 0.01, \*\*\**P* < 0.001, significantly different from sham rats. <sup>†</sup>*P* < 0.05, significantly different from CYP-treated rats.

**Fig. 2.** Representative cystometric traces of bladder pressure and micturition volume for sham (A), CYP-treated (B), and CYP + GTE-treated (C) rats.

### Effects on urinary cytokine levels

Urinary concentrations of cytokines (IL-1 $\beta$ , IL-6, and IL-17) were measured in rats. As shown in Table 5, the levels of IL-1 $\beta$  and IL-17 were significantly (9.4- and 5.5-fold, respectively) higher in CYP-treated rats than in sham rats. This increase was completely attenuated by the repeated oral administration of GJG (1000 mg·kg<sup>-1</sup>·day<sup>-1</sup>, 7 days). IL-6 levels were approximately 3.7-fold higher with the CYP treatment. The GJG treatment was more likely to suppress. An elevation in IL-1 $\beta$  levels was completely alleviated in CYP-treated rats by the GTE treatment (Table 5).

### Discussion

In urology clinical practice, GJG is used to treat patients with urinary frequency and incontinence due to



**Table 4.**  $K_d$  and  $B_{max}$  for specific binding of [ $^3H$ ]NMS and [ $^3H$ ] $\alpha\beta$ -MeATP in sham, CYP-treated, and CYP + GTE-treated rats

| Variable                               | $K_d$        | $B_{max}$                        |
|--|--------------|----------------------------------|
| [ $^3H$ ]NMS binding                   | (pM)         | (fmol/mg protein)                |
| Bladder                                |              |                                  |
| Sham                                   | 235 $\pm$ 22 | 277 $\pm$ 27 (1.0)               |
| CYP                                    | 220 $\pm$ 31 | 129 $\pm$ 14 (0.46)**            |
| CYP + GTE                              | 235 $\pm$ 23 | 225 $\pm$ 33 (0.81) <sup>†</sup> |
| Submaxillary gland                     |              |                                  |
| Sham                                   | 168 $\pm$ 17 | 205 $\pm$ 16                     |
| CYP                                    | 162 $\pm$ 14 | 203 $\pm$ 15                     |
| CYP + GTE                              | 168 $\pm$ 20 | 198 $\pm$ 6                      |
| [ $^3H$ ] $\alpha\beta$ -MeATP binding | (pM)         | (pmol/mg protein)                |
| Bladder                                |              |                                  |
| Sham                                   | 859 $\pm$ 81 | 12 $\pm$ 0.97 (1.0)              |
| CYP                                    | 954 $\pm$ 77 | 5.4 $\pm$ 0.57 (0.45)***         |
| CYP + GTE                              | 918 $\pm$ 92 | 6.7 $\pm$ 0.37 (0.56)***         |

Each value represents the mean  $\pm$  S.E.M. (n = 7–9). \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, significantly different from sham rats. <sup>†</sup> $P$  < 0.05, significantly different from CYP-treated rats.

storage dysfunction (19). Tokunaga et al. reported that the administration of GJG to patients with urinary disturbance significantly decreased the frequency of urination (8). The micturition interval and mean micturition volume were significantly lower and the frequency of micturition and basal pressure were significantly higher in CYP-treated rats than in sham rats, which reflects detrusor overactivity in CYP-treated rats. The repeated oral administration of GJG alleviated urodynamic symptoms in CYP-treated hyperactive bladders by increasing the micturition interval and micturition volume and subsequently decreasing the micturition frequency and basal pressure (Table 1). GJG inhibited the bladder contraction induced by electrical stimulation of the pelvic nerve or the infusion of acetylcholine (8). These results suggest that GJG has anti-cholinergic activity. Thus, GJG has the potential to improve bladder overactivity in rats with CYP-induced cystitis.

Antimuscarinic agents are widely used as the first-line therapy for overactive bladder because parasympathetic innervation and acetylcholine are the predominant stimuli

for bladder contraction through the activation of muscarinic receptors (20). Also, it has been suggested that ATP signaling via P2X<sub>1</sub> receptors participates in the efferent control of detrusor smooth muscle excitability and that this function may be heightened in disease and aging (21). ATP also appears to be involved in bladder sensation via activation of P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors on sensory afferent neurons, both within the bladder itself and possibly at central synapses. In the current study, there was a marked decrease in the density of muscarinic and P2X receptors in the bladders of CYP-treated rats, as shown by a significant reduction in the  $B_{max}$  for specific binding of [ $^3H$ ]NMS and [ $^3H$ ] $\alpha\beta$ -MeATP. These results revealed a down-regulation in pharmacologically relevant muscarinic and purinergic receptors in the bladders of rats with cystitis, which is consistent with pharmacological subsensitivity to cholinergic and purinergic agonists in CYP-treated bladders (22, 23). The down-regulation of pharmacological receptors in CYP-treated bladders may reflect a compensatory mechanism, possibly due to the increased stimulation of receptors by acetylcholine or ATP (23–26).

Interestingly, the  $B_{max}$  for specific binding of [ $^3H$ ]NMS and [ $^3H$ ] $\alpha\beta$ -MeATP in the bladders was significantly higher in CYP + GJG-treated rats than in CYP-treated rats (Table 3). Thus, GJG showed a protective effect against the loss of muscarinic and purinergic receptors in the bladders of CYP-treated rats. GJG was shown to inhibit bladder contraction induced by stimulation of the cholinergic nerve in dogs (27), in which the inhibitory effects on the bladder appeared to be mediated by 5HT<sub>1</sub> and dopamine receptors (28). In this connection, GJG was shown to reduce the amplitude of bladder contraction and plasma levels of dopamine and serotonin (29). Therefore, GJG may inhibit the down-regulation of bladder receptors by an inhibitory effect on neurotransmitter release from nerve terminals or by an anti-cholinergic effect (8).

CYP is known to cause inflammation in the bladder by altering urinary cytokine levels (4). The levels of the inflammatory cytokines IL-1 $\beta$  and IL-17 were significantly elevated in the urine of CYP-treated rats (Table 5). Similar changes in urinary cytokines in CYP-induced

**Table 5.** Effects of the repeated oral administration of GJG and GTE on urinary cytokine (IL-1 $\beta$ , IL-6, and IL-17) levels in CYP-treated rats

| Variable                                    | Sham           | CYP               | CYP + GJG                     | CYP + GTE                    |
|---|----------------|-------------------|-------------------------------|------------------------------|
| Interleukin-1 $\beta$ (pg/mg of creatinine) | 0.77 $\pm$ 0.8 | 7.3 $\pm$ 2.1**   | 0.10 $\pm$ 0.05 <sup>††</sup> | 0.52 $\pm$ 0.5 <sup>††</sup> |
| Interleukin-6 (pg/mg of creatinine)         | 0.26 $\pm$ 0.2 | 0.95 $\pm$ 0.4    | 0.54 $\pm$ 0.3                | 0.46 $\pm$ 0.5               |
| Interleukin-17 (pg/mg of creatinine)        | 0.12 $\pm$ 0.1 | 0.66 $\pm$ 0.1*** | 0.18 $\pm$ 0.1 <sup>††</sup>  | 0.38 $\pm$ 0.1*              |

Each value represents the mean  $\pm$  S.E.M. (n = 4–6). \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, significantly different from sham rats.

<sup>††</sup> $P$  < 0.01, <sup>†††</sup> $P$  < 0.001, significantly different from CYP-treated rats.

cystitis have been previously reported (4). IL-6 has numerous biological activities and is generally considered to be a pro-inflammatory cytokine (30). Therefore, the increase in urinary cytokines observed in rats with CYP-induced cystitis suggests that cytokines are crucial for the development of bladder inflammation. Repeated treatment with GJG significantly suppressed the increase in urinary IL-1 $\beta$  and IL-17 levels in CYP-treated rats. Aconite, an ingredient of GJG, inhibited increases in pERK1/2 proteins by IL-1 $\beta$  or IL-18 (31). Hachi-mi-jio-gan (HE), a traditional Chinese herbal preparation that was shown to have a similar composition to GJG, modulated an imbalance toward Th1 predominance in MRL/lpr mice through the inhibition of IL-12 production and ameliorated autoimmune disorders (32). In addition, tachykinins are involved in inflammation and GJG reduced the level of tachykinins associated with C-fiber activation induced by acetic acid in rat urinary bladders (19). The antioxidant effect of GJG has been reported previously (10). Accordingly, the antioxidant properties of GJG may contribute to attenuate enhanced levels of urinary cytokines in rats with CYP-induced cystitis.

The GTE treatment slightly attenuated changes in urodynamic parameters in rats with CYP-induced cystitis (Table 2). Juan et al. showed that EGCG prevented ovariectomy-induced bladder dysfunction through neuroprotective effects in a dose-dependent manner (33). In contrast, GTE contains caffeine, a stimulant of the central nervous system, which has a diuretic effect and causes frequent urination (34). In our study, GTE had no significant effect on CYP-induced detrusor overactivity in rats, which may have been due to the presence of caffeine.

The down-regulation of bladder muscarinic receptors in CYP-induced cystitis can effectively be prevented by the GTE treatment (Table 4). The tea components caffeine and catechin can inhibit the response of GABA<sub>A</sub> receptors and stimulate the CNS (35). Fukuda et al. reported that the pigments of green tea leaves had the potential to protect against dioxin toxicity through the suppression of aryl hydrocarbon receptor transformation (36). Moreover, EGCG has been suggested to exert binding properties with human cannabinoid receptors and focal adhesion kinase and insulin-like growth factor-I receptors (37, 38). Thus, GTE may prevent the down-regulation of muscarinic receptors in CYP-treated bladders by affecting cholinergic neuronal activity.

GTE also significantly inhibited the enhanced level of urinary IL-1 $\beta$  in CYP-treated rats. GTE has been shown to have both anti-inflammatory and antioxidant properties in multiple cell types (34). In addition, EGCG acts as an anti-inflammatory and antioxidant agent in human corneal epithelial cells challenged with IL-1 $\beta$  and was shown to have therapeutic benefits in many

inflammatory diseases such as atherosclerosis, arthritis, and dry eye disease (39). Moreover, the anti-inflammatory activity of GTE was observed in the carrageenan-induced paw edema model of rats (40). Therefore, GTE may exhibit an inhibitory effect on urinary cytokine levels through its antioxidant and anti-inflammatory properties in CYP-induced cystitis rats.

In conclusion, the present study demonstrated detrusor overactivity, the down-regulation of bladder pharmacological receptors, and elevated urinary cytokine levels in rats with CYP-induced cystitis. These changes were effectively attenuated by repeated oral treatment with GJG at a pharmacological dose. GTE also appears to have some ameliorative effects on the altered parameters observed in CYP-treated rats. Therefore, GJG may be a pharmacologically useful phytotherapeutic agent for cystitis.

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## Conflicts of Interest

The authors declare that they have no conflicts of interest to disclose.

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