A New Method for Urinary Bladder Hyperactivity in Conscious Rats

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A New Method for Urinary Bladder Hyperactivity in Conscious Rats

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(Doctoral Program in Life Sciences and Bioengineering)

Manabu MITOBE
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>acetic acid</td>
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<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>BC</td>
<td>bladder capacity</td>
</tr>
<tr>
<td>CNS</td>
<td>central nerves system</td>
</tr>
<tr>
<td>HE</td>
<td>hematoxylin-eosin</td>
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<tr>
<td>HPMC</td>
<td>hydroxypropyl methyl cellulose</td>
</tr>
<tr>
<td>ID</td>
<td>intradouental</td>
</tr>
<tr>
<td>M3</td>
<td>muscarinic type3</td>
</tr>
<tr>
<td>MC</td>
<td>methyl cellulose</td>
</tr>
<tr>
<td>MI</td>
<td>micturition interval</td>
</tr>
<tr>
<td>MP</td>
<td>micturition pressure</td>
</tr>
<tr>
<td>MV</td>
<td>micturition volume</td>
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<tr>
<td>NEP</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>OAB</td>
<td>overactive bladder</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
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<tr>
<td>PMC</td>
<td>pontine micturition center</td>
</tr>
<tr>
<td>RV</td>
<td>residual volume</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous injection</td>
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<td>TP</td>
<td>threshold pressure</td>
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A normal voiding reflex in the adult is mediated by afferent signals from the lower urinary tract and is controlled by neural circuits in the spinal cord and brain including the cerebral cortex and pontine micturition center (PMC). These circuits coordinate the activity of smooth muscle in the detrusor and urethra with that of striated muscle in the urethral sphincter and pelvic floor. They are believed to act as on/off switches to shift the lower urinary tract between two modes of operation, storage and voiding (1) (Figure 1).

During storage, norepinephrine (NEP), from the sympathetic nerve terminal, stimulates $\beta_3$-adrenergic receptors, and leads to relaxation of bladder smooth muscle and contracts the bladder sphincter (2) once threshold tension is achieved, then afferent impulses, conveyed mainly by the pelvic nerve, reach centres in the central nerves system (CNS) (3). In contrast, voiding is induced by acetylcholine (Ach), from the parasympathetic nerve terminal, interacts with muscarinic type3 (M3) receptors and activates phospholipase C through coupling with G proteins, which generates inositol triphosphate, which in turn causes the release of calcium from the sarcoplasmic reticulum and the contraction of bladder smooth muscle and relaxing of the bladder sphincter (2).
Distension of the bladder wall is the primary stimulus for initiation of the micturition reflex (4). In addition, signals generated in the urothelium and involving suburothelial nerves may be key to both normal, voiding and various voiding disorders (5). At least two types of afferent neurons innervate the urinary bladder: Aδ- and C-fibers. Aδ afferent neurons have myelinated axons and are mechanosensitive; activated by both low (non-nociceptive) and high (nociceptive) intravesical pressure. C-fiber afferents possess unmyelinated axons and do not respond to bladder distension; they are activated by cord, heat, or chemical irritation of the bladder mucosa (6). When the balance between storing and voiding is broken, as in a urinary disorder, leading to the hypersensitization of a nociceptive afferent, C-fiber, is activated by some stimulants and the signals mediated by reflection, and is not mediated through the CNS (7) (Figure 2).

Overactive bladder (OAB), a urinary disorder disease, is characterized by urinary urgency, with or without urge urinary incontinence, usually with frequency and nocturia and the absence of obvious pathology to account for these symptoms (8). Although the pathogenic mechanisms of OAB are not fully understood, both neurogenic and myogenic theories have been proposed (3, 9-11). Increased afferent activity, decreased capacity to process afferent information, decreased suprapointine inhibition, increased
sensitivity to contraction-mediating transmitters, and changes in detrusor smooth muscle structure or function may all contribute to OAB. Therefore, pharmacological treatment of OAB is directed toward neuronal pathways and/or the detrusor muscle itself (12).

Animal models that closely resemble the pathophysiology of human OAB are critical for the development of pharmacological therapies to treat the disorder. One of the most commonly utilized methods of investigating the effects of compounds on bladder function is cystometry in either conscious or anaesthetized rats with or without the infusion of irritative agents. Irritative cystometry (produced by the infusion of chemical agents such as acetic acid or citric acid leading to the hypersensitization of C-fibers), has been used to investigate new therapies for OAB (12). However, while irritative rat cystometry models display some characteristics of human OAB (such as increased frequency and decreased void volume), the similarity to human OAB pathophysiology has been questioned in several reports (13). The implantation of catheters and intravesical infusion of chemical irritants, and the irritative cystometry model, may produce inflammatory damage in the bladder urothelium. As inflammation is not believed to be an underlying pathology in OAB, results from such models may be misleading (13-14). In addition, antimuscarinics (gold standard
treatment for OAB) do not appear to be efficacious in these types of models (15). It is likely that surgical operation, anaesthetic treatment and the continuous infusion of chemical irritants alter both myogenic and neural activities influencing urinary micturition.

In the present study, I investigated the effect of a transient intravesical infusion of acetic acid solution (under isoflurane anesthesia without surgical procedures) on micturition patterns. Then I compared the bladder morphology in female rats between the irritative cystometry method to establish a novel non-invasive hyperactive bladder model with a significant sensitivity to anti-muscarinic drugs and without bladder inflammation.
Figure 1. Pathophysiology in normal micturition. Normal voiding reflexes involve supraspinal pathways and are under voluntary control. During the voiding, the spinal parasympathetic outflow is activated, leading to bladder contraction. Simultaneously, the sympathetic outflow to urethral smooth muscle and the somatic outflow to urethral and pelvic floor striated muscles are turned off, and the outflow region relaxes. In contrast, during the storing phase, there is continuous and increasing afferent activity from
the bladder. The sympathetic outflow to urethral smooth muscle and the somatic outflow to urethral and pelvic floor striated muscles keep the outflow region closed. Whether or not the sympathetic innervation to the bladder contributes to bladder relaxation during filling (β-adrenoreceptors) in humans has not been established (3).
**Figure 2. Pathophysiology in overactive bladder.** As an example, OAB is induced by age, hypertension, spinal cord injurers and infections, the signals are mediated through c-fibers and parasympathetic nerves induce contraction of bladder smooth muscle and relax the bladder sphincter without contribution from the CNS. In laboratory, the activation of c-fiber is induced by chemical agents, acetic acid.
CHAPTER II. CONFIRMATION OF THE CHARACTERISTICS ON CYSTOMETRY MODEL

1. Summary

In this study, one of the generally used hyperactive bladder animal models, irritative cystometry method, an that is invasive condition, confirmed the effects of experimental procedures, morphological observation and the sensitivity to an anti-muscarinic agents, oxybutynin.

Intravesical infusion of 0.2% acetic acid solution induced bladder hyperactivity, as reflected by decreased bladder capacity (BC), but oxybutynin (10.0 mg/kg, ID) didn’t significantly increase (P=0.14, 91.3% change vs. vehicle) in delta BC. Histopathological inflammatory changes were observed on bladder infused by 0.2% acetic acid for 60 minutes. As to results, the irritative cystometry model induced by 0.2% acetic acid had confirmed bladder hyperactivity, however, mild inflammation on the bladder was observed and showed low sensitivity to 10 mg/kg of oxybutynin.
2. Introduction

Animal models that closely resemble the pathophysiology of human overactive bladder are important for evaluating novel therapeutics to treat the disorder. First I reviewed and confirmed the characteristics of the most useful urinary hyperactive bladder model in rats, cystometry, done with the implantation of catheters and intravesical infusion of a chemical irritant, acetic acid, under urethane anaesthesia. This model mimics OAB in human and is caused by stimulating nociceptive afferent fibers (C-fiber) with a dilute acetic acid solution into the bladder (16). The model can capture compounds which regulate autonomic nerves, afferent nerves and bladder smooth muscle. Oxybutynin is an anti-muscarinic agent that is frequently used clinically and is one of the most studied anti-muscarinics (17). The common view on the working mechanism of systemic anti-muscarinics such as oxybutynin is that they inhibit parasympathetically induced detrusor contractions by blocking the muscarinic receptors in detrusor smooth muscle (18-19). However, antimuscarinics is widely used in OAB because they improve symptoms, which are related to the storage phase, when there is normally no excitatory activity in parasympathetic efferent nerves (20).
In the present study, I implemented and confirmed the characteristics in the most useful hyperactive bladder model in rats and evaluated the efficacy of oxybutynin on urological parameters in the condition.
3. Materials and Methods

3.1. Animals

Female Sprague-Dawley rats at 9-week age weighting 180 to 250 g (Charles River Japan Inc., Japan) were used in all experiments. Rats were housed in a plastic cages (3 per cage) with wood chip bedding, allowed access to food and water ad libitum, and were maintained on a 12-hour dark: 12-hour light (7:00 to 17:00) cycle in a temperature (23 ± 2 °C)- and humidity (55 ± 10 %)-controlled room. All protocols used in the present studies were reviewed and approved by the Experimental Animal Ethical Committee of GlaxoSmithKline according to the Guide for the Care and Use of Laboratory Animals published by US Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2. Drugs

Acetic acid, oxybutynin chloride, methyl cellulose (MC) and urethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Saline and distilled water were purchased from Otsuka Pharmaceutical Co. (Japan).
Acetic acid was diluted to 0.2% with saline. Oxybutynin chloride was dissolved in a volume of 1 mL/kg with 1.0% MC solution.

3.3. Acetic acid-induced hyperactivity

All surgical procedures were performed under urethane anesthesia (1.2 g/kg, SC). Body temperature was maintained via a heating stand (37°C) throughout the study (Figure 3). The trachea was catheterized (Hibiki, size 7), and both ureters were catheterized (Natsume, size 8) to discharge urine during the experiment. The bladder was exposed with a lower midline abdominal incision and a tube (Natsume, SP65) with a cuff at the end inserted into the bladder through the bladder dome. The intravesical catheter was connected via a 3-way stopcock to a pressure transducer and syringe pump for recording intravesical bladder pressure and infusing saline into the bladder, respectively (21). Saline at room temperature (20 to 22°C) was infused at a rate of 3 mL/hr to elicit repetitive bladder contractions. Saline infusion was continued for at least 30 minutes until rhythmic bladder contractions become stable. After a control period of saline infusion for about 30 minutes, the solution was changed to saline containing 0.2% acetic acid to elicit bladder nociceptive responses, which were characterized by a
reduction in the inter-contraction intervals of repetitive bladder contraction (22).

3.4. Measurement of micturition pattern

Micturition pattern was determined according to the modified method reported by Yu et al. (22). Micturition pressure (MP) was measured through the intravesical catheter and infused solution from the urethral meatus was collected in a polypropylene beaker on an electronic scale (GX-200, AND, USA), and connected to a computer for recording of micturition volume (MV). Data were recorded, stored and calculated using data acquisition and analysis software (Notocord-hem evolution. 4.1.0.69, HEM systems, USA) (Figure 3 and 4). Bladder capacity (BC) was residual volume (RV) after voiding plus the volume of saline infused to induce the void. RV was BC minus MV. The micturition interval (MI) was also measured. All readouts were expressed as the difference between the peak and the pre-treatment values (i.e., absolute change from pre-treatment values). The values of all readouts in compound treated rats were compared with that of vehicle treated rats.
3.5. **Study design**

Study 1. To examine the hyperactivity effect and histopathological changes of 0.2% of acetic acid compared with saline on BC. At the end of the experiment animals were euthanized with CO₂ gas, and the urinary bladders were isolated for histopathological examination. Briefly, the urinary bladders were fixed in 10% neutral buffered formalin for 24 hours and processed for paraffin block. Sagittal sections (five micron thickness) of the bladder wall from the bladder dome to the trigone were cut and stained with hematoxylin-eosin (HE). Histopathological alterations in the bladder were determined under a single-blind condition.

Study 2. To determine effects of oxybutynin on the micturition parameters, BC, MV, MI, RV, micturition pressure (MP) and threshold pressure (TP), oxybutynin (10 mg/kg) was intradouental (ID) administered after stable rhythmic bladder contractions modified by 0.2% acetic acid-treatment for one hour.

3.6. **Statistics**

Statistical analysis of the difference in delta BC between vehicle treated and test compound treated groups were conducted using unpaired t test for
(n=8, single dose of control drug vs. vehicle). Results were considered effective if the test compound increases more than 80% in delta bladder capacity compared to vehicle and/or statistical significance (p<0.05 vs. vehicle).
4. **Results and Discussion**

4.1. **Effects of acetic acid on bladder capacity and morphology of urinary bladder**

As shown in Figure 5, continuous intravesical application of 0.2% acetic acid solution induced a stable decrease of bladder capacity (BC): BC was significantly decreased compared with saline infusion by 0.36 times; BC in the saline group, 1.73 ± 0.2 mL (n=7); and BC in the 0.2% acetic acid group, 0.63 ± 0.3 mL (n=7). In the histopathological examination of bladders which were infused for one hour intravesically, the saline-treatment by itself induced slight neutrophil infiltrations in the lamina propria in the urinary bladder. There were significant differences observed in the morphological alterations between the saline treated group and 0.2% acetic acid treated groups (Figure 6). In bladders from 0.2% acetic acid treated rats, inflammatory changes (neutrophil and/or macrophage infiltrations, and hemorrhages) in the lamina propria, and moderate desquamation accompanying vacuolar degeneration and necrosis in the urothelium were observed (Figure 6A and 6B), but damage was not observed on the bladder in the saline treated group (Figure 6C and 6D).
The results induced that a continuous infusion of 0.2% acetic acid caused significant inflammation on the bladder.

4.2. Effect of oxybutynin on bladder hypersensitivity induced by acetic acid

At first, the unpaired t test (delta value from baseline) was decided as the most suitable statistical method in the study to determine the vehicle’s effect on all parameters because the vehicle, 1.0% MC, tended to increase BC by itself (Figure 7).

When oxybutynin (10 mg/kg) was intradoudental (ID) administered in rats treated with 0.2% acetic acid, it only showed a tendency to increase delta BC (97.8%) (Figure 8 and Table 1): delta BC in the vehicle group, 0.46 ± 0.14 mL (n=8); and delta BC in the oxybutynin (10 mg/kg) group, 0.91 ± 0.22 mL (n=8).

Figure 9 and Table 1 show the other urological parameters. Oxybutynin in 10 mg/kg significantly decreased the delta RV (219.2%): delta RV in the vehicle group, 0.26 ± 0.15 mL (n=8); and delta RV in the oxybutynin (10 mg/kg) group, 0.83 ± 0.22 mL (n=8) as reported for anti-muscarinic effects (23). However, significant efficacy on MI and MV
including BC could not be captured under the observed anti-muscarinic effects using 10 mg/kg of oxybutynin on RV. In contrast, MP and TP could not be validated in the study, because of the large variations in rats (data not shown).

The results show that the cystometry model might decrease the sensitivity to oxybutynin because of the inflammation on the bladder induced by surgical operation and continuous infusion of acetic acid solution.
5. Figures and Tables

Figure 3. Irritative cystometry methodology. Under urethane anesthesia, catheters were implanted in the bladder for continuous infusion of saline or acetic acid solution and in the duodenum for dosing of vehicle and oxybutynin. 0.2% acetic acid solution was infused into the bladder continuously (3 mL/hour), after stability of micturition parameters by saline infusion. Data was automatically transferred from balances to HEM system and analyzed.
- **Micturition Pressure (MP)**: maximum bladder pressure during micturition
- **Threshold Pressure (TP)**: bladder pressure immediately prior to micturition
- **Micturition Volume (MV)**: volume of the expelled urine
- **Micturition Interval (MI)**: time of the infused for one micturition
- **Bladder Capacity (BC)**: \( BC(n) = RV(n-1) + MI(n) \times A \)
- **Residual Volume (RV)**: \( RV(n) = BC(n) - MV(n) \)

**Figure 4. Definition and calculation of cystometric parameters.**
Figure 5. Effect of acetic acid on bladder capacity. Saline or 0.2% acetic acid was continuously infused into bladder (3 mL/hour). Data are means ± S.E.M. of 7 animals after the infusion of each solution for one hour. *p<0.05 vs saline; Paired t test.
Figure 6. Histopathology of the bladder induced by 0.2% acetic acid or saline infusion. Bladder instillations of 0.2% acetic acid into bladder (3 mL/hour) for 60 minutes (n=4) followed by stability of saline infusion before bladder removal. Bladders were fixed in 10% buffered formalin, paraffin embedded, sectioned and processed. A and B, show a 0.2% acetic acid treated bladder. C and D, show a saline treated bladder. Desquamation (arrow) was observed in 0.2% acetic acid treated animals for 60 minutes, but not in saline treated animals.
Figure 7. **Effect of vehicle on bladder capacity.** 0.2% acetic acid was continuously infused into bladder. Vehicle, 1% MC, was administered by ID after induced the hyperactivity on the bladder. Data are means ± S.E.M. of 7 vehicle treated animals for one hour. *p<0.05 vs. control (pre-treatment); unpaired t test.
Figure 8. Effect of oxybutynin on bladder capacity. 0.2% acetic acid was continuously infused into bladder (3 mL/hour). Oxybutynin (10 mg/kg) was administered by ID. Data are means ± S.E.M. of 7 animals. A cycle showing the most pronounced changes (increase or decrease) of bladder capacity after exposure to the drug will be analyzed and compared with the vehicle (1% MC) treated group.
Figure 9. Effect of oxybutynin on residual volume. 0.2% acetic acid was continuously infused into bladder. Oxybutynin (10 mg/kg) was administered by ID. Data are means ± S.E.M. of 7 animals. A cycle showing the most pronounced changes (increase or decrease) of residual volume after exposure to the drug will be analyzed and compared with the vehicle treated group. *p<0.05 vs. control (vehicle, 1% MC); unpaired t test.
### Table 1. Effect of oxybutynin on cystometric parameters.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (1% MC)</th>
<th>Oxybutynin 10 mg/kg i.d.</th>
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</thead>
<tbody>
<tr>
<td><strong>Baseline (saline infusion)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV (ml)</td>
<td>0.26 ± 0.05</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>MI (min)</td>
<td>6.65 ± 1.19</td>
<td>4.99 ± 0.57</td>
</tr>
<tr>
<td>BC (ml)</td>
<td>1.34 ± 0.31</td>
<td>1.25 ± 0.18</td>
</tr>
<tr>
<td>RV (ml)</td>
<td>1.08 ± 0.28</td>
<td>1.09 ± 0.16</td>
</tr>
<tr>
<td><strong>AA infusion</strong></td>
<td></td>
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<tr>
<td>MV (ml)</td>
<td>0.25 ± 0.05</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>MI (min)</td>
<td>4.62 ± 0.98</td>
<td>3.57 ± 0.53</td>
</tr>
<tr>
<td>BC (ml)</td>
<td>0.84 ± 0.16</td>
<td>1.12 ± 0.26</td>
</tr>
<tr>
<td>RV (ml)</td>
<td>0.59 ± 0.14</td>
<td>0.96 ± 0.26</td>
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<tr>
<td><strong>AA infusion + dosing (i.d.)</strong></td>
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<tr>
<td>MV (ml)</td>
<td>0.45 ± 0.06</td>
<td>0.24 ± 0.04</td>
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<tr>
<td>MI (min)</td>
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<tr>
<td>BC (ml)</td>
<td>1.30 ± 0.25</td>
<td>2.03 ± 0.46</td>
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<tr>
<td>RV (ml)</td>
<td>0.85 ± 0.25</td>
<td>1.79 ± 0.45</td>
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<td><strong>Deltas (post-dosing)</strong></td>
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<tr>
<td>MV (delta ml)</td>
<td>0.20 ± 0.04</td>
<td>0.08 ± 0.04</td>
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<tr>
<td>MI (delta min)</td>
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<td>BC (delta ml)</td>
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<tr>
<td>% change</td>
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<td>97.8</td>
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<tr>
<td>RV (delta ml)</td>
<td>0.26 ± 0.15</td>
<td>0.83 ± 0.22</td>
</tr>
<tr>
<td>% change</td>
<td></td>
<td>219.2</td>
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AA: acetic acid, MC: methylcellulose

% change: (cpd dosing - vehicle dosing)/vehicle dosing × 100

Each value is mean ± S.E.M. of 8 animals

* p<0.05, ** p<0.01: compared with the data in saline infusion phase (paired t-test).

† p<0.05, †† p<0.01: compared with the data in 0.2% AA infusion phase (paired t-test).

* p<0.05: compared with the data in vehicle (1% MC) treated rats (Student's t test)
CHAPTER III. IMPLEMENTATION OF NEW URINARY HYPERACTIVE BLADDER MODEL

1. Summary

A new method to reduce the influences of secondary effects, transient infusion of a 0.5% acetic acid solution into bladder, caused a significant increase in voiding frequency (VF), without influencing total urine volume or inducing significant histopathological inflammatory alterations in the bladder urothelium. Oral administration of oxybutynin (3 and 10 mg/kg) significantly ameliorated increases in VF induced by 0.5% acetic acid. Infusion of 0.75% acetic acid induced intensive urinary inflammation and a decrease in total urine volume as well as an increase in VF. Oral treatment with oxybutynin (10 mg/kg) did not significantly improve the increased VF due to 0.75% acetic acid. Acetic acid (0.5%) infusion evoked bladder hyper-responsiveness whether applied at night or during the day. However, VF increased more by nighttime application of acetic acid, while there were no significant differences in basal levels of VF between daytime and nighttime.
In this study, the non-invasive rat urinary hyperactive bladder model indicated minimizes the secondary effects of experimental procedures such as surgical operations and anesthesia on bladder function and is sensitive to oxybutynin compared with the irritative cystometry method as the described in Chapter II. Thus, this acute model may be useful for investigating novel therapeutics for OAB treatment.
2. Introduction

To establish a non-invasive urinary hyperactive bladder model in rats, I focused on the influences of secondary effects on bladder, anaesthesia and inflammation induced by surgical operations and continuous infusion of chemical agents. First, to simplify the experimental procedures in the model, surgical operations, including implantation of catheters into bladder with cutting open the abdomen, were removed to avoid systemic anaesthesia and invasion to bladder. Second, the continuous infusion of acetic acid was shortened as much as possible, that is a transient infusion method, to reduce the damage of the bladder by flow pressure and irritation period of infused solution. After the transient infusion model was established, it was validated using oxybutynin to investigate the sensitivity to anti-muscarinics. The results indicate that it was possible to establish a non-invasive hyperactive bladder model that is sensitive to anti-muscarinic drugs and without bladder inflammation as compared to the irritative cystometry method. Finally, I evaluated the effects of acetic acid administration during the day or night on bladder hypersensitivity in the new non-invasive model.
3. Materials and Methods

3.1. Animals

Female Sprague-Dawley rats at 9-week age weighting 180 to 250 g (Charles River Japan Inc., Japan) were used in all experiments. Rats were housed in a plastic cage (3 per cage) with wood chip bedding, allowed access to food and water ad libitum, and were maintained on a 12-hour dark: 12-hour light (7:00 to 17:00) cycle in a temperature (23 ± 2 °C)- and humidity (55 ± 10%)-controlled room. All protocols used in the present studies were reviewed and approved by the Experimental Animal Ethical Committee of GlaxoSmithKline according to the Guide for the Care and Use of Laboratory Animals published by US Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2. Drugs

Acetic acid, oxybutynin chloride, mineral oil, hydroxypropyl methyl cellulose (HPMC) and Tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Isoflurane (Escain) was obtained from Merck HOEI (Japan). Saline and distilled water were purchased from Otsuka
Acetic acid was diluted to 0.2, 0.5 and 0.75% with saline. Oxybutynin chloride was dissolved in a volume of 10 mL/kg with 0.5% HPMC solution containing 0.1% Tween 80.

### 3.3. Acetic acid-induced hyperactivity

Each rat was placed in a metabolic cage (3700M071, Tecniplast, USA) with free access to food and water to acclimatize for 3 days prior to the induction of OAB. The following procedures were performed under isoflurane anesthesia (concentration 4%, flow 3 mL/min). The bladder was catheterized (the inside diameter is 0.5 mm and an external diameter is 0.8 mm) from the external urethral orifice without any surgical operation, and then this intravesical catheter was connected via a 3-way stopcock to a transducer (P23XL, Becton Deckinson, USA) to record intravesical bladder pressure. After residual urine was removed from the bladder, an acetic acid solution or saline at room temperature was injected into the bladder via the inserted catheter until the bladder pressure reached 10 cmH₂O. Five minutes later, the acetic acid solution was removed from the bladder and the inside of the bladder was gently washed twice with saline, and the rat was
placed in the metabolic cage with free access to water and food during recovery from anesthesia.

3.4. Measurement of micturition pattern

Micturition pattern was determined according to the modified method reported by Ozawa et al. (24) (Figure 10). One hour after treatment with acetic acid solution, voided urine was collected for 48 hours per the following procedures. Urine was collected in a polypropylene beaker containing 3 mL of mineral oil on an electronic scale (GX-200, AND, USA), connected to a computer for recording of micturition frequency and volume. Data were recorded and stored using data acquisition and analysis software (Notocord-hem evolution. 4.1.0.69, HEM systems, USA). Micturition parameters, voiding frequency (VF), total urine volume and micturition volume (MV) were determined.

3.5. Study design

- Study 1. To examine the concentration-dependent effect of acetic acid on micturition patterns and morphology of the bladder, saline and acetic
acid solutions at concentrations of 0.2, 0.5 and 0.75% were intravesically applied at 17:00 and the micturition pattern was determined for nine hours from 18:00 as described above. At the end of experiments animals were euthanized with CO₂ gas, and the urinary bladders were isolated for histopathological examination. Briefly, the urinary bladders were fixed in 10% neutral buffered formalin for 24 hours and processed for paraffin block. Sagittal sections (five micron thickness) of the bladder wall from the bladder dome to the trigone were cut and stained with hematoxylin-eosin (HE). Histopathological alterations in the bladder were determined under a single-blind condition.

- **Study 2.** To determine effects of oxybutynin on the micturition patterns modified by acetic acid-treatment, oxybutynin (1, 3 and 10 mg/kg) was orally administered one hour after intravesical application of 0.5% acetic acid solution (for 5 minutes at 17:00) and the micturition patterns monitored for nine hours. In a separate experiment, 10 mg/kg of oxybutynin was orally administered one hour after the intravesical injection of 0.75% acetic acid solution (for five minutes at 17:00) and the micturition patterns monitored as above.

- **Study 3.** To determine differences in the bladder responses between daytime and nocturnal applications of acetic acid, 0.5% acetic acid solution
was intravesically applied for five minutes at 9:00 or 17:00 respectively and micturition patterns monitored as described above.

3.6. Statistics

Data was expressed as mean ± S.E.M. Statistical analysis was performed after a square root transformation of VF data. Student t-test and/or Dunnett’s test for multiple comparisons were used in study 1 and 2. Two-way analysis of variance (ANOVA) followed by Fisher LSD test was used in study 3. A p-value of less than 0.05 was considered statistically significant. STATISTICA version 6.1 (StatSoft. Inc. USA) was used for all analysis.
4. Results and Discussion

4.1. Feasibility study: a measurement of micturition patterns

First, the micturition pattern of rats which were treated by transient intravesical application of 0.5% acetic acid was measured after accumulation periods for 3 days (Figure 11). The voiding frequency was increased by the treatment of acetic acid, and body weight, water intake and food intake did not show significant difference due to the acetic acid treatment (Figure 11-12). The voiding frequency was analyzed by accumulation for 9 hours in the study because the voiding frequency induced by 0.5% acetic acid was recovered after 9 hours (Figure 13).

4.2. Effects of acetic acid on micturition patterns

As shown in Figure 14, the transient intravesical application of acetic acid solutions (0.2, 0.5 and 0.75%) increased voiding frequency in a concentration-dependent manner: voiding frequency was significantly augmented by 3.5- and 4.1-times at concentrations of 0.5 and 0.75% acetic acid, respectively. Infusion of 0.5% acetic acid did not significantly affect total urine volume, while 0.75% acetic acid caused a significant reduction.
4.3. **Effects of acetic acid on morphology of urinary bladder**

As shown in Figure 15 to 18, the histopathological examination for 1, 3, 9 and 24 hours after the irritation of acetic acid, the saline-treatment by itself induced slight neutrophil infiltrations in the lamina propria in the urinary bladder. There were no significant differences observed in the morphological alterations between the saline treated group and acetic acid treated groups at concentrations of 0.2 and 0.5% (Figure 15-18B, 15-18C, 15-18D). In bladders from 0.75% acetic acid treated rats, inflammatory changes (neutrophil and/or macrophage infiltrations, and hemorrhages) in the lamina propria, and moderate desquamation accompanying vacuolar degeneration and necrosis in the urothelium were observed (Figure 15-18E and 15-18F).

4.4. **Effect of oxybutynin on bladder hypersensitivity induced by acetic acid**

Oral oxybutynin (1, 3 and 10 mg/kg) dose-dependently ameliorated acetic acid (0.5%) induced increases in VF (Figure 19). Oxybutynin at 1 mg/kg moderately improved the increased VF by 36.2%, and both 3 and 10
mg/kg of oxybutynin significantly ameliorated the VF by 66.0 and 68.1%, respectively (Figure 19). When oxybutynin (10 mg/kg) was orally administered in rats treated with 0.75% acetic acid, it showed only a tendency to ameliorate the enhanced VF (36.5% of amelioration): VF in a naive group, 6.8 ± 0.3 times/9 hours (n=4); VF in a vehicle group, 28.0 ± 1.3 times/9 hours (n=4); and VF in the oxybutynin (10 mg/kg) group, 20.3 ± 4.0 times/9 hours (n=4).

4.5. Bladder hypersensitivity induced by acetic acid administered during the day or at night

Acetic acid (0.5%) treatment at night (17:00) or in the morning (9:00) significantly increased VF by 2.9 and 3.9 times, respectively, without affecting total urine volume (Figure 20). The increase in VF after nighttime administration was remarkably higher than after daytime administration (p<0.01), although there were no significant differences in the basal levels of VF between daytime and nighttime.
5. Figures and Tables

Figure 10. Transient infusion methodology. Acetic acid solution or saline was transient infused into bladder for 5 minutes under isoflurane inhalation. Oxybutynin (10 mg/kg) was administered by PO. Data are means ± S.E.M. of 8 animals. Voiding frequency was analyzed by measurement of voiding times for 3 hours and compared with the vehicle treated group.
Figure 11. Frequency Volume Chart on voiding patterns in conscious rats. Female SD rats received a single intravesical injection of saline or 0.5% acetic acid for 5 minutes at 17:00, and then their micturition pattern (voiding frequency, number of micturitions every three hours) was recorded and analyzed in a metabolic cage before and after acetic acid treatment using HEM System.
Figure 12. Effect of a transient intravesical application of acetic acid on body weight, food intake and water intake in conscious rats. Eight female SD rats received a single transient intravesical injection of acetic acid (0.5% AA for 5 minutes) at 17:00, and then their body weight, food intake and water intake were measured before and after acetic acid treatment. No significant changes were observed in these parameters.
Figure 13.  Effect of a transient intravesical application of acetic acid on voiding frequency in conscious rats.  Eight female SD rats received a single intravesical injection of saline, 0.2%, 0.5% and 0.75% acetic acid for 5 minutes at 17:00, and then their micturition pattern (voiding frequency, number of micturitions every three hours) was recorded and analyzed.  *p<0.05, **p<0.01 compared with the saline treated group by the Dunnett’s test.
Figure 14. Effect of a transient intravesical application of acetic acid on voiding patterns in conscious rats. Under isoflurane anesthesia, acetic acid solutions (0.2, 0.5 and 0.75%) were infused into the bladder for 5 minutes at 17:00. After recovery from anesthesia, the micturition patterns were monitored for 9 hours from 18:00 to 3:00. Each bar represents the mean ± S.E.M (n=4). *p<0.05 and **p<0.01, compared with the saline treated group by the Dunnett’s test.
Figure 15. Microscopic examination of typical bladders after the treatments for 1 hour (n=4). A, intact bladder. B, saline treated bladder. C, 0.2% acetic acid treated bladder. No significant histopathological changes were observed in saline or 0.2% acetic acid treated group. D,
0.5% acetic acid treated bladder with minimal edema. E and F, 0.75% acetic acid treated bladder with moderate vacuolar degeneration (arrows), cytoplasmic swelling, necrosis of epithelial cells, minimal edema, hemorrhage and neutrophil infiltration were observed in the bladder.
Figure 16. Microscopic examination of typical bladders after the treatments for 3 hours (n=4).  A, intact bladder.  B, saline treated bladder.  C, 0.2% acetic acid treated bladder.  D, 0.5% acetic acid treated bladder.  Minimal neutrophil infiltration and edema were observed in saline, 0.2 and 0.5% acetic acid treated group.  E and F, 0.75% acetic acid treated
bladder with moderate desquamation, mild vacuolar degeneration, cytoplasmic swelling and necrosis of epithelial cells, minimal edema, hemorrhage and neutrophil infiltration. Mild fibrin debris (*) and hemorrhage, minimal neutrophil infiltration were observed in the lumen.
Figure 17. Microscopic examination of typical bladders after the treatments for 9 hours (n=4). A, intact bladder. B, saline treated bladder. C, 0.2% acetic acid treated bladder. D, 0.5% acetic acid treated bladder with minimal edema, neutrophil infiltration. In addition, minimal cytoplasmic swelling and vacuolar degeneration of epithelial cells were
observed in only one case. E and F, 0.75% acetic acid treated bladder with moderate desquamation, mild vacuolar degeneration and necrosis of epithelial cells, neutrophil infiltration, hemorrhage, minimal macrophage infiltration and edema. Moderate fibrin debris (*) and hemorrhage, mild neutrophil infiltration (arrow) were observed in the lumen.
Figure 18. Microscopic examination of typical bladders after the treatments for 24 hours (n=4). A, intact bladder. B, saline treated bladder. C, 0.2% acetic acid treated bladder. D, 0.5% acetic acid treated bladder with minimal edema, neutrophil infiltration. In addition, minimal cytoplasmic swelling and vacuolar degeneration of epithelial cells were
observed in only one case. E and F, 0.75% acetic acid treated bladder with mild desquamation, vacuolar degeneration of epithelial cells, neutrophil infiltration, macrophage infiltration, minimal hemorrhage and edema. Minimal fibrin debris, hemorrhage and neutrophil infiltration were observed in the lumen.
Figure 19. Effect of oral administration of oxybutynin (1, 3 and 10 mg/kg) on micturition patterns modified by transient intravesical treatment of 0.5% acetic acid in rats. Acetic acid was intravesically infused for 5 minutes at 17:00, and 1 hour later oxybutynin was administered and the micturition patterns monitored for 9 hours. Each bar represents the mean ± S.E.M (n=4-8). ++p<0.01, compared with the saline treated group by the student’s t test. *p<0.05, compared with the vehicle treated group by the Dunnett’s test.
Figure 20. Bladder hyperactivity induced by a transient intravesical application of 0.5% acetic acid solution during the day or at night in rats. Acetic acid solution was intravesically infused for 5 minutes at 9:00 or 17:00. Open bar: Saline treated group. Closed bar: Acetic acid treated group. Each bar represents the mean ± S.E.M (n=4). *p<0.05 and **p<0.01, compared with the corresponding group by the Fisher LSD test.
CHAPTER IV. CONCLUDING REMARKS

Animal models that closely resemble the pathophysiology of human OAB are critical to developing pharmacological therapies for the treatment of the syndrome. Irritative cystometry in rats is one of most commonly used animal models in which the intravesical infusion of irritative agents, like acid, causes an increase in micturition frequency, a decrease in bladder capacity or voided volume and a reduction of bladder compliance via activation of the sensory component in the micturition reflux pathway (13, 15). Although available evidence supports the usefulness of irritative cystometry in the identification of compounds for treatment of bladder dysfunction, results should be interpreted with caution as these protocols often result in inflammatory responses in the bladder wall and substantial damage in the bladder urothelium which are not associated with OAB (13, 14).

To reduce the potential impact of inflammation on bladder function (in addition to the influence of surgical operation and anesthesia on both myogenic and neurogenic functions of the urinary bladder) a protocol using
a transient intravesical infusion of acetic acid solution under light anesthesia was used in the current study.

A five-minute intravesical application of a 0.75% solution of acetic acid produced profound increases in VF, along with a reduction in total urine volume and significant inflammatory alterations in the urinary bladder. This finding is consistent with literature reports on the cystitis model, where irritative intravesical stimulation causes not only an increase in VF but also a reduction in total urine volume and inflammatory changes in the urothelium (25). It is possible that increased bladder damage (and subsequent inflammation) induced by higher concentrations of acetic acid results in pain at lower filling volumes, ultimately leading to a reduction in fluid intake. Pain at lower filling volumes is a primary component of cystitis and therefore the bladder effects produced by the application of 0.75% acetic acid appear to more closely resemble cystitis (rather than OAB) as same as the cystometry method induced by cystometry with 0.2% acetic acid.

In contrast, the transient application of 0.5% acetic acid produced a significant decrease in VF without inflammatory changes or changes in total urine volume. Therefore the effects on bladder function induced by lower concentrations of acetic acid appear to more closely resemble symptoms associated with OAB.
The only oral agents approved for the treatment of OAB are anti-muscarinics. In clinically, new medicines are expected for patients who have adverse events, dry mouth, headache and residual urinary volume, caused by anti-muscarinics (26). The effects of the relatively non-selective anti-muscarinics, oxybutynin, were significantly different between the 0.5% and 0.75% treated groups. The increased VF by 0.5% acetic acid was significantly ameliorated by oral dosing of oxybutynin (3 and 10 mg/kg), whereas that induced by 0.75% and cystometry of 0.2% acetic acid did not significantly improved VF (data not shown) or BC by oxybutynin administration even at a dose of 10 mg/kg. The impairment of functional responses to cholinergic stimulation and the alterations of muscarinic receptor expression in the bladders of rats in cystitis models, including the cystometry model induced by chemical agents, have been reported (27, 28).

The intensive inflammation in the urinary bladder may cause substantial alterations in the functional response to oxybutynin in rats treated with 0.75% and cystometry with 0.2% acetic acid. In addition, multiple studies suggest that a high proportion of interstitial cystitis patients are refractory to treatment with antimuscarinics (29, 30).

Therefore the current findings that oxybutynin was ineffective in rats treated with 0.75% and cystometry with 0.2% acetic acid is consistent with
the relative ineffectiveness of oxybutynin in cystitis patients, while the effectiveness of oxybutynin in affecting micturition in rats treated with 0.5% acetic acid, not cystometry with 0.2% acetic acid, is consistent with its effectiveness in treating OAB.

Nocturia, one of the symptoms of OAB, profoundly influences general health and the quality of life of OAB patients. Although micturition is closely associated with arousal state (31, 32), how the sleep/awake cycle affects urodynamics is poorly understood. In the present study, the 0.5% acetic acid treatment significantly increased VF during both day and night application. Interestingly, increases in VF after nighttime application were remarkably higher than daytime application, although there were no significant differences in the basal levels of VF between daytime and nighttime. The current findings suggest that basal bladder compliance does not exhibit diurnal differences in the rat and that the sensitivity of sensory pathways against irritative stimuli is enhanced during nighttime, the active phase, compared with daytime, the sleeping phase in rats.

The present studies demonstrate that a transient infusion of 0.5% acetic acid without the use of surgical procedures may be a useful model for investigating the efficacy of potential pharmacological treatments for OAB as shown in Figure 21. The transient infusion of 0.5% acetic acid produces
increases in VF without inducing apparent histopathological inflammatory alterations. The described protocol minimizes the secondary effects of experimental procedures such as surgical operations and anesthesia on bladder function and is sensitive to the only approved pharmacological agents for treating OAB, antimuscarinics (Table 2). Thus, the non-invasive rat urinary hyperactive bladder model, induced by a transient intravesical application of acetic acid, may be useful for investigating novel therapeutics for OAB treatment.
1. Figure and Table

**overactive bladder**

(BPH, age, obstructions hypotensions, cancers spinal cord injuries, infections)

uroepithelium

lamina propria

smooth muscle

bladder sphincter<br><internal><br>relax<br>regulation

<external>

contraction

voiding

stimulants<br>acetic acid

c-fiber (unmyelinated)

Aδ-fiber (myelinated)

M3-R<br>antimuscarinics<br>(oxybutynin)

Parasympathetic nerve

 PMC

cerebral cortex

Figure 21. Mechanism of anti-hyperactivity effects by oxybutynin in transient infusion model.
<table>
<thead>
<tr>
<th></th>
<th>transient infusion</th>
<th>cystometry</th>
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<tbody>
<tr>
<td>anesthesia (permanent)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>surgical operation</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>intravesical infusion (continuous)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>bladder inflammation</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>sensitivity to oxybutynin</td>
<td>+</td>
<td>±</td>
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Table 2. Summary of differentiations between a new model, transient infusion, and a standard model, cystometry.
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CHAPTER VI. REFERENCES


