Combined Treatment With a β₃-Adrenergic Receptor Agonist and a Muscarinic Receptor Antagonist Inhibits Detrusor Overactivity Induced by Cold Stress in Spontaneously Hypertensive Rats

Tetsuya Imamura,* Teruyuki Ogawa, Tomonori Minagawa, Takashi Nagai, Toshiro Suzuki, Tetsuichi Saito, Hitoshi Yokoyama, Masaki Nakazawa, and Osamu Ishizuka
Department of Urology, Shinshu University School of Medicine, Matsumoto, Japan

Aims: This study determined if combined treatment with the muscarinic receptor (MR) antagonist solifenacin and the β₃-adrenergic receptor (AR) agonist mirabegron could inhibit detrusor overactivity induced by cold stress in spontaneously hypertensive rats (SHRs). **Methods:** Thirty-two female 10-week-old SHRs were fed an 8% NaCl-supplemented diet for 4 weeks. Cystometric measurements of the unanesthetized, unrestricted rats were performed at room temperature (RT, $27 \pm 2°C$) for 20 min. The rats were then intravenously administered vehicle, 0.1 mg/kg solifenacin alone, 0.1 mg/kg mirabegron alone, or the combination of 0.1 mg/kg mirabegron and 0.1 mg/kg solifenacin (n = 8 each group). Five minutes later, the treated rats were exposed to low temperature (LT, $4 \pm 2°C$) for 40 min. Finally, the rats were returned to RT. After the cystometric investigations, the β₃-ARs and M₃-MRs expressed within the urinary bladders were analyzed. **Results:** Just after transfer from RT to LT, vehicle-, solifenacin-, and mirabegron-treated SHRs exhibited detrusor overactivity that significantly decreased voiding interval and bladder capacity. However, treatment with the combination of solifenacin and mirabegron partially inhibited the cold stress-induced detrusor overactivity patterns. The decreases of voiding interval and bladder capacity in the combination-treated rats were significantly inhibited compared to other groups. Within the urinary bladders, there were no differences between expression levels of M₃-MR and β₃-AR mRNA. The tissue distribution of M₃-MRs was similar to that of the β₃-ARs. **Conclusions:** This study suggested that the combination of solifenacin and mirabegron act synergistically to inhibit the cold stress-induced detrusor overactivity in SHRs. *Neurourol. Urodynam. 36:1026–1033, 2017.* © 2016 The Authors. *Neurourology and Urodynamics* Published by Wiley Periodicals, Inc.

Key words: β₃-adrenergic receptor agonist; cold stress; lower urinary tract symptoms; muscarinic receptor antagonist; spontaneously hypertensive rat

INTRODUCTION

Cold stress is brought on by a sudden decrease in environmental temperature or by continuous exposure to low temperature. It induces lower urinary tract symptoms (LUTS) involving urine storage that manifest as urinary urgency and/or increased urinary frequency. For patients with LUTS, cold stress is one of the factors that exacerbate their symptoms. To investigate mechanisms, treatments, and prevention for cold stress-exacerbated LUTS, we established an animal model with detrusor overactivity induced by cold stress.[1] Cold stress-induced detrusor overactivity, which is characterized by increased basal pressure and decreased micturition volume, voiding interval, and bladder capacity, is considered as a model of storage symptoms.

The bladder has two main functions, that is, urine storage and voiding. Both are controlled by complex neural systems including sympathetic and parasympathetic nerves. During the storage phase, β-adrenergic receptors (ARs) mediate relaxation of urinary bladder smooth muscle by the release of noradrenalin from postganglionic sympathetic nerve terminals. The β₃-ARs are predominant in human bladders.[2–4] During the voiding phase, the postganglionic parasympathetic nerve terminals release acetylcholine that modulates detrusor contractions through both M₃- and M₂-muscarinic receptors (MRs). The M₃-MRs are the primary physiological mediator of the detrusor contraction.[5] Thus, β₃-AR agonists and MR antagonists are clinically used to improve storage symptoms of LUTS

patients.[6,7] In addition, the combination of β₃-AR agonists and MR antagonists is considered to be an effective treatment for LUTS and has fewer side effects than each type of agent alone because of the lower doses that are generally used.[6,7]

Previously, in Goto–Kakizaki (GK) rats, which serve as disease model for diabetes mellitus, we showed that the MR antagonist imidafenacin could inhibit the detrusor overactivity induced by sudden drops to low temperature (LT).[8] Similarly, a high dose (1.0 mg/kg) of the β₃-AR agonist CL316243 could also inhibit the cold stress-induced detrusor overactivity in healthy Sprague–Dawley (SD) rats.[9] These results suggested that the M₃-MRs and β₃-ARs might partially mediate pathways of cold stress-induced detrusor overactivity. In this study, we determined if combined treatments with a β₃-AR agonist and a MR antagonist, which have different mechanism of actions to

improve the urine storage function, could inhibit the cold stress-induced detrusor overactivity in spontaneously hypertensive rats (SHRs).

MATERIALS AND METHODS

Animals

Ten-week-old female SHRs (SHR/Izm, n = 32, 180–200 g, Japan SLC, Inc., Shizuoka, Japan) were used for these experiments. The rats were fed a standard lab chow that was supplemented with 8% NaCl (CLEA Japan, Inc., Tokyo, Japan) to reduce individual differences in hypertension among the animals,[10] and water was freely available. They were maintained under a 12 hr alternating light-dark cycle for 4 weeks. The animals were treated in accordance with National Institutes of Health Animal Care Guidelines and guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine.

Drugs

The MR antagonist solifenacin succinate (solifenacin, HY-A0002, MedChem Express, Princeton, NJ) was dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO). Just prior to administration, the dissolved solifenacin solution was diluted with 0.9% saline to deliver 0.1 mg/kg. The β_3-AR agonist mirabegron, kindly provided by Astellas Pharma Inc. (Tokyo, Japan), was dissolved in 0.9% saline. Just prior to administration, the dissolved mirabegron solution was diluted with 0.9% saline to deliver to 0.1 mg/kg. The final volume of these drug solutions was 0.2 ml, and they were administered intravenously. The mixture of solifenacin and mirabegron was also prepared in a final volume of 0.2 ml to yield the same concentrations as each drug delivered alone.

Cystometric Investigations

After 4 weeks of maintenance on the hypertension-inducing diet containing 8% NaCl, the SHRs were anesthetized with pentobarbital sodium solution (40 mg/kg-body weight, Kyoritsu Seiyaku Co., Tokyo, Japan), and the urinary bladders were exposed and incised at the center of the dome. A polyethylene catheter (PE50, Becton Dickinson and Company, Sparks, MD) was inserted through the incision and fixed at that site with a 5-0 suture. The free end was tunneled subcutaneously and exteriorized at the back of the neck. The cannulated rats were kept for 3 days as above.

Three days after the cannulation, just prior to cystometric investigation, a polyethylene catheter (PE10, Becton Dickinson and Company) was inserted into the jugular vein of rats anesthetized by inhalation of 3% sevoflurane (Abbot Japan Co., Ltd., Tokyo, Japan). Each rat was allowed to recover from the anesthesia in a metabolic cage for 2 hr. After the recovery, the bladder catheter was connected through a T-tube to a pressure transducer (P23 DC; Nihon Kohden, Tokyo, Japan) and a syringe pump (TE-351, Terumo, Tokyo, Japan). To measure micturition volume, a fluid collector connected to a force displacement transducer (Type 45196; NEC San-ei Instruments, Tokyo, Japan) was placed under the metabolic cage. Throughout the experiments, saline kept at room temperature was pumped into the bladder at a rate of 10 ml/hr. The bladder pressure and micturition volume were recorded continuously on a pen oscillograph (10 mm/min recording speed; Recti-Horiz-8K; NEC San-ei Instruments). The following cystometric parameters were measured: basal pressure (cmH_2O), micturition pressure (cmH_2O), voiding interval (min), micturition volume (ml), residual volume (ml), and bladder capacity (ml). Bladder capacity was calculated by adding the micturition volume and residual volume that was determined as the difference between the saline infusion volume and micturition volume. The rats were not given food and water during the cystometric investigations.

Cystometric measurements of the unanesthetized, unrestricted rats were taken under the following environmental temperature conditions. The rats were placed singly in metabolic cages at room temperature (RT, $27 \pm 2°C$) for approximetarly 20 min during which the first cystometric measurements were made to estimate baseline values. They were then intravenously administered with vehicle (0.9% saline), 0.1 mg/kg solifenacin alone, 0.1 mg/kg mirabegron alone, or the combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron (n = 8 in each). Five minutes later, two micturitions were verified. Then the rats were gently and quickly transferred in their metabolic cage to a cold room maintained at $4 \pm 2°C$. Cystometry was continued for two 20 min periods, Phases I and II. Afterward, the rats were gently and quickly returned to the RT room for the final approximately 20 min of cystometry.

After the cystometric investigations, the rats were re-anesthetized by pentobarbital sodium, and the urinary bladders were removed, trimmed, and processed for real-time reverse transcription-polymerase chain reaction (PCR) and immunohistochemical investigations (described below). After the bladders were removed, the rats were euthanized by inhalation of diethyl ether.

Real-Time Reverse Transcription Polymerase Chain Reaction

Expression levels of M_3-MR and β_3-AR mRNAs were semi-quantitatively estimated by real-time reverse transcription PCR. Total RNA was extracted from approximately one-third of the bladder tissue (bladder dome to trigone) with a RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 0.1 μg of total RNA with SuperScript VILO Master Mix (Thermo Fisher Scientific K.K., Foster City, CA). The synthesized cDNA was mixed in TaqMan Universal PCR Master Mix (Thermo Fisher Scientific K.K.) with the following gene assay probes: M_3-MR (Chrm3, Rn00560986_s1, Thermo Fisher Scientific K.K.), β_3-AR (Adrb3, Rn00565393_m1, Thermo Fisher Scientific K.K.), or beta-actin (Actb, Rn00667869_m1, Thermo Fisher Scientific K.K.). Real-time reverse transcription PCR of the mixed cDNA-probe solution was performed at 50°C for 2 min followed by 95°C for 10 min. These were followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Gene expression was calculated by the delta–delta method as the ratio to threshold cycle value of the internal standard gene beta-actin.

Immunohistochemistry

M_3-MRs and β_3-ARs within the urinary bladders were visualized by immunohistochemistry. The harvested urinary bladders were fixed with 4% paraformaldehyde and embedded in paraffin. Serial sections (5 μm) were deparaffinized and treated with 10 mM sodium citrate (pH 6.0, 100°C, 5 min) for antigen retrieval. The specimens were then coated with 1.5% normal donkey serum (Chemicon International, Inc., Temecula, CA) and 1.5% non-fat milk in phosphate buffered saline for 1 hr at 4°C. They were incubated with antibodies against uroplakin III (UPIII (M-17), sc-15186, 1:100, goat polyclonal, Santa Cruz Biotechnology Inc., Santa Cruz, CA), calcitonin gene-related peptide (CGRP, 16013, 1:800, guinea pig polyclonal, Progen

Biotechnik GmbH, Heidelberg, Germany), smooth muscle actin (SMA, 61001, 1:100, mouse monoclonal, Progen Biotechnik GmbH), muscarinic acetylcholine receptor M_3 (ab87199, 1:200, rabbit polyclonal, Abcom, Cambridge, UK), or beta-3 adrenergic receptor (SP4073P, 1:100, rabbit polyclonal, Acris Antibody, Inc., San Diego, CA) for 12 hr at 4°C. The sections were rinsed with 0.01 M phosphate buffered saline (PBS), and then incubated with donkey anti-goat, -guinea pig, or -mouse IgG secondary antibody conjugated with Alexa Fluor 594 (1:250, Thermo Fisher Scientific K.K.), and donkey anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (1:250, Thermo Fisher Scientific K.K.) for 1 hr at 4°C. Finally, after rinsing, cell nuclei were counterstained with 5 μg/ml 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Thermo Fisher Scientific K.K.). The stained samples were observed with a Leica DAS Microscopethe (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical Analysis

The results were expressed as means ± standard error of the means. Two-way repeated measures analysis of variance (ANOVA) and Scheffe's test were used within each group. To compare values for the parameters measured in vehicle-, mirabegron-, solifenacin-, and the combination of solifenacin and mirabegron-treated rats, two-way non-repeated ANOVA and Scheffe's test were used. Differences with $P < 0.05$ were considered significant.

RESULTS

Cold Stress-Induced Detrusor Overactivity in SHRs

Just after transferring from RT to LT, vehicle-treated SHRs exhibited detrusor overactivity patterns characterized by increased frequency of micturition and decreased micturition volume during the first 20 min of LT exposure (Fig. 1A, Phase I). The cold stress-induced detrusor overactivity patterns were maintained during the second 20 min of LT exposure (Fig. 1A, Phase II). After the return to RT, the cold stress-induced detrusor overactivity patterns gradually disappeared (Fig. 1A).

During Phase I, basal pressure of the vehicle-treated SHRs significantly increased ($P < 0.05$, Fig. 2A), while micturition pressure did not change (Fig. 2B). Compared to the Phase I values, the changes in both basal and micturition pressures in Phase II of the vehicle-treated SHRs were not significant. After return to RT, the basal and micturition pressures significantly decreased (Table I). The voiding interval of the vehicle-treated SHRs at RT decreased during Phase I ($P < 0.01$, Fig. 2C). The rate of reduction, determined by dividing the LT Phase I values by the RT baseline values, was 0.41 ± 0.05. Similarly, the bladder capacity also decreased during Phase I ($P < 0.01$, Fig. 2D). The reduction rate of the bladder capacity was 0.41 ± 0.06. During Phase II, the voiding interval and bladder capacity in the vehicle-treated SHRs did not change compared to Phase I (data not shown). After the return to RT, the voiding interval and bladder capacity of the vehicle-treated SHRs significantly increased to the baseline RT values (Table I).

Effect of Antimuscarinic Agent and β₃-Adrenargic Receptor Agonist on Cold Stress-Induced Detrusor Overactivity in SHRs

At the first RT, the baseline micturition patterns of SHRs were not affected with treatment of solifenacin or mirabegron (Fig. 1B and C). During Phases I and II of LT exposure, the SHRs treated with solifenacin alone or mirabegron alone exhibited the cold stress-induced detrusor overactivity patterns that were similar to the patterns of the vehicle-treated SHRs (Fig. 1B and C, respectively). Upon returning to RT following LT Phase II, the cold stress-induced detrusor overactivity patterns for both treatment groups gradually disappeared (Fig. 1B and C).

During LT Phase I exposure, the basal (Fig. 2A) and micturition pressures (Fig. 2B) of the solifenacin-treated SHRs increased ($P < 0.05$ each). These values did not change during the Phase II. Upon returning to RT, both basal and micturition pressures of the solifenacin-treated SHRs significantly decreased (Table I).

After transferring from RT to LT, both basal and micturition pressures of the mirabegron-treated SHRs tended to increase over RT values, but the changes were not significant (Fig. 2A and B, respectively). These values also did not change during Phase II. Upon returning to RT, the basal pressure of the mirabegron-treated SHRs significantly decreased, but the micturition pressure did not change significantly (Table I).

During Phase I, the voiding interval in both solifenacin- and mirabegron-treated SHRs decreased ($P < 0.01$, $P < 0.05$, respectively, Fig. 2C). The reduction rates of solifenacin- and mirabegron-treated SHRs were 0.46 ± 0.03 and 0.38 ± 0.04, respectively. At the same time, the bladder capacity in the solifenacin- and mirabegron-treated SHRs also decreased ($P < 0.01$, $P < 0.05$, respectively, Fig. 2D). The reduction rates of solifenacin- and mirabegron-treated SHRs were 0.46 ± 0.02 and 0.38 ± 0.06, respectively. During Phase II, the voiding interval and bladder capacity in both solifenacin- and mirabegron-treated SHRs did not change compared to Phase I (data not shown). After returning to RT, both voiding interval and bladder capacity significantly increased to the baseline RT values (Table I).

Effects of Combination Treatment With Antimuscarinic Agent and β₃-Adrenargic Receptor Agonist on Cold Stress-Induced Detrusor Overactivity in SHRs

Under RT condition, treatment with the combination of solifenacin and mirabegron did not significantly alter the micturition patterns of the SHRs (Fig. 1D). After transfer to LT, the combination-treated SHRs, in contrast to the vehicle-, solifenacin-, and mirabegron-treated SHRs, did not show an increase in micturition frequency and a decrease in micturition volume (Fig. 1D). As with the vehicle- and single drug-treated SHRs during Phase II, the micturition patterns of the combination-treated SHRs did not change (Fig. 1D). After return to RT, the micturition patterns were similar to the baseline RT patterns (Fig. 1D).
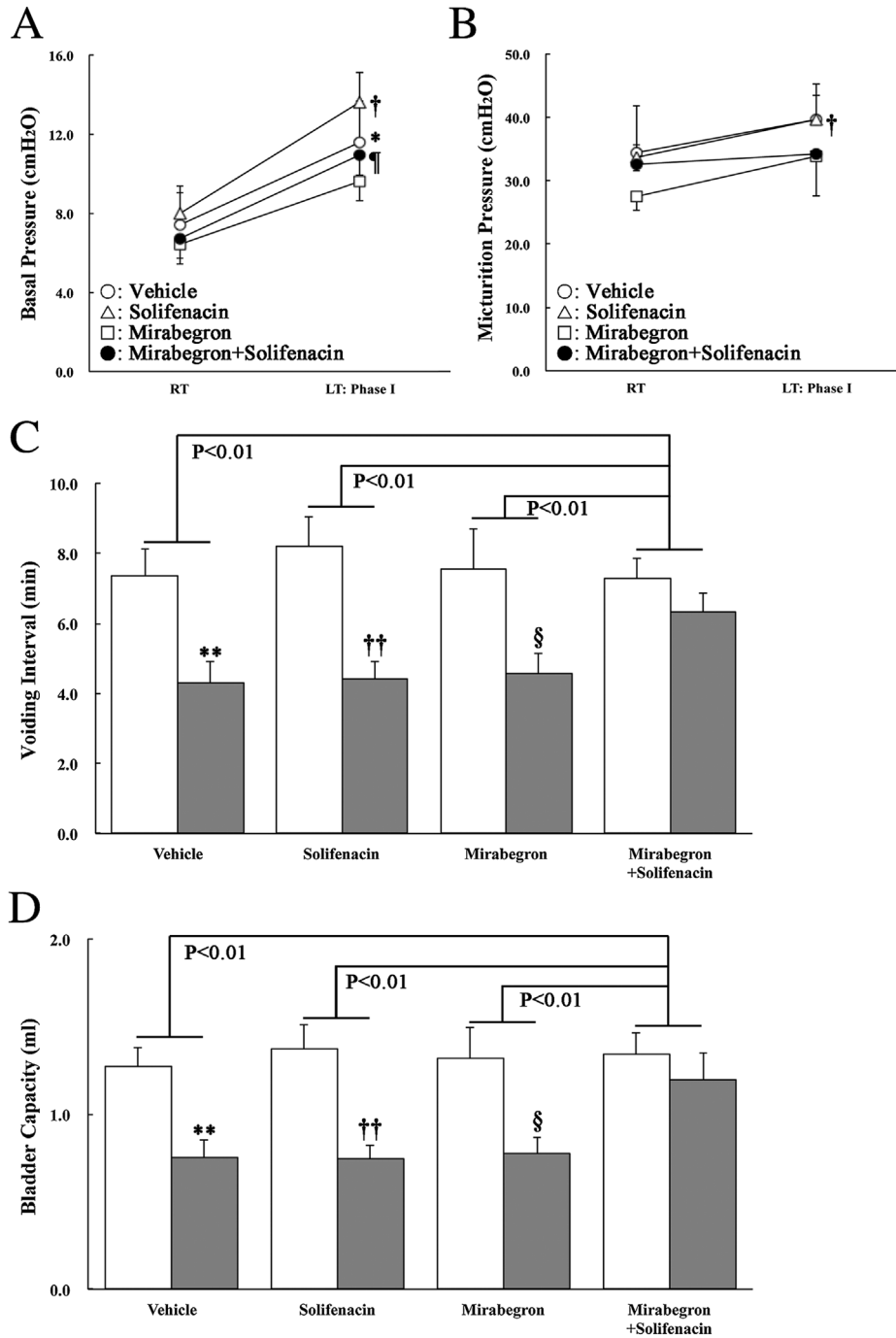
After transferring from RT to LT, the basal pressure of the combination-treated SHRs increased ($P < 0.05$, Fig. 2A), while the micturition pressure did not change (Fig. 2B). During Phase II, neither the basal nor the micturition pressure changed significantly compared to Phase I. Upon returning to RT, both basal and micturition pressures of the combination-treated SHRs significantly decreased (Table I).

During LT Phase I, both voiding interval and bladder capacity of the combination-treated SHRs tended to decrease, but the changes were not significant (Fig. 2C and D, respectively). These values also did not change during Phase II (data not shown). Upon returning to RT, while the voiding interval increased significantly compared to Phase II, the bladder capacity did not change significantly (Table I).

After transfer to LT, the reduction rate of voiding interval in the combination-treated SHRs was 0.14 ± 0.04, which was lower than that for the vehicle-, solifenacin-, and mirabegron-treated SHRs ($P < 0.01$ each). Similarly, for the combination-treated SHRs, the reduction rate of bladder capacity was
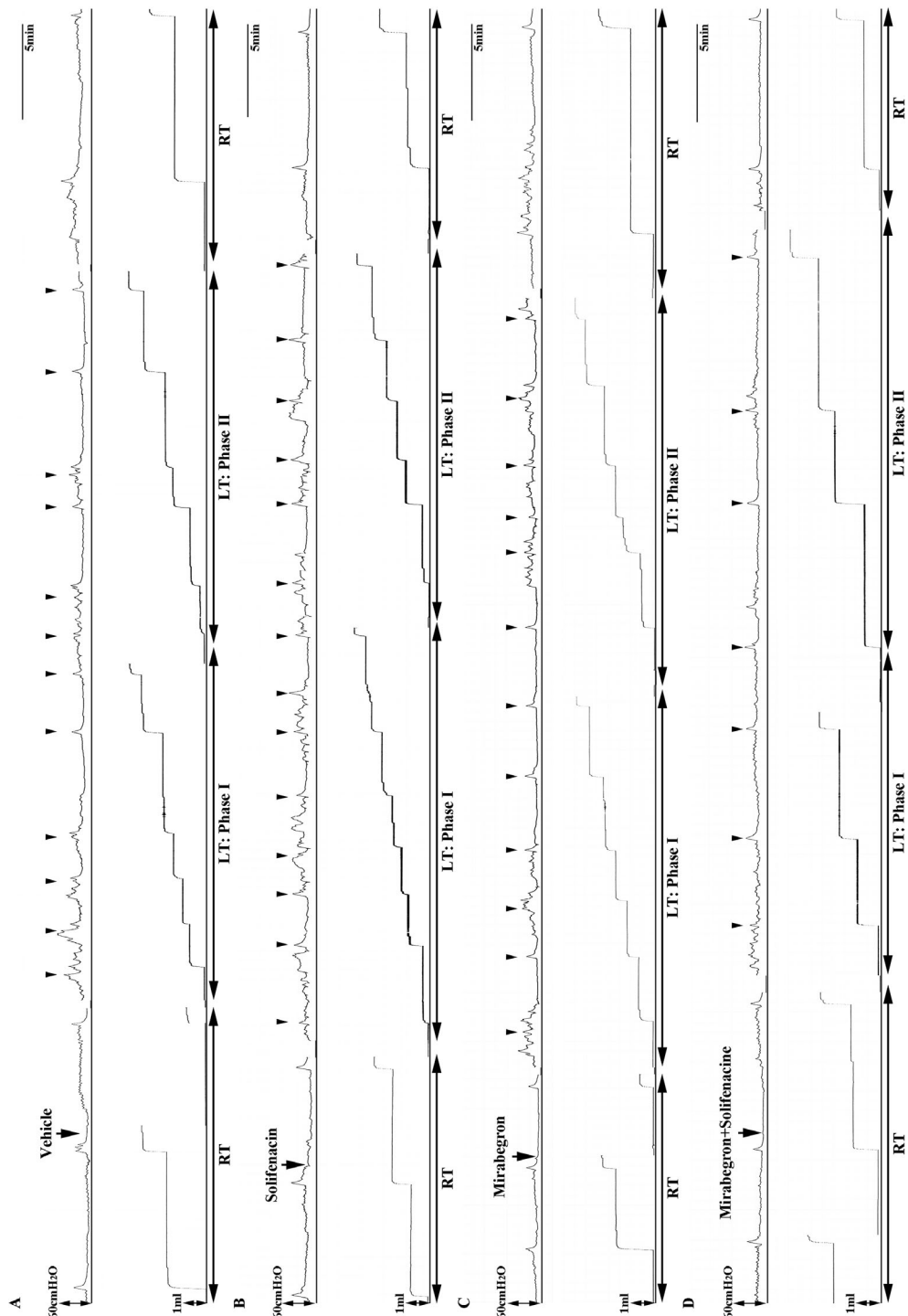
Fig. 1. Changes in micturition patterns during cystometric investigations in representative treated SHRs. In each panel, the top tracing is bladder pressure, and the bottom is micturition volume. (**A**) Just after transfer from RT to LT, the vehicle-treated SHRs exhibited detrusor overactivity patterns. The cold stress-induced detrusor overactivity patterns were maintained during both Phase I and Phase II of LT exposure. Upon return to RT, the cold stress-induced detrusor overactivity patterns gradually disappeared. (**B and C**) During the first RT exposure, treatment with solifenacin (**B**) or mirabegron (**C**) had no effect on micturition patterns. During both phases of LT exposure, the (**B**) solifenacin- and (**C**) mirabegron-treated SHRs exhibited cold stress-induced detrusor overactivity patterns that were similar to the vehicle-treated SHRs (**A**). Upon return to RT, the cold stress-induced detrusor overactivity patterns also gradually disappeared. (**D**) The treatments with combination of solifenacin and mirabegron did not alter the micturition patterns at RT. After transfer to LT, the combination-treated SHRs did not show the cold stress-induced detrusor overactivity patterns. After return to RT, the micturition patterns remained similar to the baseline RT patterns. Arrowheads: micturition points during LT exposure.

Fig. 2. Changes in basal and micturition pressure, voiding interval, and bladder capacity after transfer from RT to LT. (**A**) Upon transfer to LT, the basal pressure of vehicle-, solifenacin-, and combination-treated SHRs increased significantly. While the basal pressure of the mirabegron-treated animals also increased, the change was not statistically significant. (**B**) At LT, micturition pressure increased significantly only for the solifenacin-treated SHRs. (**C and D**) Upon transfer to LT, both voiding interval (**C**) and bladder capacity (**D**) in vehicle-, solifenacin-, and solifenacin-treated SHRs decreased significantly. In the combination-treated SHRs, the decreases in these parameters were significantly inhibited compared to the vehicle-, solifenacin-, and mirabegron-treated SHRs. White bar; RT value, Gray bar; LT Phase I value. $^*P < 0.05$, $^{**}P < 0.01$; compared to RT values in the vehicle-treated rats, $^\dagger P < 0.05$, $^{\dagger\dagger}P < 0.01$; compared to RT values in the solifenacin-treated rats, $^\S P < 0.05$; compared to RT values in the mirabegron-treated rats, $^\P P < 0.05$; compared to RT values in the combination-treated rats.

0.11 ± 0.06, which was also lower compared to the vehicle-, solifenacin-, and mirabegron-treated SHRs ($P < 0.01$ each). Therefore, decreases of both voiding interval and bladder capacity from RT to LT in the combination-treated SHRs were significantly inhibited compared to the vehicle-, solifenacin-, and mirabegron-treated SHRs (Fig. 2C and D, respectively).

Expression of M₃-Muscarinic and β₃-Adrenergic Receptors Within SHR Urinary Bladders

Within the urinary bladders of the SHRs, the expression level of M_3-MR mRNA tended to be higher than that of β_3-AR mRNA; however, the differences were not significant (Fig. 3A). By

TABLE I. Micturition Parameter Changes in SHRs Returned From Low Temperature to Room Temperature

	Vehicle-treated	Solifenacin-treated	Mirabegron-treated	Solifenacin + mirabegron-treated
Basal pressure				
LT: Phase II	11.03 ± 1.29	12.83 ± 1.67	10.72 ± 1.61	11.36 ± 1.61
RT	6.22 ± 1.23**	9.77 ± 1.94*	7.33 ± 3.25*	6.26 ± 1.63**
Micturition pressure				
LT: Phase II	37.28 ± 4.99	43.98 ± 3.99	35.05 ± 5.23	35.98 ± 3.75
RT	29.06 ± 5.99**	35.37 ± 4.13**	27.47 ± 2.70	22.30 ± 1.91**
Voiding interval				
LT: Phase II	4.05 ± 0.38	4.65 ± 0.61	4.40 ± 0.26	6.23 ± 0.26
RT	7.04 ± 0.66**	7.86 ± 0.86**	7.84 ± 1.11**	7.80 ± 0.89*
Bladder capacity				
LT: Phase II	0.72 ± 0.06	0.79 ± 0.10	0.75 ± 0.04	1.17 ± 0.07
RT	1.23 ± 0.09**	1.33 ± 0.14**	1.35 ± 0.18**	1.36 ± 0.17

$^{*}P < 0.05$, $^{**}P < 0.01$, compared to Phase II; LT: low temperature; Phase II: LT exposure 20–40 min; RT: room temperature following Phase II; basal pressure and micturition pressure, cmH$_2$O; voiding interval, min; bladder capacity, ml.
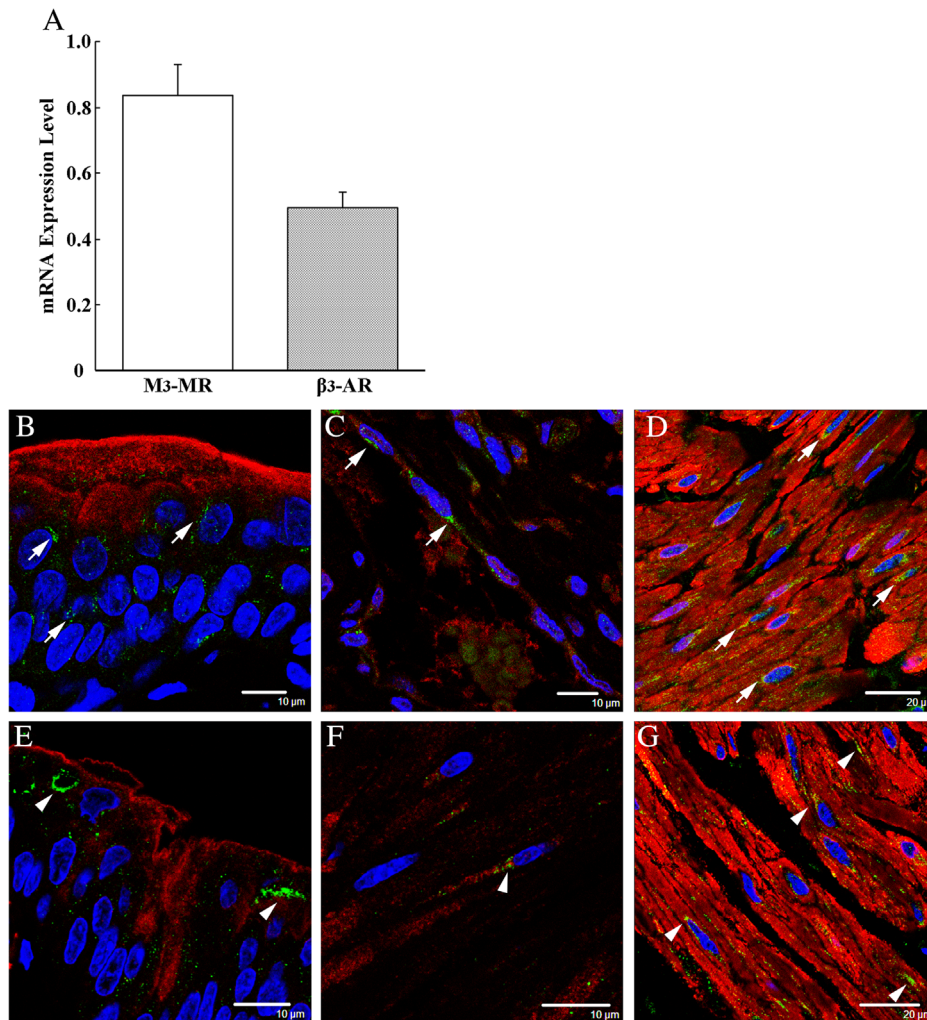

Fig. 3. Expression of M$_3$-muscarinic and β$_3$-adrenergic receptors within the urinary bladders. (**A**) Within the urinary bladders of the SHRs, there was no difference between expression levels of M$_3$-MR and β$_3$-AR mRNA. The mRNA expression levels were calculated as the ratio to the internal standard gene, beta-actin mRNA. (**B–D**) M$_3$-MRs (green, arrows) were expressed within (**B**) the uroplakin III-positive urothelium (red), (**C**) the CGRP-positive afferent nerve cells (red), and (**D**) the SMA-positive detrusor (red). (**E–G**) Similarly, β$_3$-ARs (green, arrowheads) were also detected within the (**F**) urothelium (red), (**G**) afferent nerve cells (red), and (**H**) detrusor (red). Blue: nuclei.

immunohistochemistry, M_3-MRs were detected within the uroplakin III-positive urothelium (Fig. 3B), CGRP-positive afferent nerve cells (Fig. 3C), and SMA-positive detrusor (Fig. 3D). Similarly, β_3-ARs were also detected within the urothelium (Fig. 3F), afferent nerve cells (Fig. 3G), and detrusor (Fig. 3H).

DISCUSSION

In rats, cold stress associated with a sudden drop to LT induces detrusor overactivity that increases micturition frequency and decreases micturition volume due to decreased voiding interval and bladder capacity.[1] In this study, we also showed that the cold stress-exposed SHRs exhibited similar detrusor overactivity. In healthy normal SD rats, the cold stress-induced detrusor overactivity is slowly mitigated with cold-exposure time during Phase II. However, the cold stress-induced detrusor overactivity of the SHRs was not diminished with time during the LT exposure. These continued cold stress-induced detrusor overactivity patterns were recognized in latent LUTS rats, such as GK rats.[8]

In this study, we used SHRs maintained on a diet containing 8% NaCl to exacerbate their LUTS,[10] and then determined if the combination treatment with the MR antagonist solifenacin and the β_3-AR agonist mirabegron could inhibit the cold stress-induced detrusor overactivity. SHRs treated with solifenacin alone exhibited cold stress-induced detrusor overactivity patterns that were similar to the vehicle-treated SHRs. We previously showed that many kinds of MR antagonists also do not inhibit the cold stress-induced responses in the SD or Wister–Kyoto (WKY) rats.[11] However, one of the MR antagonists, imidafenacin, does inhibit the cold stress-induced detrusor overactivity in GK rats in which the ratio of M_3-MR to M_2-MR was higher compared to the WKY rats.[8] Thus, we suggested that pathways through M_3-MRs, at least in part, could mediate the cold stress-induced responses, and the effects of the MR antagonists on the cold stress-induced detrusor overactivity depended on the expression level of M_3-MRs.

Similarly, treatment of the SHRs with mirabegron alone did not inhibit the cold stress-induced detrusor overactivity patterns. We previously showed that the same low dose (0.1 mg/kg) of the β_3-AR agonist CL316243 did not inhibit the cold stress-induced responses in SD rats.[9] However, a higher dose, 1.0 mg/kg, did inhibit the cold stress-induced detrusor overactivity.[9] Thus, we suggested that while the pathways involving β_3-ARs could mediate the cold stress-induced responses, the effects were dose dependent.

In the present study, we showed that the combination of a MR antagonist and a β_3-AR agonist, which were each ineffective alone, partially inhibited the cold stress-induced responses in SHRs. The MR antagonist solifenacin is used for symptomatic treatment of urinary frequency, urgency, and/or incontinence because it inhibits detrusor contractions modulated through muscarinic receptors activated by postganglionic parasympathetic nerve terminals that release acetylcholine.[5] The β_3-AR agonist mirabegron facilitates the storage of urine by activating β_3-adrenergic receptors present within the detrusor muscle cells.[7] Therefore, we hypothesized that the different mechanisms of action for solifenacin and mirabegron could act synergistically to inhibit the cold stress-induced detrusor overactivity in SHRs.

The distribution in human bladder tissues of β_3-ARs is similar to that of MRs.[12–14] By immunohistochemistry of the SHR bladders, we showed that, as in humans, the presence of

the M_3-MRs was similar to that of the β_3-ARs in the urothelium and detrusors of SHRs. During urine storage, bladder afferent nerves are activated by the binding of acetylcholine, which is released from the urothelium, to the MRs.[15,16] MR antagonists can effectively inhibit the activation of afferent nerves by blocking the MRs. In addition, several animal studies suggested that the MR antagonists might desensitize the bladder afferent nerves.[17–19] In contrast, the β_3-AR agonists bind to receptors within the urothelium, stimulating the release of nitric monoxide (NO) by the urothelium.[20,21] The urothelium-released NO inhibits the activation of the afferent nerves.[22] Some studies report that activation of β_3-ARs in rats has inhibitory effects on bladder afferent activity.[23,24] Furthermore, the combination of antimuscarinic agents and β_3-AR agonists inhibits a part of the Rho kinase pathway in vitro.[25] Therefore, the combination of solifenacin and mirabegron might effectively promote the bladder functions during the storage phase through inhibition of afferent nerve activation. In turn, this would inhibit the cold stress-induced detrusor overactivity.

Our study suggested that the combination of MR antagonists and β_3-AR agonists have the potential to be more effective treatments for LUTS than either type of agent alone. While MR antagonists are the ones mainly used to treat overactivity bladder, these agents can give rise to adverse events, such as dry mouth, constipation, and voiding difficulty resulting from an increase of residual urine.[26] The β_3-adrenergic receptor agonists also have a few side effects, such as cardiovascular events due to changes in heart rate and blood pressure.[26] However, the side effects associated with both agents might be controlled by reducing the doses.[27] In addition, there were no relevant differences in frequency of these adverse events between the combination- and single-treatments using the same doses.[27,28] Therefore, the combination of antimuscarinic agents and β_3-AR agonists may maximize the efficacy while minimizing the side effects.

This study had some limitations. Our cold stress model reflects systemic reactions due to "whole body" cooling. Thus, the analysis of partial responses that may have occurred within the urinary bladders following drug administration is limited. However, detrusor overactivity due to intravesical instillation of oxotremorine methiodide was inhibited by an intravenous combination of antimuscarinic agents and β_3-AR agonists.[29] This is consistent with our results that suggest that the combined treatments might have direct effects on the urinary bladder. We tested only one dose of each drug alone and in combination. We selected 0.1 mg/kg for each agent, which alone would not inhibit the cold stress-induced detrusor overactivity in SHRs. We estimated mRNA expression levels of β_3-ARs and M_3-MRs within the whole urinary bladder tissues; however, we did not compare the expression of these receptors within either the urothelium or detrusor. Finally, we have not yet investigated the mechanism by which cold stress-induced detrusor overactivity in SHRs was undiminished during Phase II of LT exposure.

CONCLUSIONS

Just after transfer from RT to LT, cold stress-exposed SHRs exhibited detrusor overactivity that significantly decreased voiding interval and bladder capacity. Treatment with 0.1 mg/kg solifenacin- or mirabegron did not inhibit the cold stress-induced detrusor overactivity. However, the combination of the solifenacin and mirabegron at the same doses partially inhibited the cold stress-induced responses. After transfer to LT, the decreases of both voiding interval

and bladder capacity in the combination treated-rats were significantly inhibited compared to other groups. This study suggested that the different mechanisms of action of solifenacin and mirabegron are synergistic in inhibiting the cold stress-induced detrusor overactivity in SHRs. Therefore, the combination drug treatments might provide significant improvements over single drug treatment and be without serious side effects in patients with cold stress-exacerbated LUTS.

REFERENCES

1. Imamura T, Ishizuka O, Aizawa N, et al. Cold environmental stress induces detrusor overactivity via resiniferatoxin-sensitive nerves in conscious rats. Neurourol Urodyn 2008;27:348–52.
2. Fujimura T, Tamura K, Tsutsumi T, et al. Expression and possible functional role of the beta3-adrenoceptor in human and rat detrusor muscle. J Urol 1999;161:680–5.
3. Igawa Y, Yamazaki Y, Takeda H, et al. Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle. Br J Pharmacol 1999;126:819–25.
4. Takeda M, Obara K, Mizusawa T, et al. Evidence for beta3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: Analysis by molecular biological and pharmacological methods. J Pharmacol Exp Ther 1999;288:1367–73.
5. Ohtake A, Saitoh C, Yuyama H, et al. Pharmacological characterization of a new antimuscarinic agent, solifenacin succinate, in comparison with other antimuscarinic agents. Biol Pharm Bull 2007;30:54–8.
6. Cardozo L, Lisec M, Millard R, et al. Randomized, double-blind placebo controlled trial of the once daily antimuscarinic agent solifenacin succinate in patients with overactive bladder. J Urol 2004;172:1919–24.
7. Yamaguchi O. Beta3-adrenoceptors in human detrusor muscle. Urology 2002;59:25–9.
8. Imamura T, Ishizuka O, Ogawa T, et al. Muscarinic receptors mediate cold stress-induced detrusor overactivity in type 2 diabetes mellitus rats. Int J Urol 2014;21:1051–8.
9. Imamura T, Ishizuka O, Ogawa T, et al. Pathways involving beta-3 adrenergic receptors modulate cold stress-Induced detrusor overactivity in conscious rats. Low Urin Tract Symptoms 2015;7:50–5.
10. Imamura T, Ishizuka O, Zhong C, et al. An extract (THC-002) of Ba-Wei-Die-Huang-Wan inhibits expression of tachykinins, and $P2 \times 3$ and TRPV1 receptors, and inhibits ATP-induced detrusor overactivity in spontaneously hypertensive rats. Neurourol Urodyn 2009;28:529–34.
11. Chen Z, Ishizuka O, Imamura T, et al. Role of alpha1-adrenergic receptors in detrusor overactivity induced by cold stress in conscious rats. Neurourol Urodyn 2009;28:251–6.
12. Bschleipfer T, Schukowski K, Weidner W, et al. Expression and distribution of cholinergic receptors in the human urothelium. Life Sci 2007;80:2303–7.
13. Otsuka A, Kawasaki H, Matsumoto R, et al. Expression of β-adrenoceptor subtypes in urothelium, interstitial cells and detrusor of the human urinary bladder. Low Urin Tract Symptoms 2013;5:173–80.
14. Tyagi S, Tyagi P, Van-le S, et al. Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. J Urol 2006;176:1673–8.
15. Lips KS, Wunsch J, Zarghooni S, et al. Acetylcholine and molecular components of its synthesis and release machinery in the urothelium. Eur Urol 2007;51:1042–53.
16. Yoshida M, Inadome A, Maeda Y, et al. Non-neuronal cholinergic system in human bladder urothelium. Urology 2006;67:425–30.
17. De Laet K, De Wachter S, Wyndaele JJ. Systemic oxybutynin decreases afferent activity of the pelvic nerve of the rat: New insights into the working mechanism of antimuscarinics. Neurourol Urodyn 2006;25:156–61.
18. Iijima K, De Wachter S, Wyndaele JJ. Effects of the M3 receptor selective muscarinic antagonist darifenacin on bladder afferent activity of the rat pelvic nerve. Eur Urol 2007;52:842–7.
19. Yokoyama O, Yusup A, Miwa Y, et al. Effects of tolterodine on an overactive bladder depend on suppression of C-fiber bladder afferent activity in rats. J Urol 2005;174:2032–6.
20. Birder LA, Nealen ML, Kiss S, et al. Beta-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. J Neurosci 2002;22:8063–70.
21. Otsuka A, Shinbo H, Matsumoto R, et al. Expression and functional role of beta-adrenoceptors in the human urinary bladder urothelium. Naunyn Schmiedebergs Arch Pharmacol 2008;377:473–81.
22. Andersson KE, Persson K. Nitric oxide synthase and the lower urinary tract: Possible implications for physiology and pathophysiology. Scand J Urol Nephrol Suppl 1995;175:43–53.
23. Hatanaka T, Ukai M, Watanabe M, et al. Effect of mirabegron, a novel β3-adrenoceptor agonist, on bladder function during storage phase in rats. Naunyn Schmiedebergs Arch Pharmacol 2013;386:71–8.
24. Sawada N, Nomiya M, Hood B, et al. Protective effect of a β3-adrenoceptor agonist on bladder function in a rat model of chronic bladder ischemia. Eur Urol 2013;64:664–71.
25. Frazier EP, Peters SL, Braverman AS, et al. Signal transduction underlying the control of urinary bladder smooth muscle tone by muscarinic receptors and beta-adrenoceptors. Naunyn Schmiedebergs Arch Pharmacol 2008;377:449–62.
26. Yamaguchi O. Latest treatment for lower urinary tract dysfunction: Therapeutic agents and mechanism of action. Int J Urol 2013;20:28–39.
27. Abrams P, Kelleher C, Staskin D, et al. Combination treatment with mirabegron and solifenacin in patients with overactive bladder: Efficacy and safety results from a randomised, double-blind, dose-ranging, phase 2 study (Symphony). Eur Urol 2015;67:577–88.
28. Yamaguchi O, Kakizaki H, Homma Y, et al. Safety and efficacy of mirabegron as 'add-on' therapy in patients with overactive bladder treated with solifenacin: A post-marketing, open-label study in Japan (MILAI study). BJU Int 2015;116:612–22.
29. Furuta A, Suzuki Y, Kimura S, et al. Combination therapy with β3-adrenoceptor agonists and muscarinic acetylcholine receptor antagonists: Efficacy in rats with bladder overactivity. Int J Urol 2016;23:425–30.