下部尿路閉塞ラットの冷えストレス排尿筋過活動に対するα1受容体拮抗薬とホスホジエステラーゼ5阻害薬の併用によるレジニフェラトキシン感受性求心性神経を介した抑制機序

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The Combination of α1-adrenergic Receptor Antagonist and Phosphodiesterase 5 Inhibitor Mitigates Cold Stress-induced Detrusor Overactivity through Resiniferatoxin-Sensitive Nerves in Bladder Outlet Obstructed Rats

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Key words: cold stress, bladder outlet obstruction, α1-adrenergic receptor antagonist, phosphodiesterase 5 inhibitors, resiniferatoxin

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Abstract

Background: We determined if the α1-adrenergic receptor (AR) antagonist naftopidil, the phosphodiesterase 5 (PDE5) inhibitor tadalafil, or the combination inhibited cold stress-induced detrusor overactivity in bladder outlet obstructed rats. We also investigated the role of resiniferatoxin (RTX)-sensitive nerves in detrusor overactivity.

Methods: The urethras of 10-week-old female Sprague-Dawley rats were loosely ligated to create a partial bladder outlet obstruction. After 4 weeks, at room temperature (RT, 27℃), the rats were randomly assigned to receive an intraperitoneal infusion of vehicle control (n=11), 0.15 mg/kg-body weight naftopidil (n=7), 0.5 mg/kg-body weight tadalafil (n=7), or the combination of naftopidil and tadalafil (n=11). The treated rats were then exposed to low temperature (LT, 4℃) for cystometry. Other rats were subcutaneously injected with 0.3 mg/kg RTX (n=8), and then two days later underwent cystometric investigations. The number of calcitonin gene-related peptide (CGRP)-positive neurons was examined by immunohistochemistry.

Results: After transfer from RT to LT, the vehicle-, naftopidil-, and tadalafil-treated rats had decreased voiding intervals and bladder capacity. These decreases were inhibited by the combined naftopidil-tadalafil treated rats. RTX caused similar cystometric decreases as the combination-treated rats. The number of the CGRP-positive afferent nerves in the RTX-treated rats was significantly reduced.

Conclusion: The combination of an α1-AR antagonist and a PDE5 inhibitor mitigated the cold stress-induced detrusor overactivity in bladder outlet obstructed rats. RTX treatment also inhibited the cold stress responses while reducing the presence of CGRP in afferent nerves. α1-AR antagonists and PDE5 inhibitors could act efficiently, and may affect RTX-sensitive nerves, to reduce cold stress-induced detrusor overactivity.

（和訳）

目的： 我々は、ラット下部尿路閉塞モデルにナフトピジル、ホスホジエステラーゼ5（PDE5）阻害薬のタダラフィルまたはそれを併用することで冷えストレスによって誘発される排尿筋過活動を抑制するか検討した。また、併用による冷えストレス排尿筋過活動の抑制機序のひとつとしてレジンフェラトキシン（RTX）感受性　心性神経の関与について検討を行った。

方法： 10 週齢雌 Sprague-Dawley (SD) ラットの尿道を結紮した後、4 週間飼育したものを下部尿路閉塞ラットとした。尿道結紮を開放したラットを生食(n=11)、0.15mg/ g-ナフトピジル(n=7)、0.5mg/ g-タダラフィル(n=7)、同用　のナフトピジルとタダラフィル併用(n=11)の 4 群に　　た。最初に、　　（RT：7± ℃）で　脱内圧を行った後、それぞれの薬剤を　腔内　与した。　与　0　後、　　（LT:4± ℃）に速やかに移して　脱内圧測定を行った。また、　脱内圧測定　日前に 0.3 mg/ g の RTX を皮下与した下部尿路閉塞ラット(n=8)に対して、同様の　脱内圧測定を実施した。　疫染色にて　ルシトニン遺伝子関連ペプチド（CGRP）陽性神経細胞の個数を調べた。

結果：　　下では、各群間での　脱機　に変化は認められなかった。　　から　に移行したとき、生食、ナフトピジル、タダラフィル　与群で 1 回排尿間隔時間と　脱容　が　下する排尿筋過活動を呈した。しかし、併用群での 1 回排尿間隔時間と　脱容　の　下は、ほかの群と比較して、抑制される傾向があった。RTX　与群でも併用群と同様な傾向を示した。また、RTX　与群では、CGRP 陽性　心性神経細胞数が有意に減少していた。

結論： α1 受容体遮断薬と PDE5 阻害薬の併用は、下部尿路閉塞ラットの冷えストレスによって誘発される排尿筋過活動の一部を抑制することが示唆された。RTX　与群では、他の群と比較して CGRP 陽性神経細胞がより少なくなる傾向がみられた。ナフトピジルとタダラフィルは RTX 感受性神経を介しており冷えストレスによる過活動を部的に抑制していると思われる。

I Introduction

Patients with benign prostatic hyperplasia (BPH) often complain about lower urinary tract symptoms (LUTS) that occur due to bladder outlet obstruction. Thus, patients with BPH are treated with an α1-adrenergic receptor (AR) antagonist and/or a phosphodiesterase 5 (PDE5) inhibitor to release the obstruction. In clinical practice, combination therapy with both an α1-AR antagonist and a PDE5 inhibitor is often effective for overactive bladder symptoms[1)-4)]. One of the factors that exacerbates LUTS is cold stress due to a sudden drop in temperature or repeated exposure to a low temperature environment. We have established a rat model for cold stress LUTS that elicits detrusor overactivity[5)]. The cold stress-exacerbated LUTS is mediated by cross talk among neurological pathways, including unmyelinated C fibers within the urinary bladder[5)], enhancement of sympathetic nerve activity[6)], and expression of transient receptor potential cation channel, subfamily M, member 8 (TRPM8) in the skin[7)].

We have developed a second rat model for testing the pharmacological effects of drugs on cold stress-induced LUTS[8)]. The model, which is based on partial obstruction of the bladder outlet, mimics the human form of LUTS associated with BPH. In that model, the cold stress-induced detrusor activity and changes in bladder storage characteristics are mitigated by treatment with an α1-AR antagonist[8)]. In addition, we showed that PDE5 inhibition reduces unmyelinated C fiber-related detrusor overactivity elicited by acetic acid in nicotine-treated rats[9)]. In normal healthy rats, treatment with resiniferatoxin (RTX), a capsaicin analogue that reduces the content of calcitonin gene-related peptide (CGRP) in unmyelinated C fibers, suppresses C fiber activation and inhibits cold stress-induced detrusor overactivity[5) 10)-13)],

Based on these previous findings, in this study we determined if the combination of an α1-AR antagonist and a PDE5 inhibitor, at lower doses than either alone, could inhibit the cold stress-induced detrusor overactivity in rats with partial bladder outlet obstruction. We also investigated the effects of RTX treatment on the CGRP content of the bladder unmyelinated C fibers and on cold stress-induced detrusor overactivity.

II Material and Methods

A Animals

Ten-week-old female Sprague-Dawley (SD) rats (Japan SLC Inc., Shizuoka, Japan) were housed for 4 weeks under a 12-hour alternating light-dark cycle with freely available food and water. The animals were treated in accordance with National Institutes of Health Animal Care Guidelines and the protocol was approved by the Animal Ethics Committee of Shinshu University School of Medicine

B Preparation of bladder outlet obstructed rats

The SD rats were anesthetized with midazolam (2.0 mg/kg-body weight, Sandoz International GmbH, Tokyo, Japan), medetomidine hydrochloride (0.15 mg/kg-body weight, Kyoritsu Seiyaku Co., Tokyo, Japan), and butorphanol tartrate (2.5 mg/kg-body weight, Meiji Seika Pharma Co., Ltd., Tokyo, Japan). A midline incision was made to expose the urethra, and a metal rod with an outer diameter of 1.1 mm was placed alongside it. To produce bladder outlet obstruction, the urethras were loosely ligated to the metal rod with 5-0 silk. Afterwards, the metal rod was carefully removed, leaving the ligature to create a partial bladder outlet obstruction. The incision was then closed. The rats with bladder outlet obstruction were housed for 4 weeks (as above). Based upon cystometric investigation (see below) to identify effectively ligated rats with bladder obstruction, only animals with a bladder volume between 2 and 5 ml at room temperature (RT, 27±2℃) were selected for the following experiments.

C Drugs

We used naftopidil, kindly provided by Asahi Kasei Pharma Co. (Tokyo, Japan), as an α1-AR blocker. The naftopidil powder was completely dissolved with 0.1 M phosphate buffer solution in half of the final volume. Then several drops of 0.1 M sodium dihydrogen phosphate solution were slowly added with vortexing and ultrasonication to achieve the final volume and pH 4.0. The dissolved naftopidil solution was diluted to the desired concentration with 0.9% saline. Tadalafil powder (Toronto Research Chemical Inc., Toronto, Canada), a PDE5 inhibitor, was completely dissolved with dimethyl sulfoxide (DMSO, Fujifilm Wako Pure Chemical Co., Osaka, Japan). The dissolved tadalafil solution was diluted to the desired concentration with DMSO. Resiniferatoxin powder (RTX, Sigma-Aldrich, Steinheim, Germany), a capsaicin analogue, was completely dissolved with DMSO. The dissolved RTX solution was diluted with DMSO to the deliver 0.3 mg/kg by subcutaneous injection.

D Cystometric investigations

Four weeks after creating the partial bladder outlet obstruction and 2 days prior to the cystometric investigations, the animals were anesthetized (as above) to insert a catheter for cystometric investigations. The urinary bladder and ligated urethra were exposed, and the ligature thread was then removed. A polyethylene catheter (PE50, Becton Dickinson and Company, Sparks, MD, USA) was inserted at the center of the bladder dome. The catheter was fixed at that site with a 5-0 suture. For delivery of vehicle or drugs during the cystometry experiments, another catheter (PE90, Becton Dickinson and Company), was inserted into the intraperitoneal space. Both catheters were brought out subcutaneously to the back and fixed with 3-0 silk sutures. After the operation, each rat was caged individually for two days.

For cystometry, 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). Saline (0.9% NaCl) was infused continuously into the bladder at a rate of 10 ml/hr. A urine collector connected to a force displacement transducer (type 45196; NEC San-ei Instruments, Tokyo, Japan) enabled measurement of micturition volume. The bladder pressure and micturition volume were continuously recorded with LabChart system (AD Instruments, BRC Bioresearch, Inc., Nagoya Japan) through a PowerLab system (AD Instruments).

The following cystometric parameters were measured: basal pressure (cmH_2O), micturition pressure (cmH_2O), voiding interval (min), and bladder capacity (ml). The bladder capacity was calculated by adding the micturition volume and the residual volume that was determined as the difference between the saline infusion volume and micturition volume. The rats were not given food or water during the cystometric investigations.

Cystometric measurements of the unanesthetized, unrestricted rats were made under the following environmental temperature conditions. They were randomly separated into the control and three experimental groups as follows: (1) vehicle control (n=11), (2) 0.15 mg/kg-body weight naftopidil (n=7), (3) 0.5 mg/kg-body weight tadalafil (n=7), and (4) 0.15 mg/kg-body weight naftopidil and 0.5 mg/kg-body weight tadalafil (n=11). Cystometric measurements were then conducted to obtain baseline measurements for approximately 20 minutes at RT. Through the intraperitoneal catheter, each rat received either the control vehicle, naftopidil, tadalafil, or the combination of naftopidil and tadalafil. Twenty minutes after the treatments, the rats were quickly and smoothly transferred in the metabolic cages to a refrigerator (MPR-513, SANYO Tokyo Manufacturing Co., Ltd., Tokyo, Japan) for exposure to low temperature (LT, 4±2°C). The

bladder pressure and micturition volume of the rats were again recorded for 40 min. After the cystometric investigations, the rats were anesthetized as above, and then the urinary bladders were removed, and the rats were then euthanized by inhalation of diethyl ether.

Two days prior to the cystometric investigations, other rats with partial bladder outlet obstructions were catheterized as above and then subcutaneously injected with 0.3 mg/kg-body weight RTX. Two days later, the RTX-treated rats underwent the same LT-exposure cystometric investigations.

E Immunohistochemistry investigations

The harvested urinary bladders were fixed in 4% paraformaldehyde phosphate buffer solution (Nalkalai Tesque, Inc., Kyoto, Japan) for 12 hours at 4ºC. The tissues were embedded in paraffin, and cut into 5-μm thick serial sections. The sections were deparaffinized, and then antigen retrieval was achieved by immersion of the sections in 0.01 M sodium citrate (pH 6.0, Mitsubishi Chemical Medience Co., Tokyo, Japan) and microwaving at 100ºC for 5 minutes. The specimens were coated with 1.5% normal donkey serum (Chemicon International Inc., Temecula, CA, USA) and 1.5% non-fat milk in 0.01 M phosphate buffered saline (PBS, pH 7.4, Mitsubishi Chemical Medience Co.) for 1 hour at 4ºC. The sections were then incubated with primary antibodies, for CGRP (1:800, guinea pig polyclonal, Progen Biotechnik GmbH, Heidelberg, Germany) as a marker of afferent nerves, and smooth muscle actin (SMA, 1:100, mouse monoclonal, Progen Biotechnik GmbH, Heidelberg, Germany) for 12 hours at 4ºC. The sections were rinsed with PBS at 4ºC, and then incubated with donkey anti-guinea pig IgG secondary antibody conjugated with Alexa Fluor 594 (1:250, Life Technology Co., Molecular Probes, Eugene, OR, USA) and donkey anti-mouse IgG secondary antibody conjugated with Alexa Fluor 488 (1:250, Life Technology Co.) for 1 hour at 4ºC. Following rinsing, cell nuclei were counterstained with 5 μg/ml 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Life Technology Co.). The slides were coated with Fluorescent Mounting Medium (Dako Cytomation, Carpinteria, CA, USA) and observed with a fluorescence microscope (Keyence, Osaka, Japan). CGRP-positive cells were detected and counted among the SMA-positive smooth muscle layers. With a x60 objective lens, the counting areas were randomly viewed from the top of the bladder to the trigone, and the number of CGRP-positive cells were counted in 5-10 locations per tissue sample.

F Statistical analysis

The results were expressed as means ± standard error of the means. The significance of statistical differences between cystometric variables were determined by Student's

paired t-tests before and after the drug administration at RT, or between RT and LT. Two-way non-repeated measures analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test for multiple comparisons were performed for comparison of variables among groups. P-values less than 0.05 were considered significant.

III Results

A Effects of naftopidil and tadalafil alone and in combination on cold stress-induced bladder overactivity in bladder outlet obstructed rats

At RT, there were no differences in either bladder pressure or micturition volume among the rats in the control or experimental groups (Fig. 1). These were not altered after intraperitoneal administration of the vehicle control, naftopidil, tadalafil, or the combination of naftopidil and tadalafil.

After transfer from RT to LT, basal pressure in all groups significantly increased, but micturition pressures did not change (Table 1). As seen for individual rats (Fig. 1), during LT exposure, the vehicle-, naftopidil-, tadalafil-, or combination-treated rats exhibited increased micturition frequency and lower micturition volume compared to RT condition. Voiding intervals at RT were significantly decreased at LT in the vehicle-treated (-62.24±5.48%), naftopidil-treated (-66.25±0.56%), tadalafil-treated (-67.99±2.05%), and combination-treated (-34.54±12.31%) rats (Fig. 2A). Similarly, bladder capacities at RT in the vehicle-, naftopidil-, tadalafil-, and combination-treated rats were also less at LT (-64.84±4.13%, -64.08±10.46%, -69.35±2.10%, -42.98±6.72%, respectively; Fig. 2B). However, the decreases of both voiding interval and bladder capacity in the combination-treated rats were significantly less than in the vehicle-, naftopidil-, or tadalafil-treated rats.

B Cold stress-induced bladder overactivity in resiniferatoxin-treated bladder outlet obstructed rats

Two days prior to cystometric investigations, another group of bladder obstructed rats (n=8) was treated with RTX. At RT, both bladder pressure and micturition volume in the RTX-treated rats did not differ from the control and experimental groups described above. After transfer from RT to LT, unlike the vehicle-, naftopidil-, tadalafil-, and combination-treated rats, the RTX-treated rats did not have increased micturition frequency and lower micturition volume (Fig. 3A). During LT exposure, the basal pressure of the RTX-treated rats also increased significantly, while micturition pressure did not change (Table 1). Both the voiding interval (Fig. 3B) and bladder capacity (Fig. 3C) tended to decrease, but the changes were not statistically significant. However, the percent decreases of voiding interval and bladder capacity in the LT RTX-treated rats, 30.15±9.99% and 24.33±11.24% respectively (Fig. 3A, B), were not significantly different from the decreases in the combined naftopidil- and tadalafil-treated rats (Fig. 2A, B).

C Expression of urinary bladder CGRP-positive afferent nerves

We examined the expression and distribution of CGRP-positive afferent nerves among the SMA-positive smooth muscle layers of each group. There were numerous GCRP-positive afferent nerves in the vehicle- (Fig. 4A), naftopidil- (Fig. 4B), tadalafil- (Fig. 4C), and combination- (Fig. 4D) treated rats. However, there were many fewer CGRP-positive afferent nerves in the RTX-treated rats (Fig. 4E). The number of afferent nerves detected by CGRP antibody among the SMA-positive smooth muscle layers in the RTX-treated rats was significantly lower than in any of the other treatment groups (Fig. 4F).

IV Discussion

It is well established that cold stress induces LUTS in a large number of mammals, including rats and humans. We verified this effect again in our LUTS rat model that had cold stress-induced detrusor overactivity following partial obstruction of the bladder outlet. The decreased voiding interval and bladder capacity elicited by exposure to LT in this model were not inhibited by 0.15 mg/kg-body weight of the α1-AR antagonist naftopidil or by 0.5 mg/kg-body weight of the PDE5 inhibitor tadalafil. However, the combined treatment with the same naftopidil and tadalafil dosages partially inhibited the cold stress-induced detrusor overactivity. A previous study showed that 0.3 mg/kg-body weight naftopidil inhibits the cold stress-induced detrusor overactivity of the same rat model[8]. In addition, 1.0 mg/kg-body weight tadalafil improved bladder storage functions in rats with nicotine-induced bladder hypoxia[9]. The combined therapy, in which half the dose of each drug was used in our study, partially inhibited the cold stress responses. Thus, our data suggest that some effects of these two drugs efficiently inhibit the cold stress-induced detrusor overactivity.

To investigate the mechanisms by which cold stress detrusor activity can be treated, we focused on RTX-sensitive nerves. A previous study of RTX-treated normal rats showed that activation of unmyelinated C fibers is one of the neuronal pathways that mediates detrusor overactivity[5]. The current study also showed that RTX inhibited the cold stress response in rats with bladder outlet obstruction. In addition, the urinary bladders of the RTX-treated rats had fewer CGRP-positive afferent nerves compared to the controls. Thus, our evidence suggests that the cold stress-induced detrusor overactivity of the bladder in outlet obstructed rats is also mediated by bladder unmyelinated C fibers that include RTX-sensitive nerves.

In general, either naftopidil or tadalafil is used to improve clinical voiding symptoms. Also, these drugs are reported to pharmacologically suppress afferent nerve (myelinated Aδ and/or unmyelinated C fibers) activity[14)-16)]. These findings suggest a neurological mechanism by which cold stress storage symptoms are improved, and our data are consistent with these observations. We found that treatment with the combination of naftopidil and tadalafil, each at half the effective dose alone[8),9)], partially reduced the cold stress-induced LUTS symptoms of the bladder outlet obstructed rats. A previous study showed expressions of α1A- and α1D-AR within the CGRP-positive afferent nerves[8]. Also, expressions of PDE5 activity were detected within the CGRP-positive cells[17]. This suggests that the combination of naftopidil and tadalafil suppressed activity of the afferent nerves, possibly including the RTX-sensitive nerves. The pharmacological

effects of the combination treatments might efficiently inhibit the cold stress-induced detrusor overactivity in bladder outlet obstructed rats.

We recognize some limitations within this study. First, naftopidil and tadalafil have anti-inflammatory, anti-oxidative, anti-fibrotic, and vascular endothelial protective properties that could be caused by chronic ischemia related with BPH[18),19)]. We did not investigate these potential modes of action in reducing LUTS symptoms. Secondly, while both drugs are known to improve pelvic blood flow[20)-22)], we did not directly investigate any improvement of blood flow within the urinary bladders as a result of the pharmacological treatments. Finally, we did not estimate the expression levels of endothelial and/or nerve-associated nitric oxide synthase or cyclic guanosine monophosphate, which were metabolites related with the PDE5 inhibitor. Even with these limitations, our results suggest that the combination of naftopidil and tadalafil has the potential to effectively treat cold stress-exacerbated LUTS due to BPH.

V Conclusions

Neither the α1-AR antagonist naftopidil (0.15 mg/kg-body weight) nor the PDE5 inhibitor tadalafil (0.5 mg/kg-body weight) alone inhibited detrusor overactivity in cold stressed, bladder outlet obstructed rats. However, the combination of naftopidil and tadalafil inhibited the decrease in voiding interval by 35% and the decrease in bladder capacity by 43%. The cold stress response in bladder outlet obstructed rats was also inhibited by subcutaneous injection of RTX two days before cystometry. RTX treatment reduced the presence of CGRP in detrusor afferent nerves, and inhibited the decrease in cold stress voiding intervals and bladder capacity in the bladder outlet obstructed rats. This indicates that the cold stress-induced detrusor overactivity in the bladder outlet obstructed rats was mediated, at least in part, by RTX-sensitive nerves. We hypothesize that the combination of naftopidil and tadalafil acts efficiently to inhibit cold stress-induced detrusor overactivity in bladder outlet obstructed rats by suppressing afferent nerve activity, some of which may include RTX-sensitive nerves. Thus, we conclude that the combination of an α1-AR antagonist and a PDE5 inhibitor has the potential to effectively treat clinical cases of cold stress-induced LUTS in patients with BPH.

Figure Legends

Fig. 1 Representative cystograms of changes in bladder pressure, micturition frequency, and micturition volume upon transfer from RT to LT. (A) After transfer from RT to LT, vehicle-treated rats exhibited cold stress-induced bladder overactivity. Micturition frequency increased (upper tracing) and micturition volume decreased (lower tracing) compared to RT condition. (B and C) At RT, naftopidil (B) or tadalafil (C) treatments did not alter micturition. During exposure to LT, (B) naftopidil- and (C) tadalafil-treated rats also exhibited cold stress-induced bladder overactivity that were similar to the vehicle-treated ones. (D) At RT, combined treatment with naftopidil and tadalafil did not alter micturition. After transfer to LT, the combination-treated rats also exhibited increased micturition frequency and lower micturition volume compared to RT conditions; however, these changes were partially inhibited compared to the vehicle-, naftopidil-, or tadalafil-treated rats. Top: bladder pressure; Bottom: micturition volume. Triangles: micturition during LT exposure. *, Twenty minutes of waiting time after intraperitoneal injection of vehicle, 0.15 mg/kg-body weight naftopidil, 0.5 mg/kg-body weight tadalafil, or the combination of naftopidil and tadalafil; **, approximately 3 minutes of transfer time from RT (room temperature) to LT (low temperature). Arrowheads, micturition events during LT exposure.

Fig.2 Decreased voiding interval and bladder capacity during LT exposure. (A and B) After transfer to LT, voiding interval (A) and bladder capacity (B) in all groups were significantly decreased. However, the decreases in voiding interval and bladder capacity of the combination-treated rats were significantly inhibited compared to the vehicle-, naftopidil-, and tadalafil-treated rats. White bar: RT (room temperature). Gray bar: LT (low temperature); naftopidil, 0.15 mg/kg-body weight; tadalafil, 0.5 mg/kg-body weight; *$P<0.05$, **$P<0.01$; compared to RT baseline values.

Fig. 3 Change of micturition and voiding interval and bladder capacity in RTX-treated rats with transfer from RT to LT. (A) After transfer to LT, RTX-treated rats partially inhibited the cold stress-induced bladder overactivity that increased micturition frequency (upper tracing) and decreased of micturition volume (lower tracing). *, approximately 3 minutes of transfer time from RT to LT. (B) During LT exposure, the voiding interval of the RTX-treated rats tended to decrease, but the change was not statistically significant. (C) Bladder capacity of the RTX-treated rats was decreased compared to the RT. The decreases of voiding interval and bladder capacity were the same as for the rats given the combination of 0.15 mg/kg-body weight naftopidil and 0.5 mg/kg-body weight tadalafil

(see Figure 2). RTX, resiniferatoxin, 0.3 mg/kg; RT, room temperature; LT, low temperature;*P<0.05, compared to RT. Arrowheads, micturition events during LT exposure.

Fig.4 CGRP-positive afferent nerves within the detrusor of bladder outlet obstructed rats. (A-D), There were numerous CGRP-positive afferent nerves (red, arrowheads) in the (A) vehicle-, (B) naftopidil-, (C) tadalafil-, and (D) combined naftopidil- and tadalafil-treated rats. (E) Within the detrusor of the RTX-treated rats, there were fewer CGRP-positive nerve cells (red, arrowheads). (F) Expression numbers of the CGRP-positive afferent nerves in the RTX-treated rats were the lowest among the groups. CGRP, calcitonin gene-related peptide; Green: SMA-positive detrusor. Blue: nuclei. Bar: 30 μm.

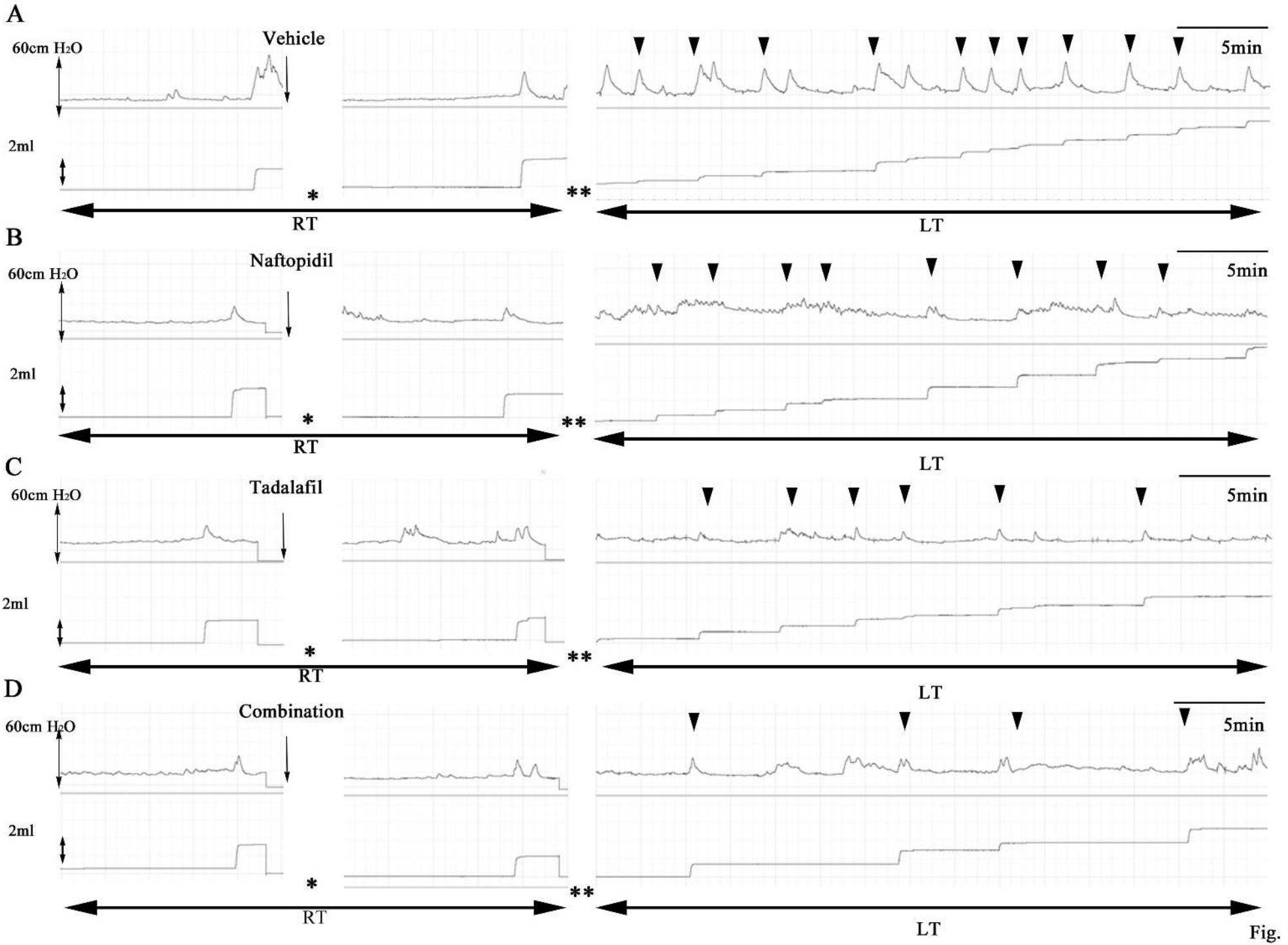
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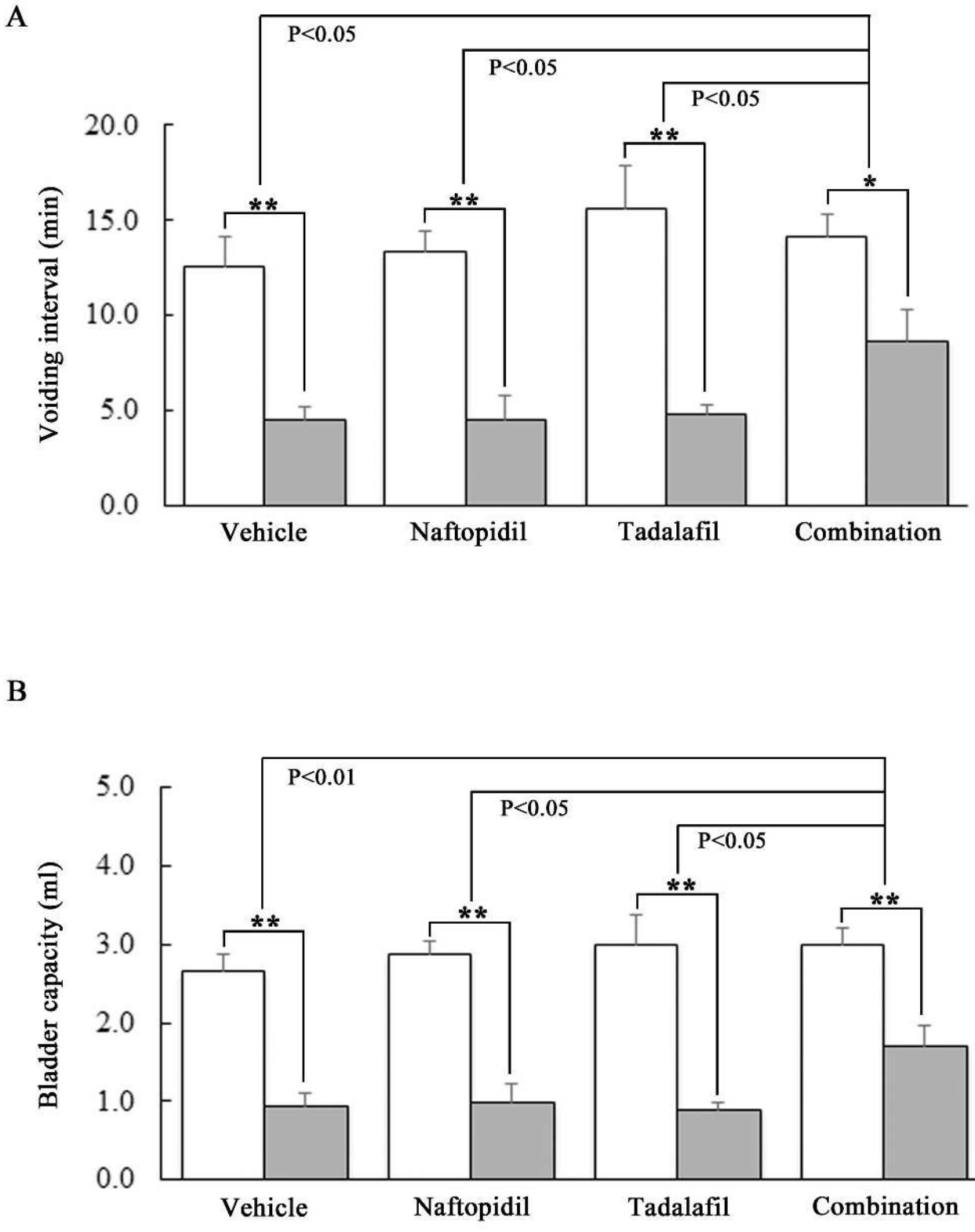
Fig. 1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P<0.05
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Voiding interval (min)
20.0
15.0
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Vehicle
Naftopidil
Tadalafil
Combination
B
P<0.01
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Bladder capacity (ml)
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4.0
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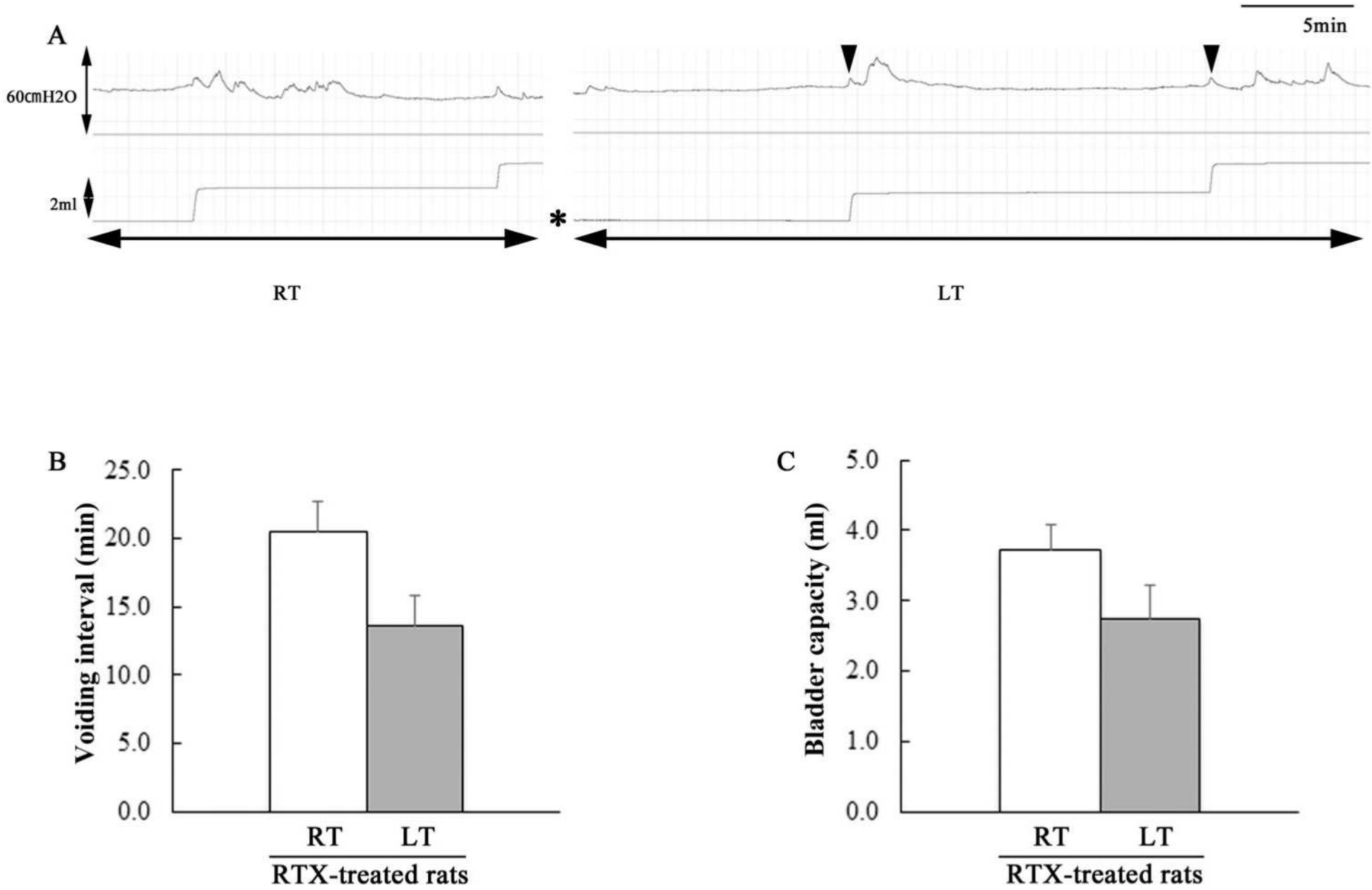
0.0
Vehicle
Naftopidil
Tadalafil
Combination

Fig. 2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5min
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2ml
*
RT
LT
B
Voiding interval (min)
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20.0
15.0
10.0
5.0
0.0
RT
LT
RTX-treated rats
C
Bladder capacity (ml)
5.0
4.0
3.0
2.0
1.0
0.0
RT
LT
RTX-treated rats

Fig. 3

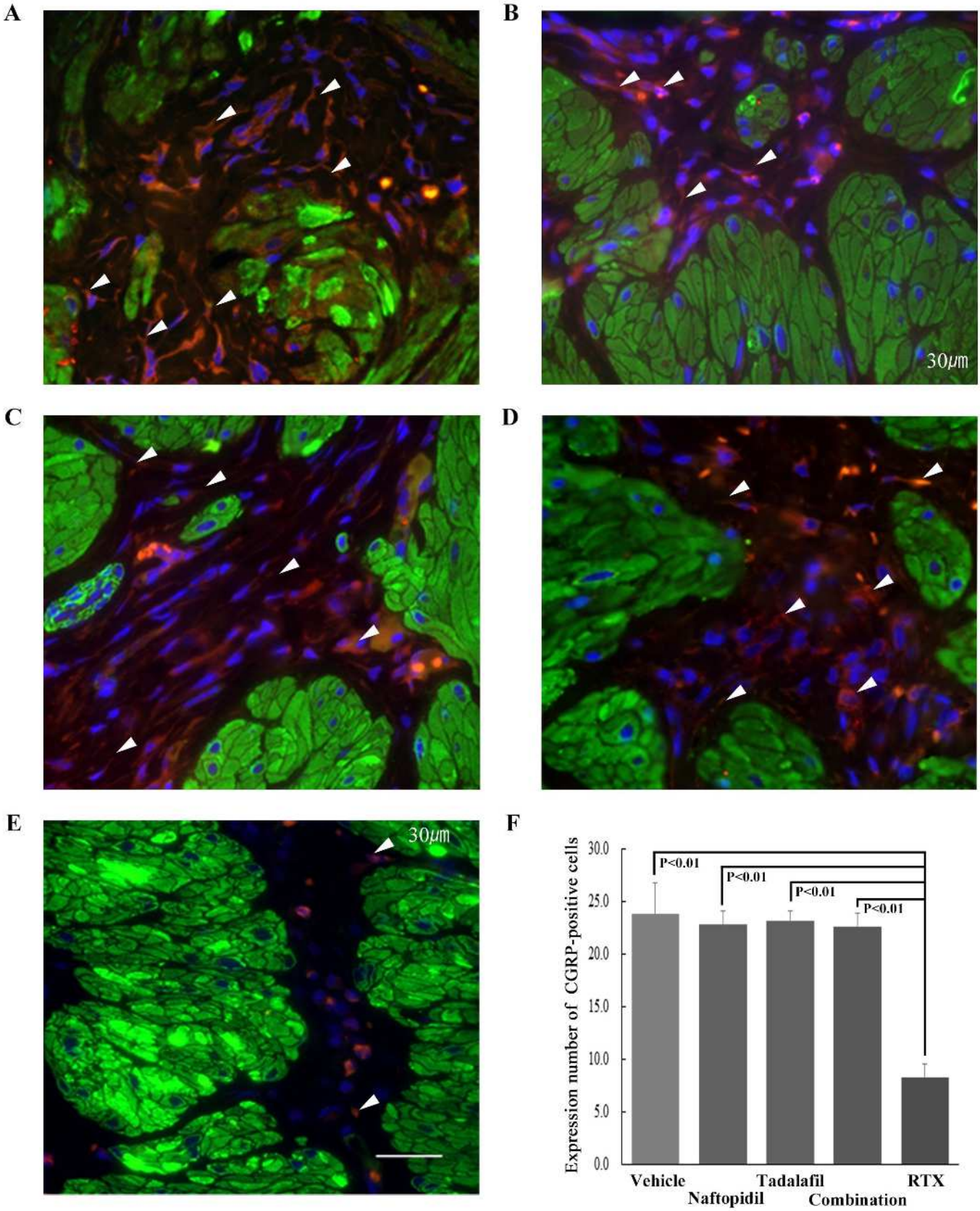
A
B
30μm
C
D
E
30μm
F
Expression number of CGRP-positive cells
30.0
25.0
20.0
15.0
10.0
5.0
0.0
P<0.01
P<0.01
P<0.01
P<0.01
Vehicle
Naftopidil
Tadalafil
Combination
RTX

Fig. 4

Table 1 Effects of temperature on basal and micturition pressures in obstructed bladder outlet rats.

Parameter	Condition	Vehicle	Naftopidil	Tadalafil	Naftopidil + Tadalafil	RTX
Basal pressure (cmH_2O)						
	RT	4.47±0.74	5.79±1.06	4.45±0.52	3.68±0.39	10.14±1.99
	LT	8.54±0.79	11.79±1.24	9.20±1.25	7.92±0.68	13.25±2.03
	(RT-LT)	-4.13±1.03**	-5.18±1.10 **	-4.75±1.16**	-4.24±0.63**	-3.11±1.26 *
Micturition pressure (cmH_2O)						
	RT	22.92±3.58	26.03±3.51	28.95±4.02	24.64±2.28	35.75±3.28
	LT	22.33±2.16	26.38±2.59	23.00±3.72	25.89±2.87	36.55±3.11
	(RT-LT)	0.38±2.42	-0.38±3.05	5.95±2.64	-1.25±1.20	-0.78±4.56

RTX, resiniferatoxin; RT, room temperature, 27±2°C; LT, low temperature, 4±2°C; *P<0.05, **P<0.01 compared to RT in each group.