Original article

Therapeutic effects of Choreito, a traditional Japanese (Kampo) medicine, on detrusor overactivity induced by acetic acid in rats

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List of Abbreviations and Acronyms

AA = acetic acid

CRT = Choreito

DO = detrusor overactivity

HIF1 = αhypoxia-inducible factor 1α

LUTS = lower urinary tract symptoms

OAB = overactive bladder

UPIII = uroplakin III

Abstract (247 words/limit: 250 words)

Choreito (CRT), a traditional Japanese (Kampo) medicine, is widely used for the treatment of overactive bladder (OAB) and other lower urinary tract symptoms in Japan. This study aimed to identify the effects and therapeutic mechanism of CRT on the improvement of detrusor overactivity (DO) using an experimental rat model. Forty-five female Sprague-Dawley rats were equally divided into 3 groups: intravesical saline instillation with normal food (normal group), intravesical acetic acid (AA) instillation with normal food (AA group), and intravesical AA instillation with CRT (AA with CRT group). To induce a decrease in bladder capacity, instillation of 0.2% AA was used based on prior studies. Cystometric investigation was employed to clarify the effects of AA and CRT. Microcirculation was performed using a laser blood flowmeter, and the localization of hypoxia-inducible factor 1α (HIF1α) was assessed by immunohistochemistry. The bladder capacities of the normal, AA, and AA with CRT groups were 1.2±0.3 mL, 0.4±0.1 mL, and 0.8±0.1 mL, respectively. CRT significantly attenuated AA irritation of the urinary bladder and exerted protective effects on basal pressure, micturition pressure, micturition interval, and micturition volume. Furthermore, CRT could prevent the excess blood flow and edematous change under the urothelium induced by intravesical AA instillation. No obvious changes in immunohistochemical HIF1α staining were observed among the groups. CRT attenuated DO induced by intravesical AA instillation in a rat experimental model. CRT might impart therapeutic effects on OAB via the mitigation of urothelial damage and regulation of excess blood flow.

***Keywords*:** acetic acid, Choreito, complementary alternative medicine, overactive bladder, rat

1. Introduction

Lower urinary tract symptoms (LUTS), which include overactive bladder (OAB), are persistent conditions that markedly decrease quality of life.[1,2] Although many conservative and surgical treatment options have been established for OAB, some patients continue to experience refractory symptoms to current therapies. Accordingly, the role of complementary alternative medicines requires clarification as a possble treatment option for LUTS or OAB. Kampo medicine, also known as traditional Japanese medicine, has gained a unique status following approval by the Ministry of Health, Labour, and Welfare of Japan.[3-5] Many Kampo formulations are now manufactured on a modern industrial scale, in which the quality and quantity of ingredients are standardized under strict, scientific quality controls. There currently exist Kampo drugs for numerous illnesses and forms of organ dysfunction, including LUTS and OAB.[6] One such formulation, Choreito (CRT; Tsumura Co., Ltd., Tokyo, Japan), has been widely used for the treatment of LUTS, OAB, hemorrhagic cystitis, refractory urinary tract infection, and spontaneous excretion of urinary stones.[6-9] The agent is considered effective for LUTS treatment via its anti-inflammatory and diuretic effects.[6-8] However, there is insufficient evidence of CRT in the clinical and basic scientific fields, and the precise mechanisms of CRT remain unclear. Intravesical acetic acid (AA) instillation has established as animmal model with detrusor overactivity induced by chemical bladder irritation in rats.[10] The aim of this study was to indentify the role of CRT on LUTS using an detrusor overactivity (DO) rat model induced by intravesical AA instillation and clarify the therapeutic mechanisms of CRT on LUTS and OAB improvement.

2 Materials and methods

2.1 Animals and ethical background

Female 10-week-old Sprague-Dawley rats (200-250 g) were used in this study. The rats were maintained under standard laboratory conditions with a 12-hour light/dark cycle and free access to food pellets and tap water. All experimental protocols were approved by the Ethics Committee of Shinshu University School of Medicine. The rats were treated in accordance with the National Institutes of Health Animal Care Guidelines and the requirements of the animal ethics committee of our university.

Forty-five rats were equally divided into three groups: saline intravesical instillation with normal food (control group), intravesical AA instillation with normal food (AA group), and intravesical AA instillation with CRT (AA with CRT group). Normal food or food containing CRT were given for 2 weeks before intravesical instillation in each group.

CRT is composed of five medical herbs including Alminum Silicate Hydrate with Silicon Dioxide, Alisma Rhizome, Polyporus Sclerotium, Poria Sclerotium, and Donkey Glue. The composition of CRT is shown in **Table 1**. The study diet was prepared that dry powdered extracts of CRT were mixed with standard laboratory food (Certified Diet, Oriental Yeast Co., LTD, Tokyo, Japan) at a final concentration of 1.0%. This concentration of CRT is equivalent to the amount of 1000 mg/kg/day that was determined by referring to the protocol of previous other Kampo study.[11]

2.2 Cystometric investigation

We performed cystometric investigations on nine rats in each group as previously described.[12] Briefly, 3 days prior to cystometric measurements, the urinary bladder was sugically exposed and a polyethylene catheter (PE50; Nippon Becton Dickinson, Tokyo, Japan) was inserted through the dome. The inserted catheter was fixed at the site with a 5-0 silk thread, and the free end of the catheter was tunneled subcutaneously and exteriorized at the back of the neck. Saline or 0.2% AA dissolved in saline were instilled via the placed catheter for 30 minutes 1 day before cystometric investigation. Cystometric assessments were performed on unanesthetized, unrestricted rats placed

in metabolic cages for 30 minutes. The catheter inserted into the urinary bladder was connected via a T-tube to a pressure transducer (P23 DC; Statham, Oxnard, CA) and a syringe pump (TE-351; Terumo, Inc., Tokyo, Japan). Saline maintained at room temperature was instilled into the urinary bladder at a rate of 10 mL/h. Bladder contractions were recorded continuously by a pen oscillograph (10 mm/min recording speed, Recti-Horiz-8K; NEC San-ei Instruments, Tokyo, Japan). Micturition volume as measured with a fluid collector connected to a force displacement a pressure transducer (P23 DC; Nihon Kohden, Tokyo, Japan) (Type 45196; NEC San-ei Instruments) was simultaneously recorded. After confirming the first and second voiding, the following cystometric parameters were directly measured: basal pressure (cmH_2O), micturition pressure (cmH_2O), voiding interval (minutes), micturition volume (mL), and bladder capacity (mL). Bladder capacity was calculated by adding the residual volume to the micturition volume.

2.3 Evaluation of microcirculation

For the evaluation of bladder microcirculation and pathological analysis, six rats in each group were equally divided into three subgroups. The rats were not used in cystometric investigations. Microcirculation was measured with a laser blood flowmeter (Omegazone; Omegawave, Tokyo, Japan) 1 day after saline or AA instillation. The rats were anesthetized as described before and then bladders were exposed through a lower midline abdominal incision.[12] The bladders were injected with 0.7 mL of physiological salt solution through the posterior wall by a 29 G needle. Bladder blood flow was recorded in arbitrary units at the bladder neck as colored image pixels. Software (Laser Speckle Blood Flow Imager-LSI version 3.4.3: Omegawave, Espoo, Finland) provided by the flowmeter converted the image pixels into relative numeric measurements of flow rates. After the bladder blood flow measurements, the bladders were removed for immunohistochemical investigations. The rats were euthanized by an overdose of pentobarbital sodium.

2.4 Histopathological and immunohistopathological analysis

Bladders that were harvested from the rats in the microcirculation measurements 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 minutes) 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 hour at 4°C. The tissues were incubated with hypoxia-inducible factor 1α (HIF1α) antibody (1:100; 20960-1-AP; rabbit polyclonal; Proteintech Inc., IL) as a tissue marker of hypoxia and uroplakin III (UPIII) antibody (1:100; M-17; sc-15186; goat polyclonal; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for assessment about localization of urothelium.

The sections were then rinsed with PBS and incubated with donkey anti-rabbit immunoglobulin G (IgG) secondary antibodies conjugated with Alexa Fluor 488 (1:250; Thermo Fisher Scientific K.K., Foster City, CA) and donkey anti-goat or -mouse IgG secondary antibodies conjugated with Alexa Fluor 594 (1:250; Thermo Fisher Scientific K.K.) for 1 hour at 4°C. After rinsing, cell nuclei were counterstained with 5 μg/mL 4′, 6-diamidino-2-phenylindole dihydrochloride (Thermo Fisher Scientific K.K.). The stained samples were observed with a Leica DAS Microscopethe (Leica Microsystems GmbH, Wetzlar, Germany).

Observers who were blinded to treatment status semi-quantitatively evaluated the HIF1α- and UPIII-positive areas within the urothelium layers. Fluorescence-labeled areas of the HIF1α- and UPIII antibodies in the urothelium layers or detrusors were averaged from 6 observed regions (×400 magnification) in each sample and expressed as the proportion of the total observed urothelium layers or detrusor areas.

Additionally, bladder samples were fixed in 10% buffered-formalin and examined under a light microscope after embedding in paraffin, sectioning, and staining with hematoxylin-eosin (HE).

2.5 Statistical analysis

All cystometric investigation and microcirculation values are expressed as the mean±standard error. Unpaired Student's *t*-tests were employed to analyze differences between two groups of rats. For multiple comparisons among groups, the closed testing procedure was performed to determine statistical significance. All analyses were performed with the Excel Statistical Program File ystat2006.xls (Igakutosho Shuppan, Tokyo, Japan), and $p < .05$ was considered to show a significant difference.

3 Results

3.1 Cystometric investigation

Mean body weight was comparable among the 3 experimental test groups (data not shown). At 1 day after intravesical AA instillation, urinary frequency increased in AA rats as compared with saline intravesical instillation in normal rats **(Figure 1A, B)**. The irritation caused by intravesical AA instillation was attenuated in the rats fed CRT **(Figure 1C)**.

In the AA group, basal pressure defined as bladder pressure during urine storage was 17.6±4.2 cmH_2O and micturition pressure defined as micturition pressure during urination was 51.0±5.8 cmH_2O **(Figure 2A, B)**. These values were significantly higher than those in the normal group (4.1±0.4 cmH_2O and 28.0±3.9 cmH_2O, respectively) **(Figure 2A, B)**. In the AA with CRT group, however, these parameters were significantly attenuated at 8.8±1.0 cmH_2O and 37.9±4.4, resepectively, versus the AA group (both $p < .05$) **(Figure 2A, B)**. The voiding interval of the rats in the AA group (2.1±0.3 minutes) was significantly shorter than that in the normal group (7.2±1.6 minutes) ($p < .05$) **(Figure 2C)**. The voiding interval of the rats in the AA with CRT group was 4.2±0.4 minutes and significantly lower than in the AA group ($p < .05$) **(Figure 2C)**. Micturition volume (0.4±0.1 mL) and bladder capacity (0.4±0.1 mL) were significantly lower in the AA group than in the normal group (1.2±0.3 mL and 1.2±0.3 mL, respectively) (both $p < .05$) **(Figure 2D, E)**. CRT could

significanlty attenuate the decreases in these parameters as well (0.7±0.1 mL and 0.8±0.1 mL, resepecively) (both $p < .05$) **(Figure 2D, E)**.

3.2 Microcirculation investigation

A schema of the intra-abdominal finding using a laser blood flowmeter is shown in **Figure 3A**. The blood flow at the bladder neck was 24.9±2.7 in the normal group **(Figure 3B,E)** and 31.4±1.3 in the AA group **(Figure 3C,E)**, indicating that AA-induced irritation could increase relative blood flow. This increased blood flow was significantly attenuated at 24.8±2.3 by CRT ($p < .05$) **(Figure 3D,E)** to imply a regulatory effect of CRT on excess of blood flow.

3.3 Histopathological and immunohistopathological analysis

To evaluate the tissue damage induced by AA, we next examined the urinary bladder after HE staining. Compared with the normal group, the interstitium under the urothelium was more edematous in the AA group **(Figure 4A,B)**. Vacuolization of urothelial cells and urothelium detachment were also apparent in the AA group. In contrast, no obvious changes were observed in the muscle layers and no cellular inflammatory reactions of neutrophilic cells were seen, indicating that 0.2% AA did not induce cytological or histopathological inflammatory insult in addition to urothelial surface and interstitial layer damage. CRT mitigated the damage to the urothelial surface and sub-urothelial edema **(Figure 4C)**.

In immunohistopathological analysis, HIF1α and UPIII were expressed within the urothelium in all groups **(Figure 4D,E,F)**. No changes in the expression of HIF1α and UPIII within the urothelium layers were detectable.

4 Discussion

In the present study, intravesical instillation of AA produced urothelial surface damage and increased blood flow in the urinary bladder to result in significantly increased urination frequency

and other cystometric changes. This confirmed AA can be used for chemical bladder irritation inducing DO in rats. The concomittant administration of CRT attenuated these effects in the experimental model, presumably by the regulation of excess blood flow and edema.

Kampo is a traditional Japanese medicine that is very common in Japan. Almost 150 Kampo formulations have been approved as prescription drugs by the Ministry of Health, Labour, and Welfare for the treatment of a wide variety of diseases. The majority of Japanese physicians trained in Western medicine continue to use Kampo formulations in daily clinical practice. For example, Dai-Ken-Chu-Tou is one of the most commonly used Kampo formulations with high levels of clinical evidence. Dai-Ken-Chu-Tou is widely prescribed for patients with gastrointestinal obstruction, such as postoperative ileus, postoperative intestinal paralysis, and chronic severe constipation, by many surgeons at medical institutions in Japan.[13-16] Previous reports addressed the unique pharmacological mechanisms of action of Dai-Ken-Chu-Tou, such as the increase of intestinal blood flow and amelioration of colitis by calcitonin gene-related peptide and/or adrenomedullin.[17,18] Moreover, several Kampo-based drugs have been established for urological conditions, including LUTS and OAB, the mechanisms of which have been investigated and reported.[19-22] Therefore, Kampo formulations are currently among the validated therapeutic options for LUTS and OAB.

CRT is used for the treatment of unidentified urological symptoms, such as LUTS and discomfort after micturition.[22] Unidentified symptoms are defined as manifestations associated with urinary tract or bladder function other than storage and voiding symptoms. Discomfort and pain in the peritoneum after micturition are common complaints in afflicted individuals. CRT can also promote the passage of ureteral stones similarly to the role of α1-blockers.[9] Mechanistically, CRT is considered to exert diuretic and anti-inflammatory effects on the urinary tract.[6] However, the results of basic and clinical resarch on CRT have been insufficient to pinpoint the therapeutic effects and modes of the drug.

OAB is regarded as a multifactorial disease of neurogenic, myogenic, and urethrogenic pathologies. It has been reported that AA infusion into the bladder induces irritation of the urothelium, stimulates nociceptive afferent fibers, and causes an inflammatory reaction. These result in a reduction in bladder capacity and consequent increases in contraction frequency and other indices of bladder hyperactivity.[10] In previous studies, intravesical AA instillation (1%) induced c-fos expression in the spinal cord via bladder irritation in test animals.[23-25] Intravesical AA instillation (0.5%) also led to bladder overactivity of neurogenic origin and increased the sensitivity of afferent sensors in the bladder wall.[26] Kashyap et al. observed the overexpression of nerve growth factor and inflammatory molecules in an overactive bladder model induced by intravesical AA instillation (0.25%) in rats,[27] while Aizawa et al. recently reported that a transient receptor potential melastatin 8 antagonist inhibited afferent overactivity induced by intravesical AA instillation (0.1%).[28] Transient receptor potential melastatin 8 channels are expressed in urothelial cells, sensory nerve fibers within the urothelium and suburothelium of the bladder, and the L6 dorsal root ganglia of rats and guinea pigs.[29,30] The above reports indicate that a higher concentration of AA, such as more than 0.5%, can induce neurogenic damage including the spinal cord, whereas a lower AA concentration may produce urothelial irritation and peripheral neurogical irritation via urothelial damage alone. The present study hypothesized that CRT could ameliorate the latter symptoms. Thus, we used 0.2% AA as an adequate concentration to elicit urothelial damage recoverable by CRT.

There are numerous systems to understand inflammation. The first description by the Roman Cornelius Celsus in the 1st century defined four cardinal signs of inflammation as redness, swellin, heat, and pain. With regard to physical findings, inflammation is characterized by vascular dilation, enhanced permeability of the capillaries, increased blood flow, and leukocyte recruitment.[31] In the current clinical field, fever elevation and laboratory examination findings can be used to interpret inflammation, especially systemic inflammation or bacterial infection. White blood cell count, C-

reactive protein level, and other blood tests are frequently used to evaluate the degree of inflammatory reactions. Concerning histopathological aspects, the infiltration of inflammatory cells can confirm cytological inflammation responses. In this study, we evaluated the change in blood flow to verify the physical findings of an inflammatory response induced by intravesical AA instillation as well as the anti-inflammatory effect of CRT. Intravesical AA instillation induced excess blood flow in the urinary bladder and a decrease in bladder capacity. Although no histopathological inflammatory cell reactions were observed, the animal model in this study was considered a suitable urothelial DO model based on chemical urothelial irritation that might also correspond to radiation or chemical forms of cystitis, such as from cyclophosphamide, in clinical situations. Acturally, Kawashima N, et al reported usefulness of CRT for hemorrhagic cystitis in the clinical case reports.[7, 8]

Omegazone, a laser blood flowmeter, can be used to measure the change of blood flow induced by chemical intervention in urinary bladder.[32-36] In this study, we observed the accelerating effect of intravesical AA instilation on bladder blood flow and demonstrated the attenuating effect of CRT in excess bladder blood flow induced by intravesical AA instillation. Therefore, CRT may affect on DO via the regulatory effect on excess of bladder blood flow.

First, we confirmed the edematous change of urothelium by HE staining. Moreover, in order to exclude hypoxia induced by edema, we evaluated the expression of the hypoxic marker HIF1α, a key indicator of hypoxic metabolic pathway activation. HIF1α was expressed within the urothelium in all groups, which indicated that 0.2% AA did not affect the bladder by means of hypoxia or ischemia and that intravesical AA instillation induced urothelial damage in this setup.

Thus, CRT attenuated the chemical irritation induced by intravesical AA instillation to result in a significant increase in bladder capacity, regulation of excess bladder blood flow, and a reduction in edematous change. Among traditional Kampo medicines in Japan, CRT is considered to reduce

inflammation including swelling (edema), fever, and other symptoms. The results of this study confirmed that CRT might improve physical and urothelial inflammation and mitigate the effects of chemical irritation.

To the best of our knowledge, this investigation is the first to show the effects of CRT on DO induced by chemical bladder irritation in rats. However, certain limitations must be considered when interpreting the results. First, the degree of inflammation and edema was uncertain. Dose and time dependency should also be investigated in the future. Second, since Kampo formulations contain many herbal components, the drug may influence multiple mechamisms in pathological conditions. In this study, we demonstrated one effect of CRT on the urinary bladder. CRT should be evaluated in other types of DO models, such as nonchemical, bacterial, and physical forms of irritation. Third, immunohistopatlogical analysis was done only by HIF1α and UPIII. More types of immunohistopathlogical staining should be carried out to show the pricise mechanisms of AA irritation and CRT, and quantitative evaluaton should also be done using a cell counter in further steps. Despite these limitations, however, our findings show that CRT may attenuate damage of the urinary bladder and can be considered a prospective treatment option for OAB patients. Further investigation considering the aforementioned limitations are being planned.

In conclusion, CRT attenuated DO induced by intravesical AA instillation through a suspected mechanism of mitigating urothelial damage and regulating excess blood flow.

5. Disclosure

The authors declare no conflicts of interest regarding the publication of this article. This work received a grant from Tsumura & Co(Grant number GAY095).

6. Acknowledgement

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Table 1. The constituents of Choreito

Constituent medical herbs	Percentage
Alminum Silicate Hydrate with Silicon Dioxide	20
Alisma Rhizome	20
Polyporus Sclerotium	20
Poria Sclerotium	20
Donkey Glue	20

Figure legends

Fig. 1

Cystometric investigation. Representative micturition charts of the (A) normal group, (B) AA group, and (C) AA with CRT group. Arrowheads indicate micturition. While the rats in the normal group did not show increased urinary frequency (blue arrowheads), those in the AA group exhibited increased urinary frequency with shorter voiding intervals and smaller micturition volumes (red arrowheads). This increase in urinary frequency was attenuated by CRT (green arrowheads). In each panel, the upper chart is bladder pressure and the bottom chart is micturition volume. AA, acetic acid; CRT, Choreito

Fig. 2

Summary of cystometric investigation results for (A) basal pressure (cmH_2O), (B) micturition pressure (cmH_2O), (C) voiding interval (minutes), (D) micturition volume (mL), and (E) bladder capacity (mL). *p< .05 and **p< .01 (Student's unpaired t-test). AA, acetic acid; CRT, Choreito

Fig. 3

Schema of intraabdominal finding using a laser blood flowmeter is shown in (A). Representative findings of the microcirculation investigation for the (B) normal group, (C) AA group, and (D) AA with CRT group. Arrow heads indicate urinary bladder. Red and orange indicate faster blood flow, while blue and green

indicate slower blood flow. After intravesical AA instillation, an increase in blood flow was observed in comparisons with saline instillation (B, C). This increased blood flow was attenuated by CRT (D). Summarized results of the microcirculation investigation are shown in E. Blood flow was significantly different between the AA and AA with CRT groups. *p< .05 (Student's unpaired t-test). AA, acetic acid; CRT, Choreito

Fig. 4

Representative histopathological findings of hematoxylin-eosin staining (200x). Compared with the (A) normal group, (B) AA group, and (C) AA with CRT group, the interstitium under the urothelium was more edematous in the (B) AA group, with vacuolization of urothelial cells (arrowheads) and detachment of the urothelium (arrow). Representative immunohistopathological findings of HIF1-α are shown for the (D) normal group, (E) AA group, and (F) AA with CRT group. No changes in the expression of HIF1α (green) and UPIII (red) within the urothelium layers were detectable. Nuclei are stained blue. AA, acetic acid; CRT, Choreito; HIF1α, hypoxia-inducible factor 1α; UPIII, uroplakin III

Figure 1

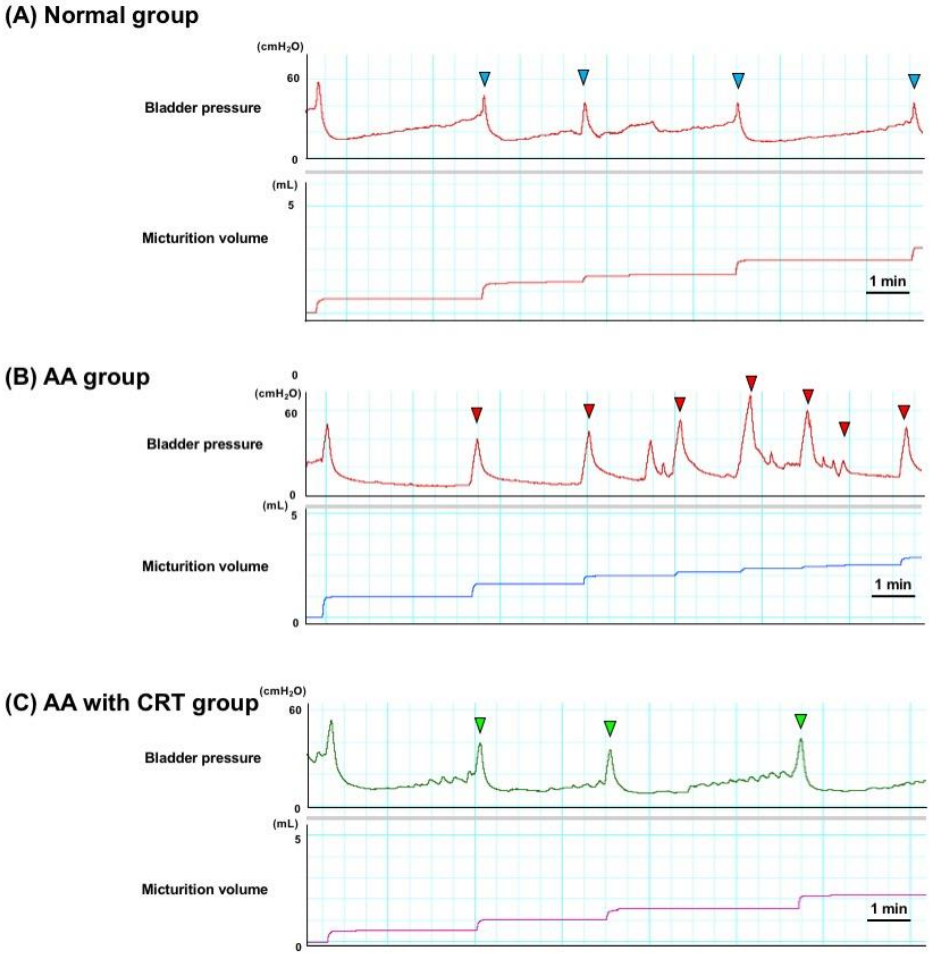
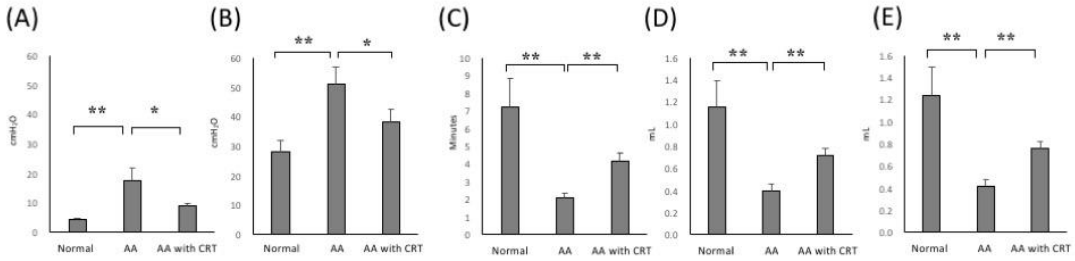
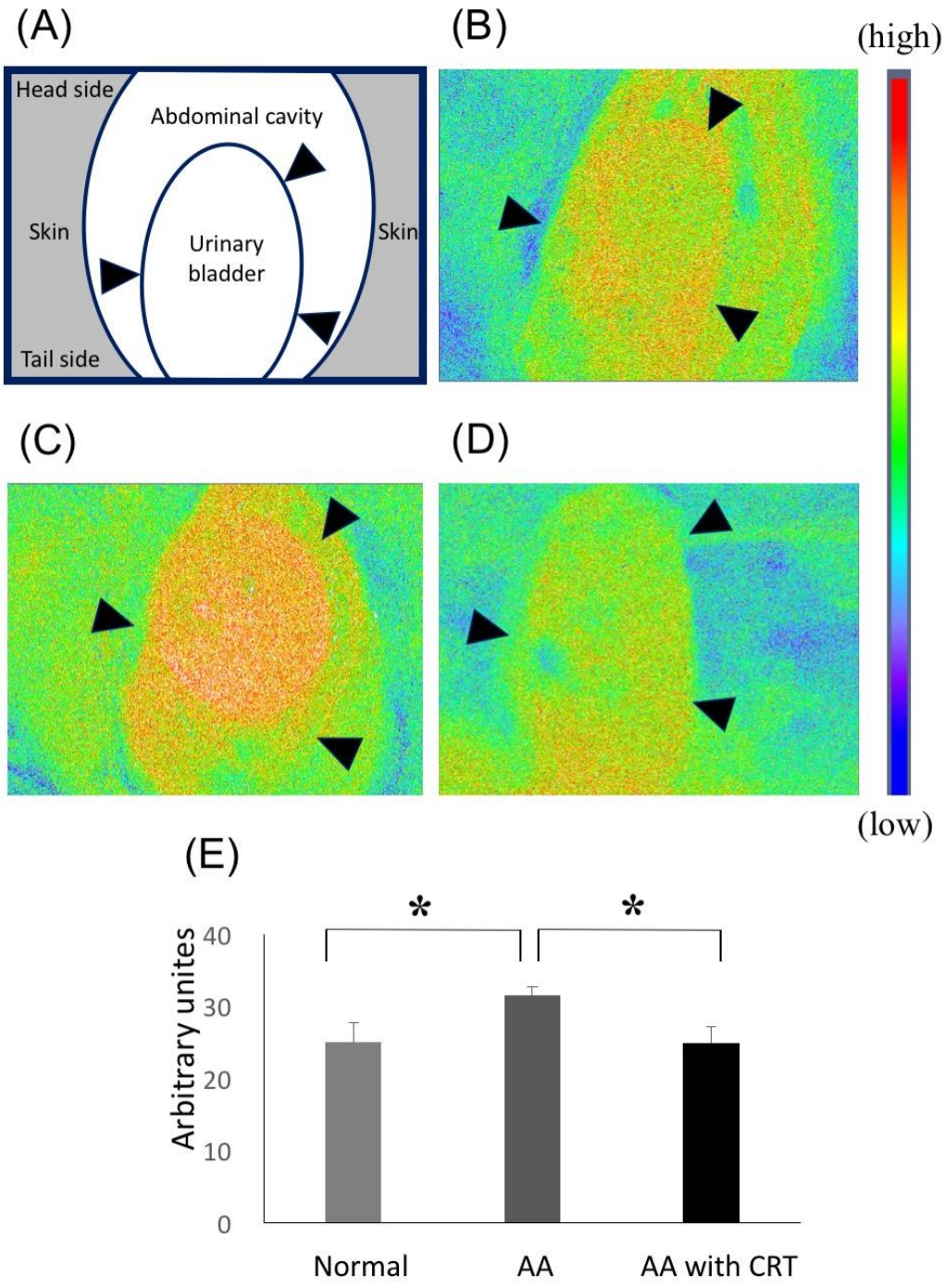

(A) Normal group
(cmH2O)
60
Bladder pressure
0
(mL)
5
Micturition volume
1 min
(B) AA group
0
(cmH2O)
60
Bladder pressure
0
(mL)
5
Micturition volume
0
1 min
(C) AA with CRT group
(cmH2O)
60
Bladder pressure
0
(mL)
5
Micturition volume
0
1 min

Figure 2



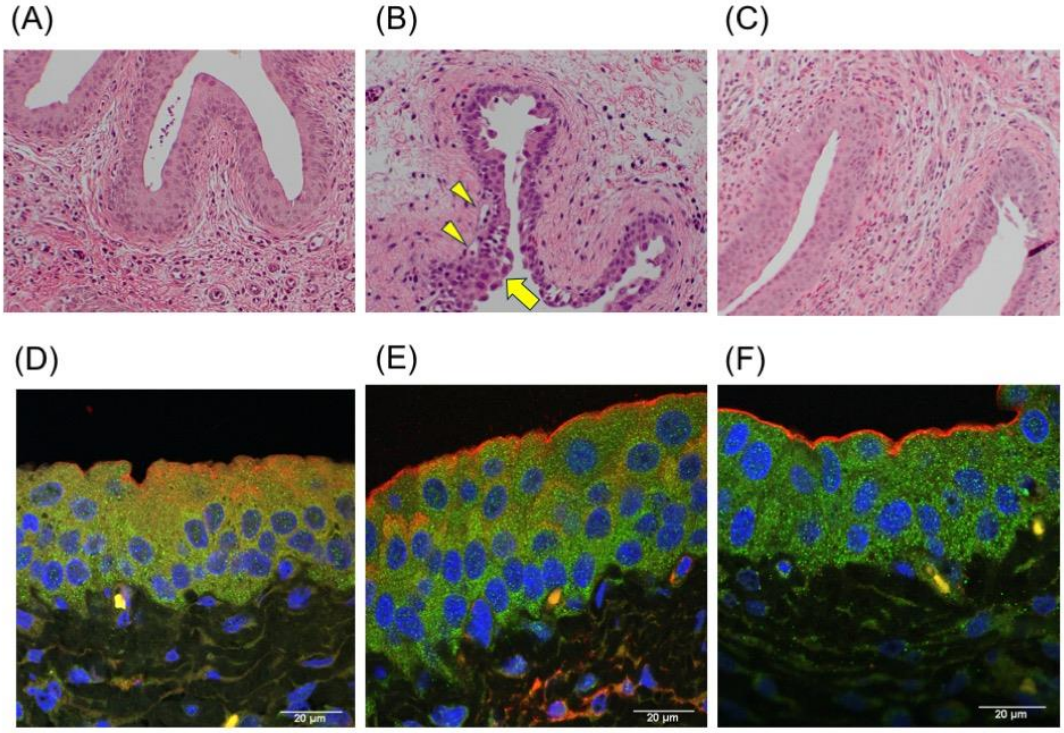
1 Figure 3

2


(A)
(B)
(high)
Head side
Abdominal cavity
Skin
Urinary bladder
Skin
Tail side
(C)
(D)
(low)
(E)
*
*
Arbitrary unites
40
30
20
10
0
Normal
AA
AA with CRT

3

1 Figure 4



2