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PII: S0022-5347(12)05195-6
DOI: 10.1016/j.juro.2012.10.014
Reference: JURO 9528

To appear in: *The Journal of Urology*
Accepted date: 4 October 2012

Please cite this article as: Noguchi, W., Ishizuka, O., Imamura, T., Kurizaki, Y., Yamagishi, T., Yokoyama, H., Lei, Z., Silwal, S.G., Nishizawa, O., Andersson, K.E., The Relationship between Alpha1-Adrenergic Receptors and TRPM8 Channels in Detrusor Overactivity Induced by Cold Stress in Ovariectomized Rats, *The Journal of Urology*® (2012), doi: 10.1016/j.juro.2012.10.014.

# The Relationship between Alpha1-Adrenergic Receptors and TRPM8 Channels in Detrusor Overactivity Induced by Cold Stress in Ovariectomized Rats

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**Running head:** COLD STRESS DETRUSOR OVERACTIVITY IN OVARIECTOMIZED RAT

(50/50)

**Key Words:** naftopidil; cystometry; skin receptors; LUTS

(5/5)

**Abstract**

**Purpose:** To study if cold stress-induced detrusor overactivity in ovariectomized rats is associated with increased thermosensitive transient receptor potential melastatin 8 (TRPM8) channel expression in the skin, and if the response can be inhibited by alpha1-adrenergic receptor blockade.

**Materials and Methods:** Twenty-four Sprague-Dawley rats at postnatal week 30 were randomly selected for ovariectomy (OVX, n=16) or sham ovariectomy (n=8). Five weeks later, cystometric measurements of the conscious, free-moving rats were performed at room temperature (RT, 28 ± 2ºC) for 20 min. Eight OVX rats were intravenously administered 1.0 mg/kg naftopidil, and the other OVX and sham-operated rats (n=8 each) were given naftopidil-free vehicle. Five min later, they were transferred to a low temperature (LT) environment (4 ± 2ºC) and micturition patterns were again recorded. TRPM8 channel expression in lumbar skin was estimated by real-time reverse-transcriptase polymerase chain reaction and immunohistochemistry.

**Results:** TRPM8 channel mRNA and protein in the skin of OVX rats were significantly higher than in sham-operated rats. At RT, micturition parameters were similar for sham-operated and OVX rats. At LT, both sham-operated and OVX rats exhibited cold

stress-induced detrusor overactivity, but the increased micturition frequency and decreased bladder capacity were significantly greater for OVX rats. Treatment of OVX rats with naftopidil inhibited the cold stress-induced detrusor overactivity.

**Conclusion:** Cold stress-induced detrusor overactivity in rats with decreased estrogen is associated with an upregulation TRPM8 channels in the skin, and is mediated by nerve pathways utilizing alpha1-adrenergic receptors.

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Cold stress associated with seasonal or continuous environmental low temperature may aggravate lower urinary tract symptoms (LUTS), including urinary urgency, urinary frequency, and nocturia.[1] A high incidence of LUTS in postmenopausal women was shown to correlate closely with the naturally occurring estrogen deficiency.[2] Anecdotally, postmenopausal women having LUTS often complain about aggravated symptoms due to cold stress in seasonally low temperatures. However, the mechanisms by which they become more sensitive to cold have not been sufficiently resolved. We have established an animal model to investigate cold stress-aggravated LUTS.[3] In this model, sudden whole body cooling of conscious healthy rats induces detrusor overactivity, decreases voiding interval, micturition volume, and bladder capacity. The cold stress-induced detrusor overactivity is mediated, in part, by a C fiber-sensitive neural pathway [3,4] and involves the calcium-permeable, non-selective, cation transient receptor potential melastatin 8 (TRPM8) channel that acts as a thermosensitive receptor in skin cells and/or sensory neurons.[5,6] The cold stress-induced detrusor activity can be inhibited by alpha1-adrenergic receptor antagonists.[7]

In the present study, we used this model to determine whether estrogen deficiency resulting from ovariectomy would increase TRPM8 channel expression levels in the

skin and increase the bladder response to cold stress. We also wanted to confirm that blockade of alpha1-adrenergic receptors could inhibit this response.

## MATERIALS AND METHODS

### Animals

Twenty-four female Sprague-Dawley rats at postnatal week 30 (Japan SLC Inc., Japan) were used for the experiments. The animals were treated in accordance with National Institutes of Health Animal Care Guidelines and the guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine (No.220034).

### Bilateral Ovariectomy

The animals were randomly divided into two groups: sham operation (sham group, n=8) and bilateral ovariectomy (OVX group, n=16). The rats were anesthetized with pentobarbital sodium solution (40 mg/kg-body weight, Kyoritsu Seiyaku Co., Tokyo, Japan), and then a midline incision was made to expose the lower abdominal cavity. In the OVX group, both ovaries were removed, and the abdomen was closed. In the sham group, the abdomen was closed without removing the ovaries. All animals were maintained for 5 weeks under a 12-h alternating light-dark cycle with freely available food and water. After 5 weeks, to estimate the effects of ovariectomy, body and uterus

weights were measured. Serum estradiol levels were determined by a commercial laboratory (SRL, Inc., Tokyo, Japan).

**Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

After cystometric investigations (see below), about 2 $cm^2$ of lumbar skin was harvested under anesthesia. The tissues were separated into samples for real-time RT-PCR and immunohistochemistry (see below). For real-time RT-PCR the skin pieces were homogenized, and total RNA was extracted with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Complementary DNA (cDNA) was synthesized from 0.1 μg of total RNA with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). To determine mRNA expression levels, real-time RT-PCR of the cDNA was performed with either Trpm8 primer for the TRPM8 channel gene (Rn00592665 m1, Applied Biosystems) or Actb primer for the beta-actin gene used as an internal amplification control (Rn00667869 m1, Applied Biosystems). The initial reactions were run at 50ºC for 2 min and then 95ºC for 10 min. These were followed by 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. Gene activity was expressed as the ratio to the internal standard gene beta-actin.

**Immunohistochemistry**

Other harvested skin pieces were fixed in 4% paraformaldehyde with 4% sucrose in 0.1 M phosphate buffer for 12 h at 4ºC and then embedded in paraffin. Each sample was cut into 5-µm thick serial sections. The sections were deparaffinized, and then antigen retrieval was achieved by immersion in 10 mM sodium citrate and microwaving at 100 ºC for 5 min. 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) for 1 h at 4ºC. The sections were then incubated with anti-TRPM8 antibody (1:50, rabbit polyclonal, Trans Genic Inc., Kobe, Japan) for 12 h at 4ºC. The sections were rinsed with PBS at 4ºC, and then incubated with donkey anti-rabbit IgG secondary antibody conjugated with Alexa fluor 594 (1:250, Molecular Probes, Eugene, OR, USA) for 1 h at 4ºC. Following rinsing, double staining of each section was achieved by incubation with anti-S100 antibody (1:50, mouse monoclonal, Abcam Ltd, Cambridge, UK), a marker of nerve fibers, for 12 h at 4ºC. After rinsing with PBS, they were incubated with donkey anti-mouse IgG secondary antibody conjugated with Alexa fluor 488 (1:250, Molecular Probes) for 1 h at 4ºC. Finally, cell nuclei were counterstained with 5 µg/ml 4’, 6-diamidino-2phenylindole dihydrochloride (DAPI, Molecular Probes). The slides were coated with Fluorescent Mounting Medium

(Dako Cytomation, Carpinteria, CA, USA) and observed with a Leica DAS Microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Observers, who were not aware of the treatment status, semi-quantitatively evaluated the proportion of TRPM8 channel-positive areas with image processing software (Image-pro Plus, Media Cybernetics, Inc., Bethesda, MD, USA). The fluorescent marker areas of the TRPM8 receptors were averaged from eight observed regions (x400 power) in each sample, and then were expressed as a proportion of the total observed tissue areas.

**Drugs**

Naftopidil, an alpha1-adrenergic receptor blocker, kindly provided by Asahi Kasei Pharma Co. (Tokyo, Japan) was dissolved with 0.1 M phosphate buffer solution to 3.5 mg/ml.

**Cystometric Investigations**

Three days prior to the cystometric investigation, the rats were anaesthetized (as above), and the urinary bladder was exposed. A polyethylene catheter (PE50, Nippon Becton Dickinson, Tokyo, Japan) was inserted through center of the bladder dome and

fixed with 5-0 silk sutures. For intravenous drug administration, the right external jugular vein was cannulated with a polyethylene catheter (PE10, Nippon Becton Dickinson, Tokyo, Japan). The free end of both catheters were tunneled subcutaneously and exteriorized at the back of the neck. After 3 days, each rat was placed without any restraint or anesthesia in a metabolic cage to measure micturition volume with a urine collector connected to a force displacement transducer (type 45196; NEC San-ei Instruments, Tokyo, Japan). The bladder catheter was connected to a pressure transducer (DX-100, Nihon Kohden, Tokyo, Japan) and a microinjection syringe pump (TE-351, Terumo, Tokyo, Japan). Throughout the experiments, room temperature (RT) saline was pumped into the bladder at a rate of 10 ml/h with the syringe pump. The following cystometric parameters were measured: basal pressure ($cmH_2O$), maximum micturition pressure ($cmH_2O$), voiding interval (min), micturition volume (ml), residual volume (ml), and bladder capacity (ml). The residual volume was determined as the difference between the saline infusion volume and micturition volume. Bladder capacity was calculated by adding the micturition volume and the residual volume.

Five weeks after bilateral OVX, the rats were separated into two groups: naftopidil-free vehicle and 1.0 mg/kg naftopidil administration groups (n=8 in each group).[7] The cystometric investigations of the catheterized, unanesthetized, unrestricted

rats were perfomed under the following environmental temperature conditions. The rats were placed singly in metabolic cages at RT (28 ± 2ºC) for 20 min. After these baseline measurements were taken, they were slowly administered 0.1 ml naftopidil-free vehicle or naftopidil solution (1.0 mg per kg body weight) through the jugular vein catheter. After 5 min, they were gently and quickly transferred in the metabolic cages to the cold room for low temperature (LT) exposure (4 ± 2ºC) for 20 min during which the cystometric measurements were again recorded. Cystometric investigations of sham-operated rats were similarly performed with intravenous naftopidil-free vehicle.

**Statistical Analysis**

The results were expressed as means ± standard error of the means. Two-way non-repeated measures analysis of variance followed by the Scheffe's test or two-way non-repeated t-test were used. Differences with $P < 0.05$ were considered significant.

## RESULTS

### Bilateral Ovariectomized Rats

Prior to bilateral OVX, there was no difference between the body weights of the sham and OVX groups, 324 ± 13 g and 320 ± 20 g respectively. At 5 weeks after the operation, the body weight of the OVX rats, 377 ± 30 g, was significantly higher than that of the sham rats, 326 ± 16 g ($P < 0.01$). In contrast, the uterus weight of the OVX rats, 0.42 ± 0.07 g, was significantly lower than that of the sham rats, 0.77 ± 0.16 g ($P < 0.01$). The serum estradiol level of the OVX rats, 3.83 ± 5.95 pg/ml, was also significantly lower than that of the sham rats, 20.83 ± 6.34 pg/ml ($P < 0.01$).

The relative expression of TRPM8 channel mRNA in the lumbar skin of the OVX rats, 2.36 ± 0.61 was significantly higher than that of the sham operated rats, 0.83 ± 0.12 ($P<0.01$, Fig. 1A). TRPM8 channels and S100-positive nerve fibers in the lumbar skin of both sham (Fig. 1B) and OVX (Fig. 1C) rats were visualized by immunohistochemistry. Distribution of TRPM8 channels was similar for the two groups; however, the proportion of TRPM8 channel-positive area in the OVX rats,

0.0063 ± 0.0011, was significantly higher than that in the sham rats, 0.0031 ± 0.0005 ($P<0.01$, Fig. 1D).

**Effect of Naftopidil on Cold Stress-Induced Detrusor Overactivity**

At RT, there were no differences in the micturition patterns of sham (Fig. 2A) and OVX rats treated with naftopidil-free vehicle (Fig. 2B), and there were no differences in the micturition parameters between the two groups (Table 1). After transfer to LT, both the sham-operated and OVX rats exhibited cold stress-induced detrusor overactivity that caused increases in micturition frequency and decreases in micturition volume. However for both parameters, the changes were greater for the OVX rats (Fig. 2B) than for the sham-operated rats (Fig. 2A). There were no differences in the basal or maximum micturition pressures between groups at either RT or LT (Table 1). Voiding interval, micturition volume, and bladder capacity of both sham-operated and OVX rats were significantly decreased at LT compared to RT (Table 1). However, the voiding interval (2.02 ± 0.26 min), micturition volume (0.43 ± 0.07 ml), and bladder capacity (0.43 ± 0.07 ml) of the OVX rats were significantly lower than in the sham-operated rats (Table 1).

At RT, administration of naftopidil to the OVX rats did not alter the micturition patterns and parameters (Fig. 2C, Table 1). After transfer to LT, naftopidil-treated OVX rats did not exhibit the typical cold stress-induced detrusor overactivity such as increased micturition frequency or decreased micturition volume. At LT, the voiding interval (4.30 ± 0.79 min), micturition volume (0.72 ± 0.14 ml), and bladder capacity (0.75 ± 0.14 ml) of the naftopidil-administered OVX rats were similar to those parameters at RT. Furthermore, these values were significantly higher than the same parameters of both the naftopidil-free vehicle-administered sham and OVX rats (Table 1).

## DISCUSSION

Estrogen receptors are present in the lower urinary tract of animals [8] as well as in humans,[9] and play an important role for LUT function, both normally and in LUT dysfunction.[10] Epidemiological studies have shown that the development of LUTS in postmenopausal women is associated with the onset of estrogen deficiency.[2] However, the effects of estrogen treatment on bladder dysfunction, including LUTS, is still controversial.[11]

Ovariectomized animals have been frequently used as an experimental model for studies on the effect of lack of estrogen, and have shown a great variety of bladder dysfunctions, including increased voiding frequency and/or altered voiding patterns.[8, 12, 13] We focused on detrusor overactivity elicited by cold stress due to sudden whole body cooling of sham-operated and OVX rats.

At RT, the micturition patterns of OVX rats did not differ significantly from sham-operated rats. Thus at RT the decrease of estrogen did not induce detrusor overactivity. Both sham and OVX rats transferred to LT exhibited cold stress-induced detrusor overactivity that caused decreased voiding interval, micturition volume, and

bladder capacity. However, the decreases in OVX rats were significantly greater than those of sham-operated rats. Thus, at LT the loss of estrogen appeared to exacerbate the detrusor overactivity.

TRPM8 channels are sensitive to moderately cold temperatures, less than 25-28ºC, with currents increasing down to 8ºC.[5,6] The cold response is mediated by these channels located in the skin and in sensory neurons of the dorsal root ganglia that are associated with cold-evoked urinary urgency.[14] In addition, we previously showed that direct stimulation of skin with menthol induces detrusor overactivity[15] that is similar to cold stress-induced detrusor overactivity in our OVX rats. Thus collectively, these results suggest that TRPM8 channels in the skin are associated with cold stress-induced detrusor overactivity. However, contributions of other TRP channels cannot be excluded.

In the current study, the loss of estrogen through ovariectomy increased the expression of TRPM8 channel mRNA and protein in the lumbar skin. Skin is an estrogen responsive organ,[16-19] but the mechanism by which ovariectomy influences TRPM8 channel expression is unknown. While we found an increase in the expression of TRMP8 mRNA and protein, we do not know how ovariectomy affected this thermosensitive channel in other tissues such as the dorsal root ganglion. There are

some reports that the loss of TRPM8 channels is associated with a decline of cold sensation.[20-23] Thus, the increased TRPM8 channel expression in OVX rats might be responsible for the greater cold sensitivity of the skin or sensory nerve endings. If our results have validity also for humans, it cannot be excluded that increased TRPM8 expression in the skin may contribute to increased cold sensitivity and to a worsening of LUTS under LT conditions in postmenopausal women.

The alpha1-adrenergic receptor blocker naftopidil reduced the cold stress-induced detrusor overactivity both in sham and OVX rats, confirming previous results in cold-stressed normal rats. Naftopidil has a higher affinity for alpha1D-adrenergic receptors compared to other alpha1-receptor subtypes.[24,25] Therefore, the cold stress-induced detrusor overactivity, both in normal rats, and rats with estrogen deficiency, may be mediated via nerve pathways that, at least partly, utilize alpha1D-adrenergic receptors.[25]

## CONCLUSION

The expression of TRPM8 channel protein and mRNA in the lumbar skin of OVX rats was significantly higher than that of sham-operated rats. At RT, the micturition patterns of OVX rats were similar to the sham-operated rats. However at LT, the OVX rats exhibited cold stress-induced detrusor overactivity that was significantly more pronounced than that found in the sham-operated rats. Both in normal rats, and rats with lack of estrogen, cold stress-induced detrusor overactivity may be mediated, at least in part, by nerve pathways that utilize alpha1D-adrenergic receptors.

(2366/2500)

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**Figure Legends**

Figure 1. Expression level of TRPM8 channels in lumbar skin. **A:** The relative expression of TRPM8 channel mRNA in the OVX rats was significantly higher than that in the sham-operated rats. **B** and **C:** The distribution of TRPM8 channels (red, arrowheads) and S100-positive nerve fibers (green, arrows) in sham-operated (B) and OVX (C) rats were similar. Blue, nuclei. **D:** The proportion of area occupied by TRPM8 channels in the OVX rats was significantly higher than that in the sham-operated rats.

Figure 2. Effect of temperature changes on micturition patterns. **A:** After transfer to LT, the naftopidil-free vehicle-administered sham-operated rats exhibited cold stress-induced detrusor overactivity patterns that included high micturition frequency and low micturition volume. **B:** Naftopidil-free vehicle-administered OVX rats transferred to LT also exhibited the cold stress-induced detrusor overactivity. The cold responses were more severe compared to sham-operated rats (A). **C:** After transfer to LT, the cold stress-induced detrusor overactivity in the naftopidil-administered OVX rats was partially inhibited. IP, intravesical pressure; MV, micturition volume; black and

white arrowheads, transfer from RT to LT; asterisks, micturition volume line returned to baseline.

Table 1. Cystometric Parameters under Room and Low Temperature

| | Basal pressure (cmH2O) | | Maximum micturition pressure (cmH2O) | | Voiding interval (min) | | Micturition volume (ml) | | Bladder capacity (ml) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RT | LT | RT | LT | RT | LT | RT | LT | RT | LT |
| Saline-administrated Sham rats | 23.45±4.75 | 26.39±5.66 | 52.13±4.97 | 52.41±6.20 | 4.16±0.36 | 2.98±0.48* | 0.83±0.09 | 0.62 ± 0.10* | 0.83±0.08 | 0.63±0.10* |
| Saline-administrated OVX rats | 23.08±4.65 | 28.50±4.87 | 52.30±5.72 | 54.95±4.63 | 4.30±0.54 | 2.02±0.26**†† | 0.72±0.11 | 0.43±0.07**† | 0.76±0.11 | 0.43±0.07**† |
| Naftopidil-administrated OVX rats | 22.50±4.50 | 22.25±3.61 | 47.75±5.64 | 45.05±5.44 | 4.18±0.59 | 4.30±0.79††## | 0.72±0.12 | 0.72±0.14††# | 0.74±0.12 | 0.75±0.14††# |

The results were expressed as means ± standard error of the means.

*P<0.05, **P<0.01; compared to RT in each group.

†P<0.05, ††P<0.01; compared to saline-administrated sham rats transferred to LT.

#P<0.05, ##P<0.01; compared to saline-administrated OVX rats transferred to LT.

Figure 1

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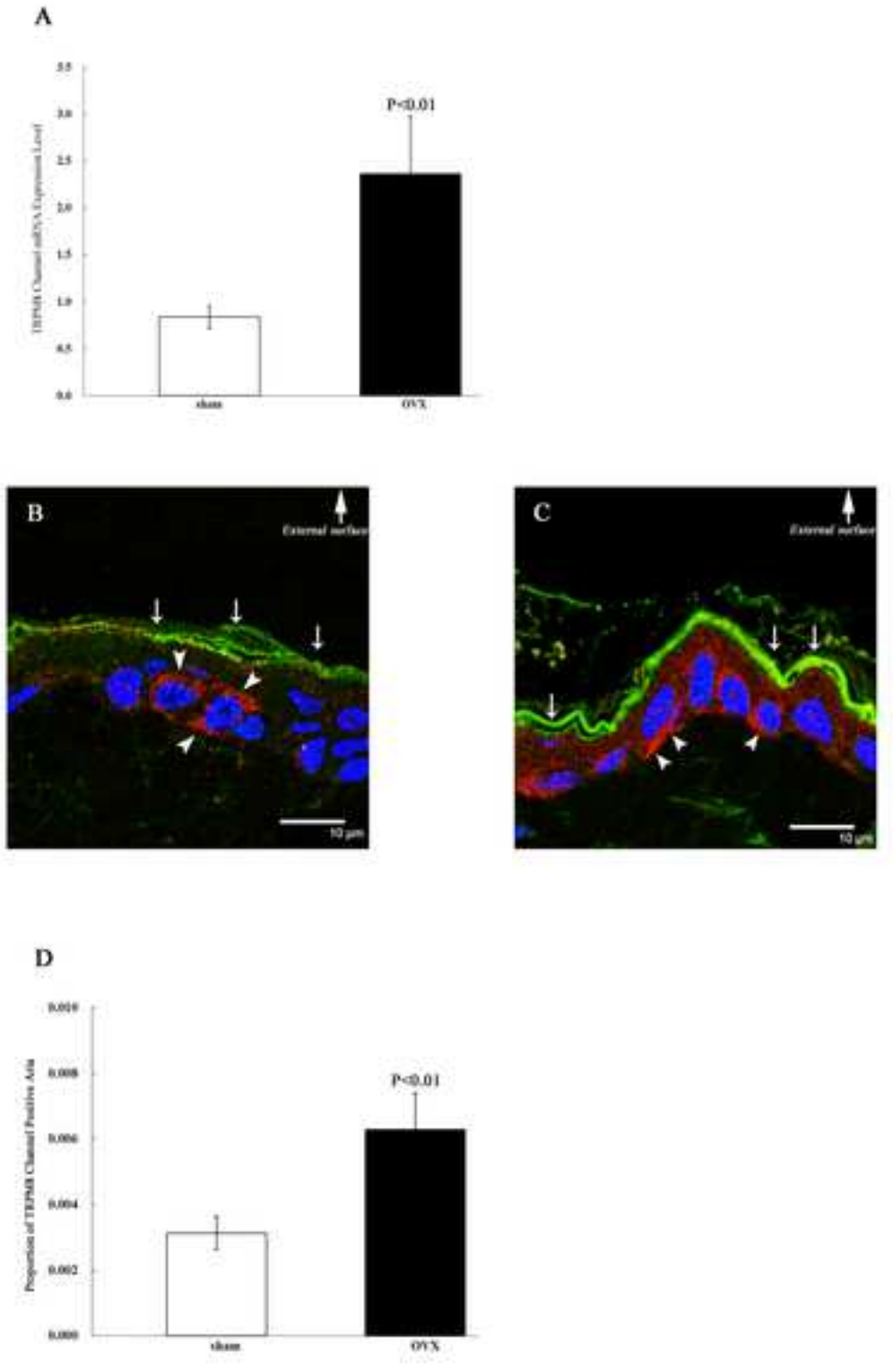


Fig. 1

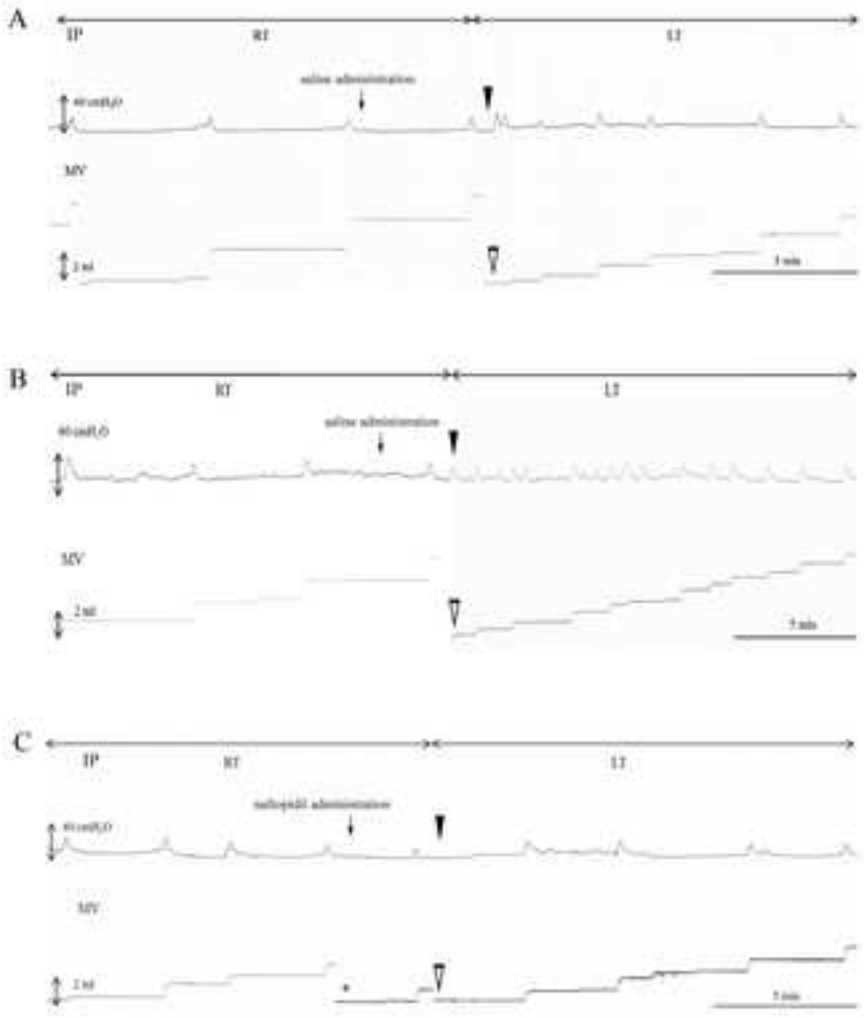


**Fig. 2**