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14. ABSTRACT
Interstitial cystitis/bladder pain syndrome (IC/BPS) is a debilitating disorder characterized by persistent pelvic pain associated with bladder symptoms including urinary frequency and urgency. Previous studies have indicated that overexpression of nerve growth factor (NGF) is a important factor in the symptom development of IC/BPS. This project evaluates the feasibility of an anti-NGF bladder drug delivery system as a potential investigational drug product. The project aims include exploration, manufacture, characterization, and nonclinical animal testing of a liposome NGF-antisense formulation targeting IC/BPS. During the project period investigators conducted nonclinical experiments and analyzed results from an animal efficacy experiment conducted during the previous period. The project is ongoing.

15. SUBJECT TERMS
Interstitial cystitis/painful bladder syndrome (IC/PBS), Liposome

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I-1. Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a debilitating disorder characterized by persistent pelvic pain with symptoms of urinary frequency, urgency, nocturia, and painful intercourse. IC/BPS can affect both genders with epidemiological data suggesting that up to 12% of women may have early symptoms of IC/BPS. IC/BPS patients are currently treated through various means: pentosan polysulfate sodium, dimethyl sulfoxide, off label use of amitriptyline, anesthetics, immunosuppressive drugs and narcotic analgesics. Each of these approaches has significant limitations. Unfortunately, there are currently no adequate medical or surgical remedies for many IC/BPS patients. IC/BPS is recognized as a significant unmet medical need.

Previous studies indicate overexpression of nerve growth factor (NGF) as a key factor in the symptom development of IC/BPS. NGF antisense oligonucleotides hold promise for IC/BPS by restricting the effect of NGF in the bladder. However, a systemic approach for NGF silencing in the bladder risks inducing systemic adverse events, suggesting a local-bladder drug delivery approach is needed. Local delivery options have traditionally been hampered by inefficient intracellular delivery of antisense. Liposomes have been used for drug delivery in a variety of applications. The investigators have previously investigated liposome drug delivery formulations and have completed studies using intravesical liposomes to demonstrate they are not systemically absorbed.

This project explores the feasibility of liposome mediated delivery of anti-nerve growth factor (NGF) for bladder pain and frequency. Investigators are developing an investigative product, called LP-11, consisting of a liposome vehicle mixed with antisense for intravesical delivery, with LP-11's formulation under development in this project. The goal of this project is to provide the foundation for future translation of LP-11 and local NGF antisense therapies for the IC/BPS indication. Lipella Pharmaceuticals Inc is collaborating with investigators located at the University of Pittsburgh to conduct the experimental program.

I-2. Keywords

Interstitial cystitis/bladder pain syndrome (IC/BPS), nerve growth factor (NGF), Small interfering RNA (siRNA), liposome, urology, drug delivery

I-3. Overall Project Summary

In this project, investigators work to develop a local therapy targeting NGF production in the bladder for the treatment of bladder pain. Aims of this project support the preclinical investigation and early product development of LP-11. The broad aims of the project are to characterize, develop manufacturing methods, and conduct formulation development of LP-11 for future clinical trials. The project aims also include preclinical evaluation of LP-11 in experimental rat animal models. The statement of work (SOW) for the experimental plan and timeline are summarized below.

II. Body

II-1. Timeline described in the SOW

<table>
<tr><td></td><td colspan="2">Year 1</td><td colspan="3">Year 2</td><td colspan="3">Year 3</td></tr>
<tr><td></td><td>0-4mo</td><td>5-12mo</td><td>13-16mo</td><td>17-20mo</td><td>21-24mo</td><td>25-28mo</td><td>29-32mo</td><td>33-36mo</td></tr>
<tr><td>AIM 1</td><td>□ Regulatory approval for animal research
□ Obtain written approval from both IACUC and ACURO</td><td>□ Order animals/
□ Prepare protocols/ documentation
□ Initiate animal study and confirmation of CYP and TNBS</td><td colspan="3">□ CYP, TNBS treatment
□ LP-NGF antisense treatment
o Pain behavior/bladder over-activity assessment
□ Tissue Analysis
o PCR/ELISA
o Patch Clamp</td><td colspan="2">Complete animal studies</td><td>Animal study data analysis/reporting</td></tr>
<tr><td>AIM 2</td><td colspan="2">□ Optimize formulation
□ Manufacture LPs
□ Analytical method development</td><td colspan="3">□ Develop GMP LP-NGF antisense manufacture process
□ cGMP documentation reporting
□ Manufacture GMP LP
□ Product specifications/batch release testing
□ Biosdistribution assessment</td><td colspan="3">□ Stability Testing
□ Ex-vivo stress testing</td></tr>
</table>

II-2. Research Accomplishments

Aim 1 (Year 3) *Animal study data reporting*
[Accomplishment-1]
Prior to this reporting period, preclinical animal studies were completed along a stepwise plan. In Aim 1 (Year 1) we concluded that cyclophoshamide (CYP) induced cystitis and trinitrobenze sulfonic acid (TNBS) induced colitis animal models exhibited bladder hyperactivity, pain and increased expression of NGF. The results of TNBS colitis rats were published in *Neuroscience* in Year 3 (Yoshikawa et al., 2015). In Aim 1 (Year 2), we studied the therapeutic effect of LP-11 in this diseased animal model, corresponding to Aim 1's "LP-NGF antisense treatment [LP-11] Pain behavior/bladder over-activity assessment" in section II-1.

The study result was summarized in the previous period's annual report. In this period we have added additional analysis to complete the study reporting. Below is a summary of previous reported findings followed by the new analysis.

Summary of previously reported methods and results:
Purpose: The purpose of the study was to evaluate the effect of bladder delivery (intravesical) of liposome conjugated with antisense oligonucleotide (LP-11) in a rat model of IC/BPS symptoms and NGF expression induced by intracolonic 2,4,6,trinitrobenzen sulfonic acid enema (TNBS) experimental colitis.

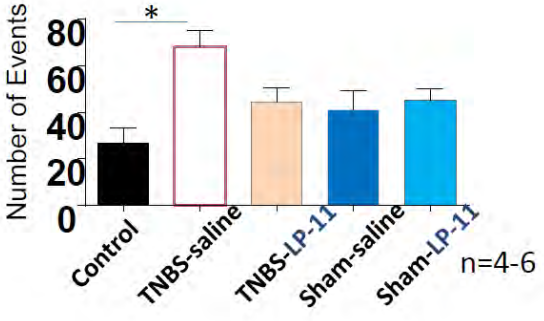
Methods : Adult female Sprague-Dawley rats were divided into five groups (n=90); (a) control group (no TNBS and no LP-11), (b) a TNBS and LP-11 treated group, (c) a TNB treated with intravesical placebo (saline) group, (d) sham (no TNBS) and LP-11 treated group and (e) sham-saline group. Intravesical administration of .2ml of LP-11 or saline was given under isoflurane anesthesia was instilled to the bladder through an inserted urethral catheter prior to TNBS administration. Twenty-four hours after instillation of LP-11 or saline, colitis was induced by the enema of 30mg TNBS.

Ten days after LP-11 or saline injection treatments, animals were subjected to either in vivo studies or bladder tissue removal. Testing involved nociceptive behavior testing for pain, cytometry for frequency (ICI), immunohistochemistry, and polymerase chain reaction for NGF analysis.

In summarizing our findings we found: (1) in the TNBS-saline group, the score of freezing behavior, defined as a lack of movement by the rats representing bladder pain sensation, was significantly higher than that of other groups including the TNBS-LP-11 group; (2) the ICI reduction rate into the bladder is significantly higher in the TNBS-saline group than that in the TNBS-LP-11 group; (3) The mRNA and protein expression of NGF in the mucosa were significantly higher in the TNBS-saline group compared to the TNBS-LP-11 group. These finding suggest a beneficial effect of LP-11 on IC/BPS according to the measures.

Newly reported results:
Lower abdominal licking
The results for animal lower abdominal licking, corresponding to pudendal nerve-dependent pain behavior (urethral pain), was examined. In previous experiments, bilateral pelvic nerve transection significantly suppressed freezing, but not licking, behavior in rats with bladder irritation. However licking was evaluated in this study to clarify if bladder pain sensation is enhanced after chronic cystitis and if LP-11 antisense treatment suppresses bladder nociceptive responses in rats.

Figure 1: Licking Behavior

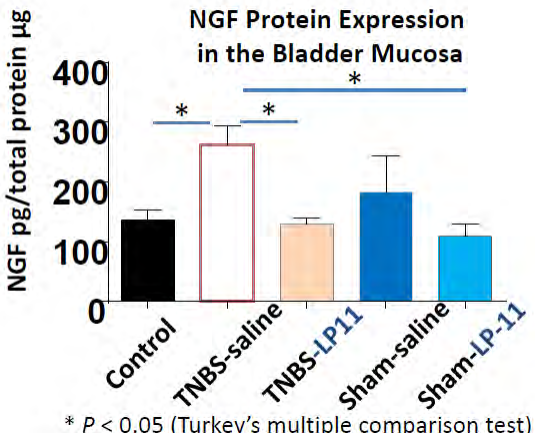


Licking events were counted by a blinded observer for 15 minutes in response to intravesical administration of resiniferatoxin (RTx, 0.3µM, 0.3ml) in a metabolic cage. We found the licking score in the TNBS-saline group of 80 was significantly higher than in the control group and tended to higher compare to other 3 groups including TNBS-LP-11 (**Figure 1**).

Protein Expression

The bladder was dissected to divide into mucosal and detrusor layers for quantification of protein of NGF. Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to measure the protein expression of NGF. We observed the protein expression of NGF in the TNBS-saline group was significantly increased in the mucosa compared to control and TNBS-LP-11 groups (Figure 2). LP-11 reduced protein levels due to the direct antisense effect of reduced transport of NGF from the bladder to bladder afferent pathways in TNBS rats

Figure 2: NGF expression



Conclusions: Our overall conclusions that LP-11 had a therapeutic effect on TNBS-induced bladder hypersensitivity as evidenced reductions in licking behavior and NGF protein expression compared to placebo are unchanged given these results. Preclinical efficacy studies suggest LP-11 may be a promising treatment for IC/BPS. The results were presented at the *Military Health Research Symposium* (MHSRS) in August, 2015 (Chancellor et al, 2015) as well as in our recent review article (Ogawa et al., 2015) and submitted as an original article (Kawamorita et al., 2015) to Journal of Urology (currently under revision)..

[Accomplishment-2]

Although we showed that CYP-induced cystitis rats exhibited bladder overactivity and increased pain sensitivity (Year 1), the expression of NGF in the bladder was not always stable. Therefore, in the Year 3, we improved the model of chronic cystitis induced by intravesical application of hydrogen peroxide (HP), which showed a more stable increase in the bladder NGF level, and tested the efficacy of LP-11 treatment targeting bladder NGF upregulation in this chronic cystitis model.

Purpose: The purpose of the study was to investigate the effect of intravesical liposome-based NGF antisense (LP-11) on bladder overactivity and nociceptive behavior in a rat model of chronic cystitis induced by intravesical HP instillation.

Methods: Adult female Sprague-Dawley rats were divided into five groups (n=48); (a) saline + vehicle (SV) group, (b) saline + LP-11 group, (c) 1.5% HP + vehicle group, (d) 1.5% HP + LP-11 group. Saline or 1.5% HP was administered into the bladder on day 0. Each rat was treated with intravesical vehicle or NGF antisense administration on day 2.

Summary of Results: (1) HP-induced cystitis rats with LP-11 treatment showed significantly less bladder overactivity (cytometry) and reduced bladder-related pain behavior (freezing behavior) compared with cystitis rats without lp-11 treatment. (2) HE staining of bladder sections showed that substantial infiltration of the inflammatory cells, submucosal bleeding, and detrusor hypertrophy seen in HP-induced cystitis rats; however these histological changes were alleviated by the LP-11 treatment. (3) In RT-PCR, HP-induced cystitis rats with LP-11 treatment showed the reductions in expression of NGF and TRPV1 in the mucosa and L6-S1 dorsal root ganglia (DRG), which was increased after HP-induced cystitis.

Conclusions: These results indicate that intravesical LP-11 therapy induces a reduction in the NGF expression in the bladder mucosa and bladder afferent pathways, which results in the improvement of bladder pain behavior and frequent urination induced by hydrogen peroxide-induced chronic cystitis. Thus, intravesical LP-11 therapy could be a novel treatment that can avoid systemic adverse events for hypersensitive bladder disorders such as IC/BPS, in which NGF has been implicated as an important mediator for inducing afferent sensitization. The results were presented at 2015 *International Continence Society* (ICS) meeting in Montreal, Canada (Majima et al., 2015; ICS abstract).

Aim 2 (Year 3) *Stability Testing*

[**Accomplishment-3**]

1. Accelerated Testing of LP-11

LP-11 accelerated stability study:

DOTAP liposomal lyophilate was removed from freezer and allowed to warm to ambient temperature. A 2 mM stock solution of SIRNA was made from dry siRNA powder with UltraPure Distilled Water (Invitrogen; RNAase and DNAase

free). The DOTAP lyophilate was rehydrated, and an aliquot of the siIRNA solution was added to the liposome suspension to make a DOTAP and siRNA solution. The resulting mixture was shaken for approximately 30 seconds. 250 uL of the resulting solution was added to 500 uL water in a 2-mL tube, and another portion was added to 500 uL synthetic urine (Ricca Chemical Company) to bring the liposome concentration to 2 mg/mL. A sample was taken from each for baseline imaging. Each sample was placed in a water bath in an incubator at 37°C. Timing was started when the tubes were added to the incubator. Samples were taken for imaging at 30, 60, 90, 120, 180, and 240 minutes. Samples were diluted in their respective solvents for imaging. Images were sampled from three 3 uL spots on the microscope slide.

Results

For the accelerated stability study, ANOVA results indicate that there was no significant difference between treatment groups (p=0.3517) or for timepoints (p=0.7355). These results suggest that there was no significant change at any point in the size distribution of the liposomes obtained via microscopy in either urine or water solvents over the course of four hours. The following are summary statistics from the accelerated study.

	Water		Synthetic Urine	
Timepoint	Mean +/- SEM	% change	Mean +/- SEM	% change
Baseline	377 +/- 27	-	400 +/- 18	-
30 min	411 +/- 26	+9.0	430 +/- 18	+7.5
60 min	406 +/- 25	-1.2	404 +/- 17	-6.0
90 min	452 +/- 27	+11.3	396 +/- 19	-2.0
120 min	413 +/- 26	-8.6	412 +/- 17	+4.0
180 min	424 +/- 29	+2.7	378 +/- 18	-8.3
240 min	414 +/- 29	-2.4	393 +/- 18	+4.0

Discussion

Over the time period monitored in the accelerated stability study (4 hours), there was no significant change in particle area at any time point, and there was no significant difference in spot area between the water and urine preparation. This time period is longer than the proposed preparation time and bladder residence time (approx. 1 hour).

2.Long-term Stability of DOTAP liposomes LP-11 admixture

[Accomplishment]

Stability of the liposomal component of the LP-11 formulation was evaluated by testing two characteristics: DOTAP concentration and particle size.

Concentration Stability Study of DOTAP Component of LP-11:

The stability of the DOTAP component of LP-11 was evaluated by quantitative ^{1}H NMR analysis at specific time points following storage of the lyophilized liposomes at -20C.

Purpose

The purpose of quantitative analysis of the DOTAP component is to ensure that during storage of the lyophilized DOTAP liposomes at -20C the purity and integrity of the DOTAP is maintained. Future additional measurements will be taken of the DOTAP liposomal powder, at time points up to 8 months post manufacture, to assess long-term stability of the liposome powder at -20C storage.

Procedure

Samples were stored at -20C until analysis. At each time point analysis procedure was kept consistent: allowing first the sample vial of LP-11 liposomes to come to room temperature followed by reconstitution with USP Sterile water to a final DOTAP concentration of 7mg/mL (vials were manufactured to contain 10mg DOTAP lipid). Deuterated chloroform was added and vials were vortexed and then centrifuged to separate the aqueous and organic layers. The deuterated chloroform layer of each sample was then transferred to an NMR tube for analysis. Analysis was performed using a Bruker AVANCE III 400mHz NMR spectrometer with cryoprobe prodigy. The proton responses were calibrated with a dioleoyl phosphatidylcholine.

Results

A minimum of 4 month stability was determined for the DOTAP component of LP-11, liposomal siRNA. DOTAP concentration was determined to be consistent for the 4 month storage period at -20C freezer conditions. Quantitative results are listed in **Table 1** below. The study is ongoing full study results will be reported in the final report.

Table 1: Concentration of DOTAP in LP-11 Liposomes in mg/mL by ^{1}H NMR

Timepoint	Concentration (mg/mL)
1 month	6.98
2 months	
4 months	

Particle Size Stability study of DOTAP Component of LP-11:
DOTAP liposomal lyophilate sample was removed from the freezer and allowed to warm to ambient temperature. Sterile water for injection (WFI) was added to to make a 2 mg/mL suspension of DOTAP. A sample from the vial was diluted to 0.2 mg/mL and three 3 uL sample spots were made on a microscope slide for imaging. This was performed at months 1, 2, 4, and the study will continue to months 6 and 8. A new vial was used at each timepoint.

Image Acquisition
All samples taken for imaging were diluted to 0.2 mg/mL liposome concentration. Images were taken with an AmScope MA1000 camera at 1280x960 resolution with 6x magnification. At least 20 images were taken at each timepoint.

Image Analysis
The purpose of the analysis is to detect changes in the liposome size distribution over time. The metric that was measured is the number of pixels per liposome spot (i.e. particle area). A program was written in the Python[[PYTHON]] programming language using the scikit-image[[SKIMAGE]] package to process the microscopy images and automatically obtain a distribution of particle areas for each image.

Error in the spot detection method has not yet been quantitatively measured (i.e. against ground-truth images); however, qualitative testing was performed by comparing input and output images. The method is robust for our particular microscopy image and it was parameterized to keep false positives at a minimum. In-focus spot areas are slightly overestimated. This error arises mainly from the mean filter. Slightly out-of-focus spot areas are slightly underestimated. This is because the peak maxima are not at the very edge of the spots but are offset from the edge closer to the center. This is reasonable, because the liposome image grows when it is slightly out-of-focus. Very out-of-focus spots are excluded via the chosen threshold. These biases are consistent throughout the samples that were taken. Future work will seek to reduce or eliminate the overestimation errors in the in-focus spots and eliminate the slightly out-of-focus spots from analysis.

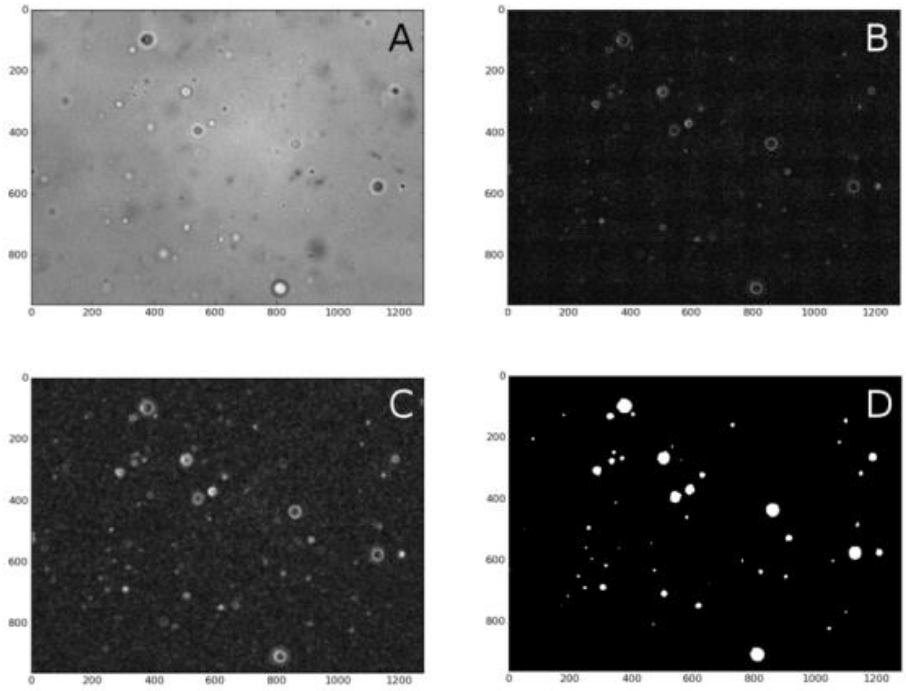


Figure 3
Example Input and Results from Spot Identification Program. A) Original microscopy image of liposomes. B) Sobel-filter of (A). C) Mean-filter of (B). D) Threshold of (C). White areas are "True" and black areas are "False."

Statistical Analysis
One-way ANOVA was performed on spot size data from the accelerated stability study. Both solvent (urine v. water) and timepoints (baseline thru 240 min) were used as model effects. As the results of the ANOVA suggested there were no significant differences, post-hoc tests were not performed.

The α-level for all tests was chosen to be 0.05. Results are reported as mean spot area +/- Standard Error of the Mean (SEM).

The ANOVA test suggested that spot size changed significantly as time from manufacture increased (p=0.0053). Results for mean spot areas and Tukey's HSD test are as follows:

Comparison	p-value, Tukey's HSD	% change
Month 1 vs. Month 2	0.1413	-11.1
Month 2 vs. Month 4	0.6570	-5.0
Month 1 vs. Month 4	0.0035*	-15.6

Timepoint	Mean Pixel Area +/- SEM
Month 1	449 +/- 17
Month 2	399 +/- 19
Month 4	379 +/- 13

These results suggest that there was a significant decrease in spot sizes from month 1 to month 4. However, there was no significant difference in mean spot size between months 1 and 2 and months 2 and 4.

Discussion
The long-term data suggest that there is a significant decrease in the mean liposome size over (at least) a four month period. Different vials were used at each timepoint. It is unlikely that the differences seen were due to variability between sample vials. Past experiments (in house; unpublished) with sphingomyelin liposomes showed no variability between liposome vials, but these experiments have not yet been performed for DOTAP liposomes.

Aim 2 (Year 3) *Ex-vivo stress testing*
[**Accomplishment-4**]
Stress testing was performed to characterize stability of LP-11 liposomes in a bladder environment.

Stress Stability Experiment

Purpose
The purpose of the *ex-vivo* stress testing was to evaluate the stability of the LP-11 liposomes in a simulated bladder environment since the product is intended for direct intravesical administration and would thus be subject to the chemical environment of the urinary bladder.

Procedure
Two critical product characteristics of the LP-11 liposomes were evaluated following exposure to the simulated bladder environment: particle size and DOTAP concentration. The testing procedure for each of these characteristics was the same as the methods listed above for the long-term stability evaluations. Vials were manufactured to contain 10mg DOTAP liposomes. Each vial was first reconstituted with 1.428mL USP Sterile water. The bladder-like environment was then modeled by mixing the reconstituted liposomes (7mg/mL) in a 2:1 (v/v) with synthetic urine (2.856mL) for a concentration of 2.33mg/mL. Vials were then incubated at 37C for 1 hour to mimic physiologic conditions (clinical protocol will include an instillation time of 1hour).

Results
The DOTAP component of LP-11 was determined to be consistent under "stress" stability conditions of dilution with synthetic urine and incubation at 37C for 1 hour. Quantitative results are listed in **Table 2** below. The study is ongoing full study results will be reported in the final report.

Table 2: Stress Stability Results—Concentration of DOTAP in LP-11 Liposomes in mg/mL by ^{1}H NMR

Timepoint	Concentration (mg/mL)
1month	2.28
2 months	
4 months	

III. Research accomplishments
- Animal study data analysis/reporting
- Interim long-term stability testing data
- Completed stress testing in a bladder environment

V. Conclusions
During the reporting period we analyzed nonclinical animal data and conducted stability and stress testing of LP-11. Analysis of the animal data continues to demonstrate potential of LP-11 on IC/BPS symptoms in rat models. Stability and stress testing studies are ongoing and full results are anticipated during the final period (extension).

In the final period, Lipella Pharmaceuticals and the University of Pittsburgh will continue to collaborate, review LP-11 formulation and manufacturing data, and take appropriate steps to wind down the project. Additionally we will be preparing to continue LP-11's experimental and regulatory plan following the conclusion of the project.

VI. Publications and Reportable Outcomes (Year 3)

Refereed
1. Yoshikawa, S., Kawamorita, N., Oguchi, T., Funahashi, Y., Tyagi, P., Chancellor, M.B., Yoshimura, N.: Pelvic organ cross-sensitization to enhance bladder and urethral pain behaviors in rats with experimental colitis. Neuroscience, 284: 422–429, 2015.
2. Ogawa, T., Ishizuka, O., Ueda, T., Tyagi, P., Chancellor, M.B., Yoshimura, N.: Current and emerging drugs for interstitial cystitis/bladder pain syndrome (IC/BPS). Expert Opinion on Emerging Drugs, in press, 2015.
3. Kawamorita, N., Yoshikawa, S., Kashyap, M., Tyagi, P., Arai, Y., Chancellor, M.B., Yoshimura, N.: Liposome-based intravesical therapy 1 targeting nerve growth factor (ngf) ameliorates bladder hypersensitivity in rats with experimental colitis. Submitted to Journal of Urology (under revision).

Abstracts

1. Chancellor, M.B., Kaufman, J., Yoshimura, N., Tyagi, P., Kawamorita, N.: Liposome-based Intravesical Therapy targeting Nerve Growth Factor (NGF) ameliorates Bladder Hypersensitivity in Rats with Experimental Colitis. 2015 Military Health System Research Symposium. Poster session 3, MHSRS-15-0089. Ft. Lauderdale, FL. 8/17-20, 2015.
2. Majima, T., Tyagi, P., Dogishi, K., Kadekawa, K., Kashyap, M., Wada, N., Takai, S., Shimizu, T, Gotoh, M., Chancellor, M, Yoshimura, N.: The effect of intravesical liposome-based NGF antisense therapy on bladder overactivity and nociception in a rat model of cystitis induced by hydrogen peroxide. 45th Annual meeting of the International Continence Society, Abstract No. 234, Montreal, Canada, October 6-9, 2015.

Neuroscience 284 (2015) 422–429

PELVIC ORGAN CROSS-SENSITIZATION TO ENHANCE BLADDER AND URETHRAL PAIN BEHAVIORS IN RATS WITH EXPERIMENTAL COLITIS

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Abstract—Neural cross-sensitization has been postulated as a mechanism underlying overlaps of chronic pelvic pain disorders such as bladder pain syndrome/interstitial cystitis (BPS/IC) and irritable bowel syndrome (IBS). Animals with experimental colitis have been used to study the underlying mechanisms for overlapped pelvic pain symptoms, and shown to exhibit bladder overactivity evidenced by frequent voiding; however, it has not directly been evaluated whether pain sensation derived from the lower urinary tract is enhanced in colitis models. Also, the cross-sensitization between the colon and urethra has not been studied previously. In the present study, we therefore investigated pain behaviors induced by nociceptive stimuli in the lower urinary tract and the involvement of C-fiber afferent pathways using rats with colitis induced by intracolonic application of 2,4,6-trinitrobenzenesulfonic acid (TNBS). In TNBS-induced colitis rats at 10 days, intravesical application of resiniferatoxin (RTx) induced a significantly greater number of episodes of both licking and freezing behaviors, which were reduced by capsaicin-sensitive C-fiber afferent desensitization. Histochemical studies using fluorescent dye tracers injected into the colon, bladder or urethra showed that dichotomized afferent neurons comprised 6.9–14.5% of L1, L6 and S1 dorsal root ganglion (DRG) neurons innervating the colon or the lower urinary tract. Transient receptor potential vanilloid 1 (TRPV1) mRNA expression was significantly increased in, the bladder, urethra and S1 DRG in colitis rats. An increase in myeloperoxidase (MPO) activity was found in the colon, but not in the bladder or urethra after intracolonic TNBS treatment. These results indicate that TNBS-induced colitis increased pain sensitivity in the bladder and urethra via activation of C-fiber afferent pathways due to colon-to-bladder and colon-to-urethral cross-sensitization, suggesting the contribution of pelvic organ cross-sensitization mechanisms to overlapped pain symptoms in BPS/IC and IBS.

Key words: pain behavior, cross-sensitization, lower urinary tract, bladder, urethra, dorsal root ganglion (DRG).

INTRODUCTION

Bladder pain syndrome/interstitial cystitis (BPS/IC) is a chronic urological disorder characterized as pelvic pain related to bladder filling, coupled with additional symptoms, such as increased urinary frequency and urgency, without proven urinary infection or other obvious pathology (Hanno et al., 2011). It is currently estimated that 3.3–7.9 million United States women 18 years old or older are suffering from BPS/IC (Berry et al., 2011). It has also been reported that over one-third of patients diagnosed with BPS/IC exhibit symptoms consistent with irritable bowel syndrome (IBS) (Alagiri et al., 1997; Novi et al., 2005), while 26–56% of patients diagnosed with IBS also have symptoms of BPS/IC (Maxton et al., 1989; Blanchard et al., 2004). In addition, BPS/IC patients often report pain in different and/or additional sites such as the urethra (Warren et al., 2008). However, the mechanisms underlying the pelvic organ cross-talk that contributes to overlapped symptoms in chronic pelvic pain syndromes such as BPS/IC, urethral pain and IBS have not been well clarified.

Previous animal studies demonstrated that 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis produced lower urinary tract dysfunction such as increased voiding frequency (Liang et al., 2007; Ustinova et al., 2007) and an increase in the firing rate of bladder afferent nerves in response to urinary bladder distension in rats (Ustinova et al., 2007). These animals showed increased expression of neuropeptides such as substance P and calcitonin gene-related peptide (Pan et al., 2010), growth factors and mast cells (Liang et al., 2007) in the bladder. Moreover, a recent report demonstrated that desensitization of the transient receptor potential vanilloid 1 (TRPV1) by intravesical application of resiniferatoxin (RTx) suppressed the

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Abbreviations: BPS/IC, bladder pain syndrome/interstitial cystitis; DRG, dorsal root ganglion; IBS, irritable bowel syndrome; MPO, myeloperoxidase; RTx, resiniferatoxin; RT-PCR, real-time polymerase chain reaction; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TRPV1, transient receptor potential vanilloid 1.

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increased excitability of bladder spinal neurons in rats with acute colitis induced by intracolonic instillation with TNBS (Malykhina et al., 2013). These results suggest that intracolonic irritation can sensitize bladder afferent pathways, resulting in bladder overactivity. Also, in previous studies, pelvic pain conditions in the colitis model were assessed by the visceromotor response elicited by colorectal distension (Greenwood-Van Meerveld et al., 2005) and referred somatic hyperalgesia in the paw and/or abdominal skin regions (Cameron et al., 2008; Claudino et al., 2010). Thus, although the rat with experimental colitis has been used as an animal model of chronic pelvic pain, pain sensation derived from the bladder has not directly been evaluated in experimental colitis. In addition, the cross-talk between the colon and urethra to induce urethral pain has not been studied previously in the colitis model. Since our laboratory developed a rat model that can be used to investigate pain sensation from the bladder and urethra under the freely moving condition by monitoring pain behaviors such as freezing and licking (Saitoh et al., 2008), we investigated whether nociceptive behaviors induced by chemical stimuli in the lower urinary tract are enhanced in colitis rats. We also investigated the number of dichotomized afferent neurons that innervate both colon and bladder or both colon and urethra; as well as changes in gene expression of TRPV1 channels in dorsal root ganglia (DRG), bladder and urethral tissues.

EXPERIMENTAL PROCEDURES

Animals

Sixty-four female Sprague-Dawley rats (206–268 g) were used in this study. Rats were divided into the following groups: (1) 20 rats for behavioral testing, (2) 24 rats for mRNA measurement with real-time polymerase chain reaction (RT-PCR), (3) eight rats for a histochemical study with fluorescent dye tracers, and (4) 12 rats for MPO activity assay. We used female rats because of the higher prevalence of BPS/IC and IBS in women than in men (Clemens et al., 2007; Ito et al., 2007; Hall et al., 2008; Lovell and Ford, 2012) and the technical easiness in urethral catheterization during behavioral studies in female rats compared to male rats. All experiments were conducted in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Induction of experimental colitis

Experimental colitis was induced as described in a previous report (Liang et al., 2007) with slight modification in the injection volume. Briefly, rats fasted for 24 h were anesthetized with isoflurane. A polyethylene catheter (PE90) was inserted from the anus and placed approximately 6 cm proximal to the anal verge, and then TNBS (50 mg/mL in 50% ethanol, 0.4 mL) was administered through the catheter and retained in the distal colon with the rats in a vertical position for several minutes. Thereafter, Surgilube® (E. Fougera & Co., Melville, NY, USA) was inserted into the anal canal to prevent the leakage of TNBS and then rats were returned to the housing facility after the recovery from anesthesia until each assay. Control animals received the vehicle treatment with 0.4 mL of 50% ethanol. In some rats used for behavioral testing, capsaicin (total 125 mg/kg) was given subcutaneously in divided doses on two consecutive days: 25 and 50 mg/kg at a 12-h interval on the first day and 50 mg/kg on the second day, to induce desensitization of capsaicin-sensitive C-fiber afferent pathways as described in previous reports (Cheng and de Groat, 2004; Kullmann et al., 2008).

Nociceptive behavior study

The measurement of nociceptive behaviors was conducted according to the previously reported method (Saitoh et al., 2008). In brief, rats were acclimated in metabolic cages (Nalgene Co., Rochester, New York) for 3 h, and then placed in a Bollman-type restraining device (KN-326; Natsume Seisakusho, Tokyo, Japan). A polyethylene tube (PE-50; Clay Adams Division of Becton Dickinson, Parsippany, NJ, USA) was inserted into the bladder through the urethra, and residual urine was withdrawn. Thereafter, RTx (0.3 μM), or the corresponding vehicle alone (10% ethanol, 10% Tween 80, and 80% physiological saline), was instilled into the bladder via the catheter at a volume of 0.3 mL and kept for 1 min. The transurethral catheter was then removed and rats were placed back into metabolic cages. Two types of behaviors, licking (lower abdominal licking) and freezing (motionless head-turning toward the lower abdomen), were scored for a 15-min interval that was divided into 5-s intervals. When licking or freezing occurred during each 5-s interval, it was scored as one positive event. The number of licking or freezing behavior events was summed for each of 5-min periods (0–5, 5–10 and 10–15 min) following the RTx treatment. The intravesical application of the solution (RTx or vehicle) was conducted in a blinded manner for assessors of animal behaviors.

Retrograde labeling of colon, bladder and urethral afferent neurons

Under isoflurane anesthesia, rats underwent a midline laparotomy to gain access to the pelvic organs. The distal colon (2.5–3.5 cm from the rectum) and the bladder or urethra were exposed, and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen Inc., Paisley, UK; 1% w/v in methanol) was injected to 6–8 different sites of the colonic wall using a 30-G needle syringe. Additionally, Fast Blue (Polysciences Inc., Warrington, PA, USA; 1% w/v in water) was injected into 4–5 different sites of the bladder or urethral wall in order to examine the presence of DRG neurons innervating the colon and bladder or the colon and urethra, respectively. The total volume of dye injected into each organ was 25 μL. To prevent leakage and labeling of adjacent tissues, the needle was left in place for 30 s after injection, and then a cotton swab was applied to prevent leaking. Abdominal incisions were closed with sutures and rats were returned to housing facility until each assay. The rats were post-operatively treated with ampicillin

(100 mg/kg, subcutaneously; Fort Dodge Animal Health, Fort Dodge, IA, USA) and buprenorphine (0.05 mg/kg subcutaneously; Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) twice a day for 3 days.

Histological assessment of labeled DRG cells

Ten days after the injection of dyes, rats were euthanized with pentobarbital, and DRG at Th13 to S2 levels were removed bilaterally. The specimens were embedded into OTC compound, frozen with dry ice/isopentane (2-methylbutane) and stored at −80 °C until used. Serial transverse sections were cut at 10-μm thickness with a cryostat, and every fourth sections were mounted on slides to avoid duplicate counting of the cells. Positively labeled cells were evaluated with an Olympus fluorescence microscope with a multiband filter set for DiI and FB. Three sections each from the right and left DRG at each level were randomly selected in a rat, and the number of dye-labeled cells per section was counted. Then, the number of dye-labeled cells in six sections per one DRG level was averaged in each rat, and the data were presented as mean ± SEM of four rats. The percentage of dual-labeled neurons was determined as a ratio against the sum of DiI- or FB-labeled neurons (Malykhina et al., 2006; Christianson et al., 2007).

Myeloperoxidase (MPO) activity assay

MPO is an enzyme found primarily contained in neutrophils; and the measurement has been used as a quantitative index of inflammation in tissues including the colon from rats with colitis (Smith and Castro, 1978; Morris et al., 1989; Yang et al., 2008). Colon, bladder and urethral tissues were rinsed with cold phosphate-buffered saline (PBS) (pH 7.4), weighed, and were homogenized for 30 s at 4 °C after addition of 200 μL radioimmunoprecipitation assay buffer (RIPA) lysis buffer (sc-24948; Santa Cruz biotechnology, CA, USA) per 10 mg of tissue and were homogenized for 30 s at 4 °C. The homogenates were incubated for 30 min on ice, and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The levels of MPO in the supernatants were measured by using a MPO assay ELISA Kit (HK105; Hycult biotechnology, Uden, Netherlands) according to the manufacturer's instruction. Quantification was performed by measuring absorbance at 450 nm using a microplate reader. The protein concentrations of supernatants were also measured by using BCA Protein Assay Kit (#23225; Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction. The MPO concentration was standardized relative to protein levels and expressed in ng/mg total protein.

RNA isolation and RT-PCR analysis

One microgram of total RNA extracted from L6-S1 DRG, bladder or urethra tissues was reverse-transcribed into cDNA using ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Quantitative PCR was performed with an MX3000P real-time PCR system (Stratagene, La Jolla, CA, USA) in a 25 μL volume using SYB Green PCR Master Mix (QIAGEN, Valencia, CA, USA). cDNA product was amplified by 40 cycles (denaturation at 95 °C for 15 s; primer annealing for 55 °C for 60 s; and elongation for 72 °C for 30 s). Primers sequences used for real-time PCR were the following: 5′-AGT AAC TGC CAG GAG CTG GA-3′ (forward) and 5′-GTG TCA TTC TGC CCA TTG TG-3′ (reverse) for rat TRPV1 and 5′-GGC CAA AAG GGT CAT CAT CT-3′ (forward) and 5′-GTG ATG GCA TGG ACT GTG GT-3′ (reverse) for rat GAPDH, which is a house keeping gene used as the internal control. All primers for PCR reaction were designed based on the NCBI database sequence of rat reference mRNA and checked for specificity with BLAST software from the NCBI website; and PCR products were validated by size determination after separation on 2% agarose gel.

Drugs

TNBS, capsaicin and RTx were purchased from Sigma–Aldrich (St. Louis, MO, USA). Capsaicin and RTx were dissolved in a vehicle consisting of 10% ethanol, 10% Tween 80, and 80% physiological saline.

Statistical analysis

All data were represented as the mean ± SEM. Statistical significance was determined by unpaired *t*-test (two-tailed) or a one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test. The P values < 0.05 were considered significant.

RESULTS

Nociceptive behaviors in conscious rats treated with TNBS

At day 10 after intracolonic administration of TNBS, rats did not show apparent pain behaviors such as freezing or licking compared with vehicle (for TNBS)-treated control rats when noxious stimulation was not applied (Fig. 1A, B; intravesical application of vehicle for RTx). When RTx (0.3 μM) was intravesically applied, TNBS-untreated control rats exhibited no apparent freezing behavior with slightly enhanced licking behavior only for the initial 5-min observation period. On the other hand, a significantly greater number of episodes of both licking and freezing behaviors were shown after intravesical application of RTx (0.3 μM) in rats with TNBS-induced colitis compared with the vehicle-treated group (Fig. 1C–F; $P < 0.001$, respectively), indicating increased pain sensitivity to noxious stimuli in the lower urinary tract following TNBS-induced colitis. In addition, these enhanced pain behaviors were significantly reduced with pre-treatment with capsaicin that induced desensitization of capsaicin-sensitive C-fiber afferent pathways (Fig. 1C–F; $P < 0.01$, respectively).

MPO activity

At day 10 after intracolonic application of TNBS, MPO activity in the colon was significantly ($P < 0.01$)

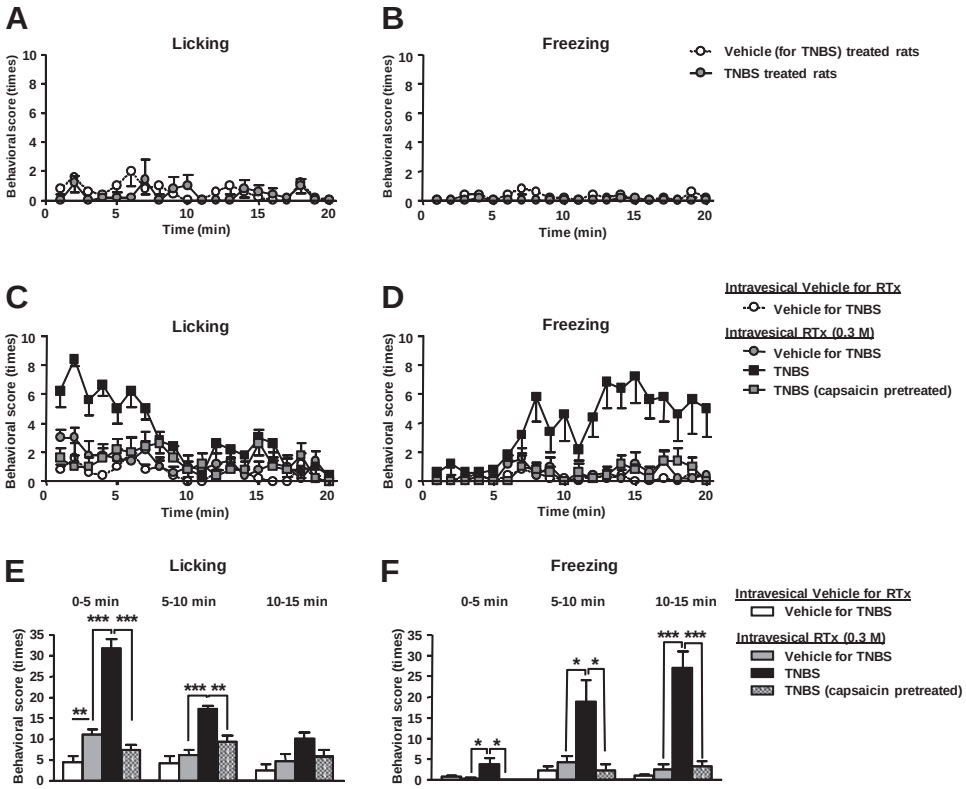

Fig. 1. Nociceptive behaviors, licking and freezing in rats with intracolonic administration of TNBS. (A and B) Effects of intracolonic administration of TNBS on licking (A) and freezing (B) behavior events in the absence of intravesical noxious stimulation in rats with vehicle (0.4 mL of 50% ethanol) or TNBS injection into the colon. Open and closed circles indicate behavioral events after intravesical application of vehicle (10% ethanol, 10% tween 80 and 80% saline used for dissolving RTx) in rats with intracolonic vehicle and TNBS injection, respectively. (C, D, E and F) Nociceptive behaviors induced by intravesical application of RTx in rats with intracolonic administration of TNBS. TNBS-untreated rats (vehicle for TNBS) with intravesical treatment of vehicle (intravesical vehicle for RTx) or RTx were also included as controls. After RTx (0.3 μM) was applied intravesically for 1 min, the number of licking and freezing behavior events was counted for 15 min, and summed for every 1 min and plotted in C and D, respectively. The total number of licking and freezing behavior events for each of 5-min periods after the instillation by RTx is shown in E and F, respectively. Some TNBS-treated rats were treated with capsaicin 4 days before the observation (capsaicin-pretreated) to investigate the effect of desensitization of capsaicin-sensitive afferent pathways. Each point or bar represents the mean ± SEM from five or six rats. Vehicle for RTx was 10% ethanol, 10% tween 80 and 80% saline. Vehicle for TNBS was 50% ethanol. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{**}P < 0.001$ (two-tailed, unpaired *t*-test or one-way ANOVA followed by Bonferroni's Multiple Comparison test).

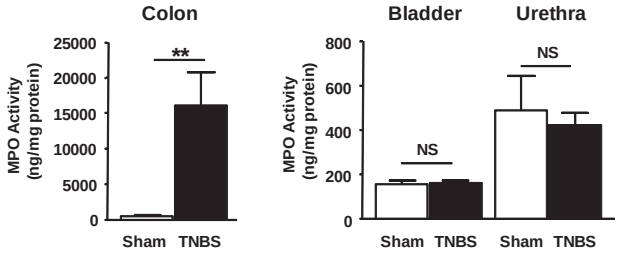

Fig. 2. Effects of intracolonic administration of TNBS on myeloperoxidase (MPO) activities in the colon, bladder and urethra in rats. MPO activities were quantified 10 days after the induction of colitis in rats. Each bar represents the mean ± SEM from six rats. Sham was treated with the vehicle for TNBS (50% ethanol). $^{**}P < 0.01$ vs. the sham group (two-tailed, unpaired *t*-test).

increased compared with the vehicle-treated group (Fig. 2). On the other hand, there was no change in MPO activities in either the bladder or urethra in rats with TNBS-induced colitis (Fig. 2).

Distribution of afferent neurons innervating colon, bladder and urethra

DRG neurons retrogradely labeled by fluorescent dyes injected into the bladder, urethra or colon are counted in DRG sections at T13 to S2 levels. Colonic afferent neurons labeled with DiI were distributed in L1, L2, L6 and S1 DRGs with abundant labeling in L1 and S1 DRG neurons whereas bladder or urethra afferent neurons labeled by FB injected into the bladder or urethra, respectively, were distributed in L1, L6 and S1 DRGs with abundant labeling in L6 DRG neurons (Fig. 4). In T13, L3–L5 and S2 DRGs, few or no cells were labeled with either FB or DiI. Dichotomized afferent neurons innervating both colon and bladder or both colon and urethra were observed in L1, L6 and S1 DRGs (Figs. 3 and 4). When DiI and FB were injected into the colon and bladder, respectively, the ratio of afferent neurons with dichotomizing projection to both colon and bladder was 6.48–14.3% among colon or bladder-innervating afferent neurons; specifically 6.48 ± 2.26% in L1 DRG, 14.0 ± 2.50% in L6 DRG and 14.3 ± 1.99% in S1 DRG

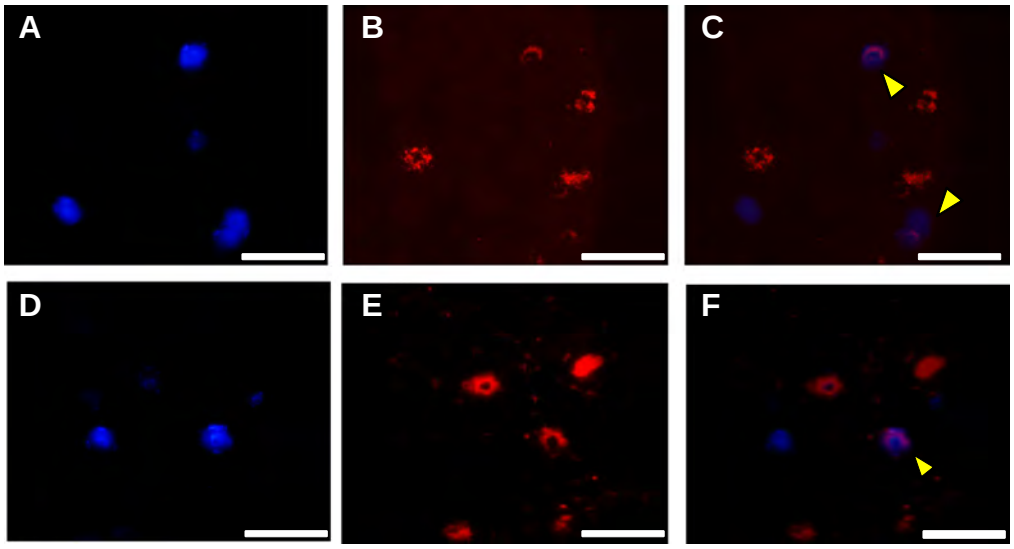

Fig. 3. Representative photomicrographs of DRG sections showing retrogradely labeled DRG neurons 10 days after the injection of DiI into the colon and FB into the bladder or urethra. Upper panels (A–C) display the fluorescent image of the same L6 DRG section showing FB-labeled bladder afferent neurons (A), DiI-labeled colon afferent neurons (B) and dichotomized afferent neurons innervating both colon and bladder indicated by yellow arrows (C). Lower panels (D–F) display the fluorescent image of the same S1 DRG section showing FB-labeled urethral afferent neurons (D), DiI-labeled colon afferent neurons (E) and dichotomized afferent neurons innervating both colon and urethra indicated by yellow arrows (F). Calibration bars = 50 μm.

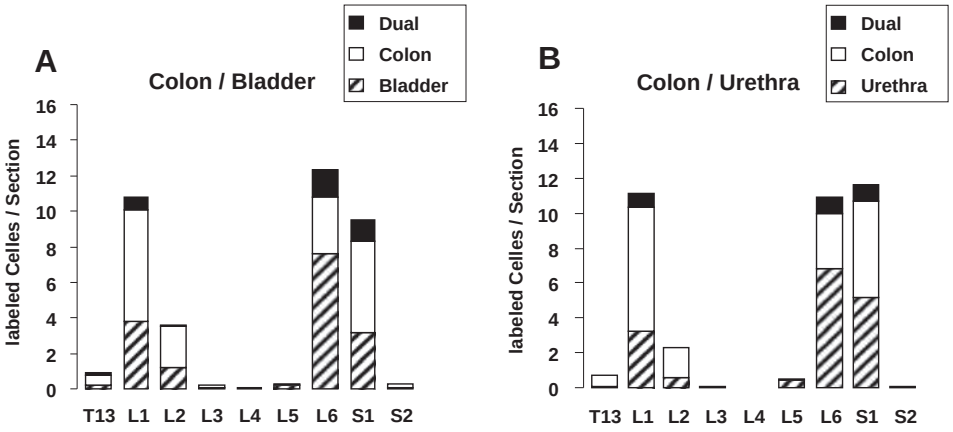

Fig. 4. Distribution of afferent neurons projected to the colon, bladder and urethra in dorsal root ganglia at the level of T3 to S2 in rats. (A) The distribution of labeled neurons after DiI and FB injection into the distal colon and bladder wall, respectively. In each bar, colonic, bladder and dually labeled afferent neurons are expressed as shown in panel A. (B) Distribution of labeled neurons after DiI and FB injection into the distal colon and urethral wall, respectively. In each bar, colonic, urethral and dually labeled afferent neurons are expressed as in panel B. At each DRG level, the number of labeled neurons was averaged in six sections (three each from light or left side) in one rat; then the data was expressed as mean ± SEM of four rats.

expressed as percentage of DiI and FB dually labeled neurons among the sum of DiI- or FB-labeled neurons in the colon/bladder dye-injected group (n = 4 rats). In another series of experiments, when DiI and FB were injected into the colon and urethra, respectively, the ratio of afferent neurons with dichotomizing projection to the colon and urethra was 7.41–9.30% among colon or urethra-innervating afferent neurons; specifically 7.41 ± 1.78% in L1 DRG, 9.30 ± 0.53% in L6 DRG and 8.43 ± 1.85% in S1 DRG, expressed as percentage of DiI and FB dually labeled neurons among the sum of DiI- or FB-labeled neurons in the colon/urethra dye-injected group (n = 4 rats).

Changes in TRPV1 mRNA in the DRG, bladder and urethra after TNBS treatment in rats

In TNBS-treated colitis rats, the mRNA levels of TRPV1 in the bladder, urethra and S1 DRG were significantly increased compared to sham rats (Fig. 5; $P < 0.05$, respectively).

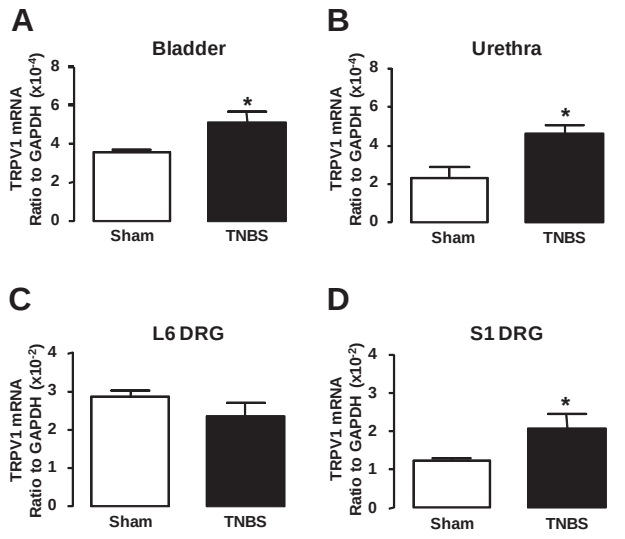

Fig. 5. Expression levels of TRPV1 mRNA against a house keeping gene (GAPDH) in the bladder (A), urethra (B), L6 DRG (C) and S1 DRG (D) in vehicle (Sham) and TNBS-treated rats. $^{*}P < 0.05$ vs. the sham group (two-tailed, unpaired *t*-test).

DISCUSSION

The results in the present study demonstrated that experimental colitis dramatically enhanced the freezing behavior induced by intravesically applied RTx (TRPV1 receptor agonist) at a low concentration (0.3 μM), which induced only a few events of nociceptive behaviors in TNBS-untreated control rats. Freezing behavior induced by intravesical application of RTx in rats was characterized as a typical nociceptive response to activation of pelvic nerve afferents innervating the bladder (Saitoh et al., 2008). Also, our previous studies showed that the delivery of therapeutic genes encoding enkephalins or anti-inflammatory cytokines to the bladder and bladder afferent pathways using non-replicating herpes simplex virus vectors is effective to reduce freezing behavior induced by intravesical RTx administration without affecting licking behavior in rats (Funahashi et al., 2013; Oguchi et al., 2013; Yokoyama et al., 2013), indicating that RTx-induced freezing behavior represents the pain behavior derived from nociceptive stimulation of the bladder. Although previous studies demonstrated that rats with colitis induced by TNBS have frequent micturition and increased afferent excitability in response to urinary bladder distention in anesthetized rats (Ustinova et al., 2007), increased bladder pain sensitivity in this colitis model had not yet been shown. In addition, von Frey testing in the paw and/or abdominal skin regions has been used to evaluate the referred pelvic pain in rodent colitis models (Cameron et al., 2008; Claudino et al., 2010); however, it is not known how the referred somatic hyperalgesia in these regions correlates to pain sensation derived from the bladder. In this regard, our current results directly demonstrated that TNBS-induced colitis at 10 days enhances bladder pain sensation elicited by nociceptive stimuli in the bladder (i.e., freezing behavior) in rats. Furthermore, we confirmed that dichotomized afferent neurons projecting to both the distal colon and bladder are identified in DRGs at the L1, L6 and S1 levels, the percentage of which (6.5–14.3%) is in accordance with the results in the previous reports (Malykhina et al., 2006; Christianson et al., 2007), supporting the previous notion that the existence of dichotomizing pelvic afferents could provide a pre-existing neuronal network for potential pelvic organ cross-sensitization (Ustinova et al., 2010).

Another new finding in this study is an existence of an organ cross-talk between the colon and urethra following TNBS-induced colitis because the cross-talk between these two organs has not been evaluated previously. The present study showed that dichotomization of sensory neurons projecting to the distal colon and urethra were shown at L1, L6 and S1 levels (7.4–9.3% of the sum of colon or urethral afferent neurons) in rats and that colitis rats showed significantly enhanced licking behavior after the intravesical application of RTx. Because the licking behavior induced after intravesical application of RTx was prevented by transection of the pudendal nerves that innervate the urethra, but not the bladder (Saitoh et al., 2008), it is assumed that RTx-induced licking behavior represents urethral pain due to afferent nerve activation in the pudendal nerve by RTx expelled from the bladder to the urethra during voiding. Therefore, these data suggest that the existence of dichotomizing pelvic afferents between the colon and urethra could contribute to pelvic organ cross-sensitization between these two organs to increase pain sensitivity in the urethra after colonic inflammation. Increased bladder and urethral pain sensitivity after colonic inflammation in this study may explain at least in part the frequent association of urethral pain in BPS/IC patients (Warren et al., 2008).

In this study, increased MPO activity, which correlates with the tissue neutrophil infiltration, was found in the colon, but not in the bladder or urethra, following TNBS-induced colitis. These results indicate that pain hypersensitivity of the bladder and urethra in rats with TNBS-induced colon inflammation is produced by the indirect mechanisms in the lower urinary tract other than inflammatory changes directly elicited by TNBS administered into the colon. Previous studies have demonstrated that pelvic organ cross sensitization is induced by activation of dichotomized afferents innervating different pelvic organs and that activation of nociceptive C-fiber afferents in one organ could sensitize afferent pathways in another organ to release neuropeptides such as substance P that trigger neurogenic inflammation and mast cell activation (Malykhina et al., 2012; Fitzgerald et al., 2013). Also, Pan et al. demonstrated that desensitization of TRPV1-expressing afferent pathways by intracolonic application of RTx, a TRPV1 receptor agonist, prior to the induction of colonic inflammation prevented the release of these peptides from the peripheral nerve terminals and reduced the development of neurogenic cross-talk between the colon and bladder (Pan et al., 2010). These results suggest that TRPV1-expressing C-fiber afferent pathways significantly contribute to the colon-to-bladder cross sensitization following colitis. Clinically, an increase in TRPV1

expression in the bladder has also been reported in patients with chronic pelvic pain syndromes including BPS/IC (Lowe et al., 1997; Liu et al., 2007; Akbar et al., 2008; Poli-Neto et al., 2009). In addition, previous preclinical studies demonstrated that immunohistochemical upregulation of TRPV1 in L6-S1 DRG innervating the colon was observed at 72 h after the induction of colitis with TNBS in rats (De Schepper et al., 2008). Our results also showed upregulation of TRPV1 mRNA expression in the bladder, urethra and S1 DRG as well as enhanced pain behaviors, which was reduced after desensitization of capsaicin-sensitive afferent pathways in rats with TNBS-induced colitis rats. These results further support the contribution of TRPV1-expressing C-fiber afferent pathways to both colon-to-bladder and colon-to-urethra cross sensitizations induced by colitis, which induce increased pain sensation in the bladder and urethra, respectively.

Previous studies reported that the combination rat model of endometriosis and ureteral calculosis shows enhanced pain behavior from both the urinary tract and female reproductive area, indicating that these two comorbidities strengthen the viscero-visceral cross-talk (Giamberardino et al., 2002; Lopopolo et al., 2014). Therefore, future studies using animal models including those of combination visceral pain could further clarify the underlying mechanisms and help explore new therapeutic targets for clinical symptoms in chronic pain syndromes.

CONCLUSION

We demonstrated that TNBS-induced colitis at 10 days enhances pain sensation derived from the bladder and urethra as evidenced by increased freezing and licking behaviors elicited by nociceptive bladder and urethral stimuli, respectively. These enhanced pain behaviors are considered to be due to activation of TRPV1-expressing C-fiber afferent pathways, possibly through dichotomized afferents innervating both colon and lower urinary tract. These pelvic organ cross-sensitization mechanisms could be involved in overlapped pain symptoms in BPS/IC and IBS.

CONFLICT OF INTEREST

None of the authors has any conflict of interest with any of the data presented in this manuscript.

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Review

EXPERT OPINION

Current and emerging drugs for interstitial cystitis/bladder pain syndrome (IC/BPS)

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Introduction: Interstitial cystitis or bladder pain syndrome (IC/BPS) is a debilitating chronic disease characterized by suprapubic pain and lower urinary tract symptoms: however, the etiology is still unknown. Therefore, the long-lasting, effective treatments of IC/BPS are still not established, and the treatment is sometimes empirically selected depending on practitioners' experience and preference.

Area covered: In this review we focus on the current treatments, ongoing clinical trials, and several potential new drugs based on the results of basic and clinical research studies. First, we discuss the potential etiologies of IC/BPS that include altered barrier lining, afferent and/or central nervous system abnormalities, possible contribution of inflammation or infection and abnormal urothelial signaling. Then, the current therapies of IC/BPS, either systemic or local, are reviewed by critical evaluation of the efficacy and shortcomings of each treatment. Finally, based on proposed etiologies of the disease, potential emerging drugs and treatments are discussed.

Expert opinion: Current therapies often fail to control the symptoms of IC/BPS. Several interventions including sustained drug release and retaining techniques, and drugs that act on afferent neural pathways are emerging and may be promising. In addition, phenotyping of IC/BPS patients based on cystoscopic findings (e.g., Hunner's vs. non-Hunner's lesion) or patients' symptoms would be important for further investigation of IC/BPS etiology and the evaluation of efficacy of new treatments.

Keywords: bladder pain syndrome, clinical trials, current research, interstitial cystitis

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1. Background

1.1. Clinical findings

Hunner initially reported [1] in 1915 that there is a rare type of bladder ulcer in women, which later became known as the well-recognized disease, interstitial cystitis (IC). IC presents clinically with the characteristic changes in bladder ulcerative lesions, also known as Hunner's lesion,[2] and discriminative symptoms such as bladder pain and urinary frequency.[1,3–5] In 1988, the National Institute of Diabetes and Digestive Kidney Diseases (NIDDK) developed the criteria for IC; however, these criteria were too strict for widespread clinical use.[6] More recently, a symptom-based definition of IC/bladder pain syndrome (IC/BPS) has been developed, and the guideline was published by the American Urological Association (AUA) in 2011.[4] In the guideline, they indiscriminate subtypes based on the existence of Hunner's lesion. IC/BPS is defined in the AUA guideline as: "An unpleasant sensation (pain, pressure and/or discomfort) associated with lower urinary tract symptoms of more than six weeks duration, in the absence of

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infection or other identifiable causes".[4] IC/BPS is a common disease in middle-aged and elderly women. The number of adult women patients with IC/BPS is estimated to be between 422,803 and 21,454,813 in the US.[7] It is also estimated that the medical care for IC/BPS costs more than $11,000 per year per patient.[8]

At present, there are two major subtypes of IC/BPS: those with or without Hunner's lesion, which are also known as ulcerative or non-ulcerative IC/BPS, respectively. Although Hunner's lesion, which is a typical finding in IC/BPS during cystoscopic examination, has been considered to be a rare occasion in IC/BPS patients, it seems that the prevalence is actually higher than expected as up to 50% of IC/BPS patients reportedly have Hunner's lesion.[2,9,10] In general, symptoms between the two subtypes are quite similar, although the patients with IC/BPS with Hunner's lesion have smaller bladder capacity.[11,12] In addition, glomerulations, which are defined as multiple petechia-like hemorrhages on bladder distension, have been used as a diagnostic criterion for IC/BPS; however, the specificity of glomerulations is in question as they are seen not only in IC/BPS but also in other conditions.[11,13] The therapies for IC/BPS are often difficult and frustrating, and the severe symptoms impair the quality of life and put restrictions on social and daily life.[14] In the guideline of European Association of Urology (EAU), IC/BPS is defined as a part of chronic pelvic pain syndrome (CPPS).[9] In CPPS, modification of peripheral organs alters both the peripheral and central nervous systems (CNS), which can lower the threshold to stimuli. There are other presumed mechanisms of the CPPS, including altered pain perception of the supraspinal sites and psychological modulation.[9] Thus, the pathophysiology of CPPS, including IC/BPS, is multifactorial and a multidisciplinary approach to the management of symptoms is essential.[9]

1.2. Pathophysiology of IC/BPS

The definitive etiology of IC/BPS is not yet clear; however, the pathogenesis of IC/BPS is thought to include various factors.[15] First, a leaky urothelium with increased permeability is present in IC/BPS patients. Lilly et al. confirmed enhanced urea absorption in IC/BPS patients after intravesical administration of a urea solution.[16] Buffington et al. showed that blood fluorescein levels were higher in IC/BPS patients than controls after fluorescein was administered orally, suggesting increased absorption of the agent from the bladder surface upon excretion.[17] Previous studies have investigated the mechanisms of increased permeability. For instance, antiproliferative factor (APF), which was later characterized as a frizzled-8 related sialoglycopeptide, inhibits urothelial proliferation, thereby adversely affecting barrier function.[18,19] Also, heparin-binding epidermal growth factor, like growth factor which is known to be decreased in the IC/BPS bladder, functionally antagonizes APF activity through mitogen-activated protein kinase signaling pathways in bladder urothelial cells.[20] Impaired tight junction proteins including ZO-1,[21] connexin43 and connexin45 [22] are also postulated as the factors inducing increased permeability. In cats with feline IC, urothelial release of nitric oxide (NO) is also enhanced.[23,24] In addition, altered expression of uroplakin, which covers the apical surface of urothelium, is found in IC/BPS without Hunner's lesion, suggesting that the change leads the functional property of the bladder.[25] The impaired barrier function induced by unknown pathogenesis, which may include bacterial infection, allows the toxic substances in urine to infiltrate into the suburothelium. Then, the toxic substances are likely to stimulate bladder afferent pathways and inflammatory responses such as cytokine production to induce bladder pain and/or urinary urgency (Figure 1).[3] In addition, there are other potential mechanisms by which chronic pelvic pain is induced in IC/BPS. Sensitization of peripheral afferent pathways due to stimulating substances and/or alterations in urothelial-afferent function lowers the threshold for sensory nerve activation in response to peripheral stimuli, resulting in pain sensation. Finally, sensitization of the CNS may be involved in the pathogenesis of IC/BPS. In the spinal cord, synaptic plasticity and repetitive activation of nociceptors induce central sensitization, leading to the altered gene expression in nociceptors to induce the persistent pain status.[26] In a functional MRI study of IC/BPS patients, changes in brain activity as evidenced by an increment of gray matter volume have been detected in several brain regions.[27] Taken together, these findings suggest that the alterations in the peripheral and central nervous systems are involved in the pathophysiological basis of IC/BPS symptoms although further studies need to identify the true contribution of each of the proposed etiologies to IC/BPS pathogenesis. In addition, because some previous clinical studies examining the pathogenesis of the disease or the efficacy of treatments, which are discussed in the following sections, utilized different patient selection criteria including the old definition of IC developed by NIDDK,[6] it is important to know which patient population was included in each study to understand the implications of trial results for each therapy, and the applicability of the previous results to newly defined IC/BPS [4] needs to be confirmed in future studies.

2. Medical need

In 2011, the AUA guideline reported the algorithm of diagnosis and management for IC/BPS.[4] Recommended treatments for IC/BPS are still limited to conservative therapies, including behavioral therapy, oral or intravesical medication, and bladder hydrodistention. Unfortunately, these treatments often fail with limited efficacy or a short duration of symptom improvement. Other treatments such as botulinum toxin and cyclosporine are optional because of a lack of long-term efficacy or a small number of patients who

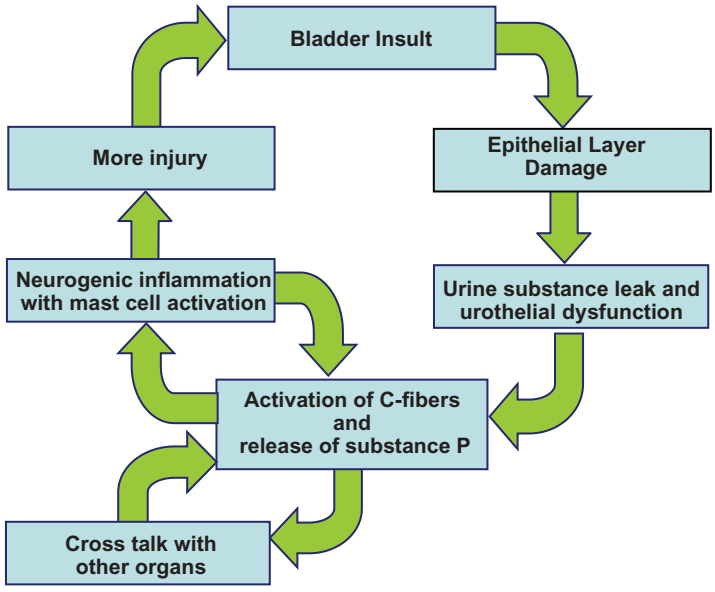


Figure 1. Proposed urothelial and neurogenic pathogenesis of interstitial cystitis/bladder pain syndrome. Reproduced with permission from [28].

participated in clinical trials.[4] At present, established IC/BPS treatments with long-term efficacy do not exist.

3. Existing treatments for IC/BPS

For the treatment of IC/BPS, conservative therapies are initially tried and then, if the symptoms are not relieved, less-conservative and/or surgical therapies are additionally performed. According to the AUA guidelines for IC/BPS, [4] the recommended treatment options are described in sequence as follows (Table 1): (1) behavioral modification with patient education, (2) physical therapies, oral agents and/or intravesical medications, (3) bladder hydrodistention including transurethral resection/fulguration of Hunner's lesions, if they exist, (4) neuromodulation, (5) oral administration of cyclosporine A (CyA) or intradetrusor injection of onabotulinum toxin A (BTX-A), and (6) surgical treatment (urinary diversion with or without cystectomy). Among them, efficacy of neuromodulation, CyA and BTX treatments are not validated because of the lack of evidence, and the US Food and Drug Administration does not approve these therapies for the treatment of IC/BPS.

3.1. Behavioral therapy

Patient education regarding fluid intake, avoidance of foods that irritate the bladder, sexual intercourse and clothes improves IC/BPS symptoms. A multicenter placebo-controlled clinical trial of amitriptyline shows that education and behavioral modifications improve the global response assessment (GRA) in 45% of IC/BPS patients in the placebo arm who

Table 1. Existing treatments for IC/BPS.

Compound/treatment	Company	Evidence level
Pentosan polysulfate (oral)	Ortho-NcNeal	1a
Amitriptyline (oral)	Multiple companies	1b
Cyclosporin A (oral)	Multiple companies	1b
DMSO (intravesical)	Multiple companies	1b
Hydroxyzine (oral)	Multiple companies	1b
Intradetrusor BTX-A +hydrodistention	Allergan	1b
Chondroitin sulfate	Multiple companies	2b
Hyaluronic acid (intravesical)	Multiple companies	2b
Patient education/behavioral modification	n/a	3
Organ removal	n/a	3
Sacral neuromodulation	Medtronic	3
Transurethral resection	n/a	3

Evidence levels: 1a, Evidence obtained from meta-analysis of randomized trials; 1b, Evidence obtained from at least one randomized trial; 2a, Evidence obtained from one well-designed controlled study without randomization; 2b, Evidence obtained from at least one other type of well-designed quasi-experimental study; 3, Evidence obtained from well-designed nonexperimental studies, such as comparative studies, correlation studies and case reports; 4, Evidence obtained from expert committee reports or opinions or clinical experience of respected authorities.

were not administered amitriptyline, although there was no control group without behavioral therapy.[29] The intervention is inexpensive and free of risk and should be incorporated into the treatment plans of all patients with IC/BPS.[4]

3.2. Amitriptyline

Amitriptyline is one of the tricyclic antidepressants that has many pharmacological effects such as anticholinergic, antihistamine and narcotic actions.[30,31] Oral administration of amitriptyline significantly improved symptoms, such as pain and urgency (greater than 30% decrease), in 42% of IC/BPS patients compared to 12.5% of the placebo group at 4 months.[30] Adverse events such as drowsiness and nausea are often severe enough to cause some patients to discontinue medication. Foster et al. also reported the efficacy of oral administration of amitriptyline or placebo in IC/BPS patients who received education and behavioral modification.[29] In this study, a GRA rate is moderately or markedly improved in 55% of patients after administration of amitriptyline although amitriptyline in combination with behavioral therapy is not significantly more effective when compared to the behavioral therapy alone group.[29] However, in patients who were able to increase the dose over 50 mg/day, higher improvement was confirmed.[29] At present, amitriptyline is considered the optional treatment for IC/BPS.[4,9]

3.3. Pentosan polysulfate sodium

Pentosan polysulfate sodium (PPS) is a heparin-like agent, which is analogous to glycosaminoglycans (GAG). The urothelium is insulated by surface mucin, which is made up of sulfonated GAG and glycoproteins. Lilly et al. reported that a loss of protective GAG layer lining in the bladder urothelium is responsible, at least in part, for the permeability changes of the IC/BPS bladder.[16] There have been some clinical trials that investigated the efficacy of the PPS for IC/BPS; however, their outcomes are not consistent. For example, it has been revealed that PPS initially improves the O'Leary-Sant Interstitial Cystitis Symptom/Problem Index in 50% of IC/BPS patients although it may take more than 6 months to confirm the efficacy.[32,33] A recent clinical trial (NCT00086684) was terminated because it could not demonstrate the efficacy (www.clinicaltrials.gov). Currently, the efficacy of combined treatment of PPS and bladder hydrodistention is now recruiting participants in a phase IV clinical trial (NCT01895153). Overall at present, PPS is considered as an optional treatment in the AUA guideline due to the lack of evidence, although PPS is an FDA-approved oral agent for IC/BPS.[4]

3.4. Intravesical treatments (DMSO, heparin and lidocaine)

Intravesical treatments with dimethyl sulfoxide (DMSO), heparin or lidocaine have been used as a popular therapeutic option for IC/BPS.[34] DMSO is the only FDA-approved drug for intravesical treatments. Perez-Marrero et al. reported that 53% of IC/BPS patients exhibited markedly improved symptoms after intravesical administration of 50% DMSO solution, compared to the placebo (18%).[35] The other trials compared the efficacy of DMSO and bacillus Calmette-Guerin (BCG). DMSO significantly reduces pain and urinary frequency in IC/BPS patients with Hunner's lesion, while it does not increase bladder capacity.[36] DMSO is more effective than BCG according to the change in the GRA; however, changes in the health-related quality of life were not observed between DMSO and BCG.[37] Recent studies also showed the efficacy of combined intravesical treatments of DMSO with other drugs such as heparin, hydrocortisone and lidocaine.[38,39]

Three clinical studies for intravesical injection of heparin reported the outcome of IC/BPS symptoms. Heparin administration (30,000 IU) improved symptoms in 56% of patients.[40] Heparin is also more effective when combined with other drugs such as lidocaine and bicarbonate. A clinical trial showed the efficacy of heparin in combination with alkalized lidocaine.[41] In 18 patients, there was a significant reduction in pain, the GRA and urinary urgency after treatments.[41] The AUA guideline indicated heparin as an optional treatment due to the paucity of placebo-controlled clinical trials.

Intravesical lidocaine has also been shown to be an effective treatment for IC/BPS. A clinical trial reported that alkalinized lidocaine and sodium bicarbonate relieve IC/BPS symptoms, as reported in the significant decrease of the GRA score, compared to the placebo (30 and 9.6%, respectively).[42] Dysuria, urethral irritation and bladder pain are the adverse events after injection. The success rate is relatively higher than other intravesical drugs, but the post-therapeutic observation period is short; therefore, the treatment is also defined as an option in the AUA guideline.[4]

3.5. Onabotulinum toxin A injection

BTX-A inhibits acetylcholine release from presynaptic cholinergic terminals, suggesting that BTX-A injection into the bladder wall could reduce the urinary symptoms of IC/BPS. Smith et al. reported the treatment efficacy of intradetrusor injection of BTX-A on IC/BPS symptoms in a pilot study.[43] The mean scores of Interstitial Cystitis Symptom Index and Interstitial Cystitis Problem Index were improved by about 70% after 100 – 200 IU BTX-A treatment. However, the effectiveness is short and diminished in 1 year.[43] A randomized clinical trial investigated the efficacy of combination of BTX-A administration with hydrodistention.[44] BTX-A combined with bladder hydrodistention improved the GRA in 70 – 80% of patients compared to 48% of patients with hydrodistention alone. However, the effectiveness was short lasting so that the improvement was seen only in 17 – 21% of patients at 1 year. Several reports

show that repeat BTX-A injections elongate the duration of activity of BTX-A.[45] Half the patients receiving 200 IU experienced dysuria, and some required intermittent self-catheterization although other adverse events were not observed. More recently, Pinto et al. showed that the injection of 100 IU BTX-A is effective to reduce symptoms such as pain intensity, frequency, nocturia in IC/BPS patients with or without Hunner's lesion, although there was no placebo group included.[46] Currently a study of intradetrusor botulinum toxin (phase II) is now recruiting participants in Taiwan (NCT01969773). At present, BTX-A injection is considered to be an optional treatment that can be performed when other treatments fail to relieve the symptoms.[4] Further large-scale, placebo-controlled studies are necessary to confirm the efficacy of BTX-A for the treatment of IC/BPS.

3.6. Cyclosporine A

A randomized clinical trial in IC/BPS patients treated with either CyA or PPS showed the advantage of CyA over PPS after 6 months treatment, and 75% of patients were satisfied with CyA medication with 50% decrease in urinary frequency.[47] Also, in pilot single-arm, non-randomized studies, 91% of IC/BPS patients experienced pain relief after 6 weeks and 87% of patients showed pain reduction and decrement of urinary frequency at 1 year.[48,49] A previous retrospective study also showed that CyA medication is more effective for improving the GRA or the Interstitial Cystitis Symptom Index score in IC/BPS patients with Hunner's lesion compared to those without.[50] Half of the patients presented with adverse events including increased serum creatinine level, hypertension and alopecia. At present, CyA treatment can be considered as an optional treatment, and a phase II clinical trial is recruiting IC/BPS patients (NCT01990898).

3.7. Bladder hydrodistention

If oral and intravesical treatments have failed to relieve the symptoms, cystoscopy, including bladder hydrodistention under anesthesia would be a next-step treatment. Three observational studies showed that bladder hydrodistention relieves urinary tract symptoms for up to a few months (18 – 56%); however, it does not last for a long duration of time;[51,52] therefore, the treatment is considered as an optional treatment. At present, the efficacy of bladder hydrodistention treatment with warm water (up to 45°C) is being investigated with recruiting participants in Israel (NCT01838486). In addition, there is the possibility of confounding of the results of intravesical treatments for IC/BPS by hydrodistention that takes place during intravesical therapies described above (Section 3.4) because hydrodistention alone may have a therapeutic effect on IC/BPS. Furthermore, because the levels of growth factors and cytokine/chemokine in the bladder could be affected by hydrodistention, one should be careful interpreting results of gene expression analysis studies of IC/BPS bladder specimens. If Hunner's lesions are found during cystoscopy, fulguration or laser ablation could be performed.

4. Market review

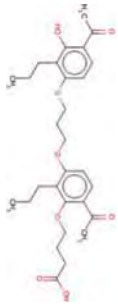
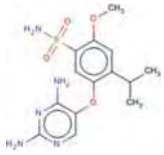
In 1975, Oravisto et al. showed the prevalence of IC/BPS in Finland. The overall prevalence was 10.6 cases per 100,000, and 1.2 female cases per 100,000 women annually diagnosed as IC/BPS.[53] In the Netherlands, the prevalence of IC/BPS was calculated to be 8 to 16 per 100,000 females.[54] Clemens et al. also showed that the prevalence of IC/BPS was 197 female and 41 male cases per 100,000 population.[55] More recently, Berry et al. investigated the prevalence estimation of IC/BPS symptoms in the US, showing that 6.53% of women have the symptoms suggestive of IC/BPS based on telephone interview of 12,752 women.[56] In other words, it is projected that 3,300,000 to 7,900,000 women over 18 years old in the US may have IC/BPS.[56] Overall, it is speculated that IC/BPS may be a common disease, and many IC/BPS patients are underdiagnosed.

Anger et al. published a paper that described the therapies for IC/BPS and their related expenditures using a national database in the US.[57] In 89 patients with IC/BPS, all patients took medication orally, 26% of them received intravesical administration and 22% received hydrodistention.[57] The cost for all treatments is calculated to be $2208 per patient, not including the clinical testing such as laboratory or radiology examination.[57] The majority of expenditures were spent on oral medical therapies. Thus, despite the high expense, the management of IC/BPS has not been achieved due to the lack of optimal treatment modalities.

5. Current research goals

As discussed above, the existing treatments often fail to improve the symptoms of IC/BPS because of unknown etiologies. Thus, we need to re-evaluate the etiology of IC/BPS, and seek new treatments based on the more detailed mechanisms of IC/BPS, including urothelial dysfunction, neural changes, neurotrophic factors, immune responses and other components. A summary of current ongoing clinical trials is shown in Table 2. Another concern is that there are no suitable animal models of IC/BPS because of the unknown and complex etiology of this disease condition. At present, various animal models – in which we can examine the consequences of each of the pathophysiologic conditions proposed for IC/BPS including increased urothelial permeability or bladder inflammation as well as the pathophysiology of IC/BPS-like symptoms – are being used for the basic research of IC/BPS. However, confirmation of the findings in animal models through clinical studies may validate new targets or modalities for the treatment of IC/BPS.

AQ13 **Table 2. Competitive environment table: current clinical trials and basic research.**

Compound	Company	Structure	Indication	Current status	Mechanism of action
Pentosan polysulfate	Johnson & Johnson	Xylan, hydrogen sulfate, sodium salt [CAS]	IC/BPS	Phase IV, terminated	Protective effect on GAG layer
Adalimumab	Pfizer	Immunoglobulin G1, anti-(human tumor necrosis factor) (human monoclonal D2E7 heavy chain), disulfide with human monoclonal D2E7 light chain, dimer	Rheumatoid arthritis, Crohn's disease, ulcerative colites	Phase III, not recruiting	TNF alpha antagonist
Calcium channel ligand (PD 0299685)	Pfizer	-	Off label	Phase II, completed	Modulating afferent nerves
Sodium chondroitin sulfate	Watson Pharmaceuticals	-	IC/PBS	Phase II, completed	Protective effect on GAG layer
Omalizumab	Roche/ Genentech and Novartis	Immunoglobulin G, anti-(human immunoglobulin E Fc region) (human-mouse monoclonal E25 clone pSVIE26 gamma-chain), disulfide with human-mouse monoclonal E25 clone pSVIE26 kappa-chain, dimer	Asthma, chronic spontaneous urticaria	Phase III, completed	IgG antibody blocks IgE
MN-001	MediciNova		Asthma	Phase II, completed	Blockade of leukotriene
Lidocaine	Biomedical, Inc.	-	Local anesthesia	Phase II, completed	Blockade of peripheral pain
BTX-A+TC-3gel	TheraCoat Ltd	-	Off label	Phase II	Local effect of BTX-A sustained release
AQX-1125	Aquinox Pharmaceuticals, Inc	-	Off label	Phase II	Activate SH2-containing inositol-phosphatase
AF-219	Afferent Pharmaceuticals, Inc.		Off label	Phase II	P2X3 antagonist
Opioid	Multiple	-	Analgesics	Va	Act on opioid receptor
Adenosine antagonist	Multiple	-	Off label	Va	Act on adenosine receptor
Gene therapy	Diamyd Inc	-	Off label	Vb	Targeting afferent nerve

Va: *in vivo* animal study; Vb: *in vitro* animal study.

6. Scientific rationale

6.1. Neural changes inducing afferent hyperexcitability

The pathophysiology of pain and lower urinary tract symptoms associated with IC/BPS is elusive; however, a neurogenic aspect of IC/BPS pathogenesis is hypothesized as follows. Urothelial injury from unknown causes induces the release of substances such as ATP and NO from bladder urothelial cells. Then, immunological responses including mast cell activation alter the condition of the bladder, which introduces production of cytokines and chemokines. These substances stimulate bladder afferent pathways and change their sensitivity and firing properties, resulting in hyperexcitability of afferent pathways, which then provokes a painful condition in IC/BPS patients. (Figure 1).[15] Bladder pain is one of the common findings in IC/BPS. It is speculated that altered bladder conditions including tissue inflammation and bladder afferent hyperexcitability are involved in pain symptoms associated with IC/BPS.[3] Some clinical studies report the evidence showing increased activity of bladder afferent pathways in IC/BPS. For example, a histological analysis reported the inflammatory changes in the bladder obtained from IC/BPS patients, showing that the edematous, rich in vasodilatation and afferent nerves and mast cells are observed,[58] although inflammatory changes of the bladder are significantly greater in IC/BPS with Hunner's lesion compared to non-Hunner-type IC/BPS, in which inflammatory cell infiltrates are reportedly not found in the bladder tissue.[59,60] Furthermore, the expression of substance P (SP), a neurotransmitter primarily affecting nociceptive C-fiber afferents, is significantly increased in bladder afferent fibers, and increased mRNA levels of SP receptors are also observed in bladder specimens from IC/BPS patients.[61] Capsaicin and resiniferatoxin, potent agonists for TRPV1 channels, desensitize bladder C-fiber afferents at high doses to relieve the painful symptoms in IC/BPS patients,[62,63] although a prospective study could not confirm the efficacy of resiniferatoxin in patients with IC/BPS.[64] Nickel et al. reported the beneficial effect of alkalized lidocaine, a local anesthetic, on irritable symptoms of IC/BPS in a placebo-controlled, randomized clinical trial.[42] Taken together, these studies may indicate that increased excitability of bladder afferents play a role, at least in part, in the pathophysiology of IC/BPS (Figure 2); although this remains to be proven, other potential pathophysiological factors could also contribute to IC/BPS pathogenesis (see Section 1.2).

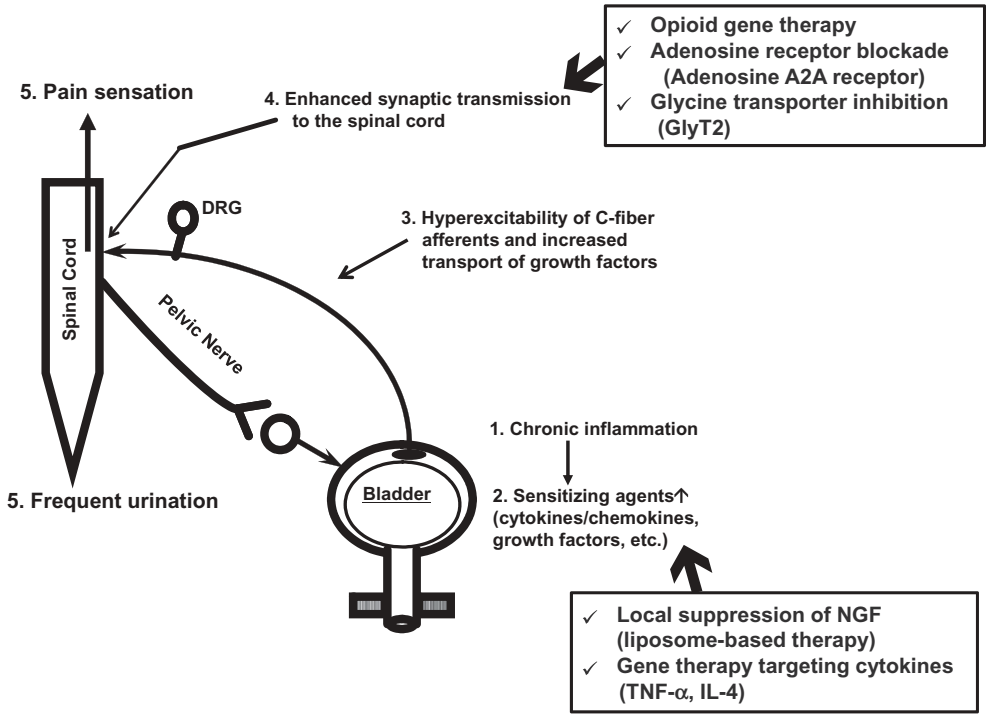


Figure 2. Proposed summary of the events involved in chronic inflammation of the bladder and hyperexcitability of C-fiber bladder afferent pathways. The events that occur following chronic bladder inflammation (1) are indicated in sequential numbers (2–5). Potential therapeutic modalities targeting enhanced synaptic transmission at the spinal cord level or inflammatory changes in the bladder, which were discussed in the text, are also shown in the boxes. DRG: dorsal root ganglia. Reproduced with permission from [28].

6.2. Nerve growth factor

Neurotrophic factors such as nerve growth factor (NGF) are observed in various inflammatory cells, such as lymphocytes and mast cells. It has been shown that NGF is involved in inflammation, an allergic reaction, and altered neurological conditions in IC/BPS.[65,66] An immunohistological study reports that expression of NGF is increased in the bladder of IC/BPS patients.[67] Neurotrophic factors including NGF are also found in urine obtained from IC/BPS patients, [68,69] and a recent meta-analysis study reported that urinary NGF could be a useful biomarker for the differential diagnosis of IC/BPS and overactive bladder as well as a predictive biomarker to help guide treatments.[69] In experimental rat studies, intravesical or intrathecal application of exogenous NGF elicits nociceptive effects and micturition frequency.[70–72] These data suggest that NGF is increased in the pathological condition and provokes bladder pain in IC/BPS.[73] Therefore, inhibition of NGF may be a therapeutic potential for lower urinary tract symptoms in IC/BPS patients (Figure 2). Evans et al. reported the clinical outcome of tanezumab, monoclonal NGF neutralizing antibody for IC/BPS in phase II study.[74] Tanezumab was administered intravenously in 68 patients with IC/BPS, and effective for self-reported pain and urinary urgency for 6 weeks compared to the placebo group, while voiding frequency and voided volume are not affected.[74] In this study, abnormal peripheral sensation is the most common adverse event, and vertigo and headache present as other adverse events. Furthermore, serious adverse events were reported by another clinical trial of tanezumab for osteoarthritis, in which bone necrosis developed and total joint replacements were needed, and several clinical trials have since been terminated (www.clinicaltrials.gov) despite that proof-of-concept evidence has been provided for effectiveness of intervention of the NGF system for the treatment of IC/BPS.[74] To avoid systemic adverse events of anti-NGF therapy, intravesical treatments may be a reasonable approach. In this regard, we recently reported that intravesical administration of liposome-NGF antisense conjugates suppresses NGF expression in the bladder mucosa and bladder overactivity in rats with acetic acid–induced cystitis.[75] Therefore, local suppression of NGF in the bladder using intravesical liposome-based delivery techniques could be an attractive approach for IC/BPS treatment, which can avoid systemic side effects although further studies are needed to clarify this point .

6.3. Cytokine and chemokine expression and IC/BPS

Inflammatory cytokines/chemokines have also been considered as potent pathophysiological factors involved in IC/BPS.[76–78] Previous studies revealed that cytokines/chemokines such as interleukin-2 (IL-2), IL-6 and IL-8 were significantly increased in urine from patients with IC/BPS compared to controls.[76,77] In contrast, the serum level of IL-4, which suppresses the secretion of inflammatory cytokines like IL-1β, TNFα and IL-6,[79–81] is decreased in IC/BPS patients and normalized by the oral treatment with suplatast tosilate.[79] Also, in IC/BPS patients with Hunner's lesion, IL-6, 10 and 17A as well as iNOS expression and the number of mast cells were significantly increased in the bladder mucosa, which was biopsied before hydrodistention compared to healthy controls.[82]

Several chemokines also act on many functions such as the recruitment of leukocytes, tumor growth, angiogenesis and organ sclerosis.[83] CXCR3 and its associated ligands, interferon-γ (INF-γ)-inducible chemokines (CXCL9, CXCL10 and CXCL11), are involved in autoimmune diseases. CXCR3 is expressed in various cells including helper T cells, epithelial cells and vascular pericytes.[84,85] Activation of IFN-γ induces the secretion of CXCL10 from endocrine epithelial cells, and then CXCL10 recruits helper T cells expressing CXCR3 to produce IFN-γ. This vicious circle may be involved in endocrine autoimmune diseases.[83] Sakthivel et al. report the increment of serum levels of CXCR3 associated ligands in IC/BPS patients.[86] They also report that the similar mRNA changes of CXCL10 and CXCL11 are observed in cyclophosphamide (CYP)-induced cystitis mice, and the altered expression is blocked by anti-CXCL10 antibodies.[86] We also investigated gene expression in bladder samples obtained from IC/BPS patients with Hunner's lesion before hydrodistention using DNA microarray. We found that the upregulation of CXCR3 associated chemokines as well as TNFSF14 in the bladder urothelium,[87] which exacerbates the inflammatory conditions, may inhibit urothelial reproduction in the Hunner's lesion of the bladder.[87] It has also been investigated that altered expression of cytokines and chemokines by multiple antigen bead assay using biopsied bladder tissues obtained after hydrodistention, showing that several cytokines such as IL-16, VCAM-1 and ICAM are increased.[88] Multivariate analysis shows that VCAM-1 and ICAM-1 are useful for distinguishing bladder tissue and urine of IC/BPS patients from those of control patients.[88] The blockade of cytokine/chemokine production in relation to IC/BPS seems beneficial for treatments of IC/BPS; however, the efficacy was not confirmed by the clinical study. Bosch et al. investigated the efficacy of adalimumab, a biological agent that blocks TNF-α, on the lower urinary tract symptoms in IC/BPS patients (phase III study: NCT01295814). Adalimumab treatment significantly improved the painful symptoms, urinary urgency and frequency symptoms at 12 weeks compared to the baseline.[89] However, statistically significant improvements could not be obtained in any outcome measures from patients with adalimumab compared to control patients.[89] Thus, at present, systemic blockade of TNF-α fails to demonstrate the treatment efficacy for IC/BPS; however, in an animal study, the local blockade of TNF-α by using herpes simplex virus (HSV) vectors expressing soluble TNF-α receptor can reduce bladder pain behavior and bladder overactivity induced by bladder irritation.[90] Further investigations are needed to elucidate the possibility of clinical translation of the HSV-based cytokine/chemokine blockade treatment for IC/BPS.

6.4 Calcium channel ligand

The α2δ subunit of ligand-gated calcium ion channels mediates activation of nociceptive afferents and is implicated in chronic pain; therefore, gabapentin and pregabalin that can block the calcium ion channel α2δ subunit have been indicated for the treatment of neuropathic pain.[91] There are some clinical studies suggesting that gabapentin may have efficacy in the refractory genitourinary pain of IC/BPS although they are open-label, uncontrolled studies with a limited number of patients.[92,93] Nickel et al. investigated the effect of PD-0299685, a calcium channel α2δ ligand, in IC/BPS patients.[94] PD-0299685 significantly reduces painful symptoms after 12 weeks compared to control patients; however, the trial could not get the proof-of-concept demonstration due to a lack of significant improvements in Interstitial Cystitis Symptom Index score.[94]

6.5. Sodium chondroitin sulfate and liposomes

Chondroitin sulfate is one important component of mucinous GAG layer in the urothelium, which may be altered in IC/BPS. Nickel et al. showed the efficacy of intravesical sodium chondroitin sulfate for symptoms of IC/BPS.[95] In this randomized controlled trial, although 38% of patients treated with chondroitin sulfate showed moderate or marked improvement, the treatment has minor benefit and its use as a monotherapy is not indicated for this condition.[95] At present, a clinical trial is recruiting participants to investigate the efficacy of a combined intravesical therapy of hyaluronic acid/chondroitin sulfate with transurethral resection of Hunner's lesion (NCT01813565: phase II).

Liposomes are the earliest prototype of nanoparticles (particles with one of the dimensions in nanometers), which consist of soluble substances enclosed by concentric bilayers.[96,97] Chuang et al. reported clinical outcomes of intravesical application of liposomes in IC/BPS patients compared to oral PPS.[98] In this open-label, unblinded prospective study of 24 IC/BPS patients, the comparable efficacy of liposomes to oral PPS was demonstrated by significant decrease in pain and urinary urgency symptoms in the liposome treatment arm. No serious adverse events and no significant worsening of symptoms were observed during follow-ups. Thus, liposome is one candidate for the treatment of IC/BPS; however, prospective trials with a large number of patients are needed to establish the efficacy of this treatment. In addition, because there is a possibility that hydrodistention of the bladder may confound the results of intravesical liposomal studies, care should be taken to avoid bladder overdistention during intravesical treatments including liposomal therapy.

7. Competitive environment

Many research and clinical studies, which evaluated various drugs for treatment of IC/PBS, were terminated due to the lack of efficacy or adverse events. Described below are other ongoing trials of drug treatments.

7.1. AF-219 (P2X3 receptor antagonist)

Urothelial cells modulate afferent signals from chemical and mechanical stimuli in the bladder, and then the signals are sent to the CNS (Figure 1).[3] Bladder distention releases ATP from the urothelium, and ATP activates P2X3 receptors in bladder afferents to modulate bladder activity evidenced by experimental studies of P2X3 knockout mice.[99,100] There is also evidence showing that the stimulatory ATP mechanism is upregulated in the bladder from IC/BPS patients because ATP release from urothelial cells in addition to urothelial expression of P2X3 ATP receptors is increased in IC/BPS patients.[101–104] It has also been reported that urothelially released ATP is enhanced in cats with feline-type IC.[105] Based on these results, a P2X3 receptor antagonist (AF-219) has been tested for IC/BPS patients in a placebo-controlled, randomized phase II study (NCT01569438) (Table 2).

7.2. Intranasal oxytocin

A previous report has shown that IC/BPS patients have reduced bladder pain during breastfeeding.[106] There is a speculation that the hormones related to the postpartum lactation relieve the symptoms of IC/BPS, because postpartum lactation decreases levels of stress, which exacerbates IC-related pain. Black et al. reported that oxytocin attenuates the stress condition induced by bladder distention in rats.[107] A prospective clinical trial of intranasal oxytocin versus intranasal saline for bladder pain of IC/BPS is now recruiting participants (NCT00919802).

7.3. Drug delivery systems

Systemic administration including oral intake often fails to treat IC/BPS. Topical application of drugs, such as lidocaine, shows efficacy with lower adverse events; however, problems such as the short duration of effectiveness remain. LiRIS® is a solid mini pellet, which encases lidocaine in a water-permeable flexible tube and releases lidocaine continuously.[108] The phase II randomized clinical trial is now recruiting participants for evaluating the efficacy of LiRIS® in female patients with IC/BPS (NCT01824303) (Table2). For sustained release of drugs, intravesical application of TC-3 gel with BTX-A is also currently being investigated (NCT01997983) (Table 2). Liquid TC-3 gel containing BTX-A is transformed into a solid state in the bladder, and the TC-3 gel dissolves in the urine to release the BTX-A. The mechanism makes it possible for the sustained release of BTX-A.

7.4. AQX-1125

AQX-1125 activates SH2-containing inositol-5′-phosphatase (SHIP1), which modulates the PI3K pathway.[109] Activation of the PI3K pathway is involved in immune cell

signaling inducing chronic inflammation. SHIP1 is predominantly expressed in immune cells derived from bone marrow tissues. It is considered that activation of SHIP1 has an anti-inflammatory effect by negatively regulating the PI3K pathway to reduce the immunological reaction.[109] Oral AQX-1125 inhibited the nociceptive pain scores in the rat model of CYP-induced cystitis; therefore, AQX-1125 is one of the candidate drugs to be studied in the phase II clinical trial (NCT01882543) (Table 2).

7.5. MN-001 (tipelukast)

MN-001 (tipelukast) is an orally bioavailable small molecule compound, which exerts its effects to produce anti-fibrotic and anti-inflammatory activity, including leukotriene (LT) receptor antagonism, inhibition of phosphodiesterases (PDEs) and inhibition of 5-lipoxygenase.[110] This compound has been tested for IC/BPS patients in the randomized phase II clinical trial (NCT00295854) (Table 2).

8. Potential development issues

Based on basic research findings, there are several suggestive mechanisms by which the painful and irritable symptoms are induced in IC/BPS patients. Among them, urothelial dysfunction with increased permeability and urothelially released substances, and sensitization of bladder afferent pathways including C-fiber afferents could contribute to the development of IC/BPS symptoms.[111] Therefore, therapies targeting the sensory activity at both peripheral and central levels may be effective for reducing symptoms of IC/BPS patients.

8.1. Opioids

Persistent bladder pain is often resistant to nonsteroidal anti-inflammatory drugs and needs more effective drugs such as morphine or oxycodone. However, these opioid treatments have potential risks such as tolerance and dependency. Therefore, the aforementioned EAU guideline of CPPS has shown that the indication of opioids is limited to selected patients. To avoid the side effects, the endogenous opioid could be one of the candidates for the treatment of bladder pain. Enkephalins, one of the endogenous opioids, are expressed in sensory and motor neurons and increased by nerve injury.[112] Supraspinal application of enkephalin inhibits the micturition reflex in rats, suggesting that upregulation of enkephalin levels in the brain and/or the spinal cord has inhibitory effects on the micturition reflex.[113] Yokoyama et al. showed that intradetrusor injection of HSV vectors encoding preproenkephalin, the precursor of enkephalins, suppresses bladder overactivity and nociceptive behavior induced by intravesical application of capsaicin, whereas the effects of vector-mediated enkephalin delivery are not observed in normal rats,[114,115] suggesting that HSV vector-mediated delivery of enkephalin has therapeutic effects on bladder nociceptive responses without affecting normal micturition. Thus, the increased levels of endogenous opioids such as enkephalin seem to be an effective modality for the treatment of IC/BPS symptoms. In humans, the efficacy of herpes virus vector NP2 encoding enkephalin on intractable cancer pain has been evaluated by the phase II clinical trial (NCT01291901).

8.2. Adenosine receptors

Adenosine is expressed in various organs including nervous systems, and acts as a neuromodulator to exert physiological effects in various organs. A number of studies have shown that activation of A1 receptors produces antinociception in several pain models, and A2A receptor knockout attenuates nociceptive responses in mouse models of somatic pain.[116–118] A recent study has also shown that systemic or intrathecal application of ZM24138 (A2A receptor antagonist) significantly reduces micturition intervals in rats with acetic acid–induced cystitis compared to control.[111] These results indicate that the blockade of A2A receptor in the spinal cord might suppress peripheral pain. In the clinical setting, adenosine is thought to be useful in the treatment of allodynia, and the efficacy of an adenosine A2A receptor antagonist for neuropathic pain was investigated in the clinical trial (NCT00349921) although the trial has been terminated because of the small number of participants and lack of data. However, at least, they reported no serious events in the trial, suggesting that adenosine receptor-targeting treatments are safe in humans. Further investigation is needed to evaluate the efficacy of this class of compounds for IC/BPS.

8.3. Glycine receptor

Glycine is one of the inhibitory amino acids, which plays a role as a neurotransmitter in the spinal cord and preganglionic neurons. Glycine also exerts inhibitory effects on bladder contractions and urethral sphincter activity and might be involved in the pathological bladder conditions associated with spinal cord injury or bladder outlet obstruction.[119–121] The extracellular level of glycine is regulated by two subtypes of the glycine transporter (GlyT), GlyT1 and GlyT2.[122] Previous studies have shown an efficacy of GlyT inhibitors for chronic pain [123,124] and bladder overactivity in rats.[125] Intrathecal application of GlyT2 inhibitor suppresses bladder overactivity induced by CYP and pain behavior induced by resiniferatoxin in rats.[125] These data suggest that inhibition of GlyT2 in the spinal cord might have a therapeutic potential for IC/BPS. Recently, VVZ-149, an inhibitor of GlyT2 and serotonin receptors, has been developed as a new analgesic agent, and the safety, tolerability and pharmacokinetics of the agent were investigated in the phase I clinical trial (NCT01905410).

8.4 Gene therapy for modulating the cytokine level

TNF-α is involved in various functions that contribute to tissue inflammation and tumor progression. For example, TNF-α induces adhesion molecules and other cytokines such as IL-1 and IL-6, and stimulates inflammatory cells.[126] It has also been recognized that TNF-α is involved in neuropathic pain [127] and acts on the peripheral tissue and the spinal cord.[127,128] The neutralization of TNF-α activity by the antibody or TNF-α soluble receptors (TNF-αsR) attenuates the pain sensation in several rat pain models.[128–130] Recently, it has been reported that gene therapy of TNF-αsR using replication-deficient HSV vectors reduces TNF-α protein levels of the bladder, leading to the suppression of cytokines such as IL-1 and IL-6 to relieve bladder pain and urinary frequency in a rat cystitis model.[90] The TNF-αsR-expressing vector treatment also suppresses the bladder overactivity induced by intravesical application of resiniferatoxin in association with the reduction in myeloperoxidase activity in the bladder.[90] Based on the results of HSV preclinical studies, a phase I trial using HSV-enkephalin gene therapy to treat chronic pain due to cancer has been completed in 10 patients with remarkable pain-relieving activity without any HSV vector-related toxicity.[131] Thus, there is the possibility that HSV vector-based gene therapy could be effective for the treatment of IC/BPS symptoms although the long-term safety of HSV vector-based gene therapy needs confirming in larger-population clinical studies.

8.5. PDE inhibitor

Cyclic nucleotide PDE1 is an enzyme that metabolizes the intracellular signal transmitters cyclic adenosine and guanosine monophosphate (cAMP and cGMP) into 5′-adenosine monophosphate (5′-AMP) and 5′-guanosine monophosphate (5′-GMP), respectively, and plays an important role in the regulation of intracellular cAMP and cGMP concentrations. PDE inhibitors can relax bladder smooth muscle and improve vascular endothelial function.[132,133] Tadalafil, a long-acting PDE5 inhibitor, is used for erectile dysfunction and lower urinary tract symptoms associated with benign prostate hyperplasia.[134] In an animal study, PDE4 inhibitor, which is expressed in inflammatory cells such as leukocytes, is effective in reducing bladder irritation induced by HCl.[135] Chen et al. also reported that the low dose of sildenafil, a PDE5 inhibitor, was effective in the treatment for IC/BPS because symptoms and bladder capacity were significantly improved after the treatment.[136]

9. Conclusion

As discussed in this review, various etiologies of IC/BPS have been postulated, which include urothelial dysfunction with increased permeability, alterations in growth factor expression, neurogenic inflammation with mast cell activation and increased NO levels, autoimmune reaction, infection, increased afferent activity and changes in CNS responses. However, there is not a pathological process that is applicable to every patient with IC/BPS. Thus, it is presumed that IC/BPS can be induced by multiple pathophysiological factors, which interact with each other, thereby leading to similar clinical manifestations. Unfortunately, at present, there is no optimal treatment for IC/BPS, but newer drugs and treatment modalities are currently under investigation in various clinical trials. Among them, several interventions, including sustained release and retaining techniques and drugs that act on afferent neural pathways, are emerging and may be promising. In addition, although sensitization of the CNS has been proposed to be involved in the pathogenesis of IC/BPS (see Section 1.2), the effort for developing new therapies targeting the brain or the spinal cord is still limited.

10. Expert opinion

Although the etiology of BPS/IC is not known, it is considered that the epithelial layer damage leads the toxic substances into the deep layer, which induces bladder pain and other symptoms. In addition, it is still not known how the "leaky urothelium" developed in IC/BPS patients. In animal studies, blocking various proposed causes discussed in this review seems to be effective for the treatments of IC/BPS; however, the results in animal studies have not come to fruition in clinical trials. One of the reasons for the discrepancy could be the subtype of IC/BPS. The European Society for the Study of Interstitial Cystitis (ESSIC) proposed that Hunner's lesion, which is often called "ulcer," is not a typical chronic ulcer, but rather a distinctive inflammatory lesion presenting a characteristic deep rupture through the mucosa. Accordingly, it is proposed that patients with Hunner's lesion are classified into the ESSIC type 3.[137] In a previous study, gene expression analysis in the bladder of IC/BPS patients with Hunner's lesion shows the overexpression of genes related to immune and inflammatory responses including helper T-cell-related chemokines, whereas similar expression changes are not found in IC/BPS without Hunner's lesion.[87] Thus, IC/BPS with Hunner's lesion might be a different entity from that without Hunner's lesion.[2] In the AUA guidelines, cystoscopy is not recommended as a diagnostic test because typical cystoscopic findings such as glomerulations are often seen not only in IC/BPS but also in other conditions. Therefore, the data from many clinical trials were obtained from the mixture of IC/BPS with and without Hunner's lesion. This situation might be one of the reasons why many drugs fail to demonstrate overall efficacy. It is possible that the cystoscopic evaluation of the existence of Hunner's lesion at least in participants of clinical trials increases the chance of successful outcomes. We consider that it is important to draw a distinction between these two conditions because the prevalence of Hunner's lesion among IC/BPS patients is higher

than previously expected.[2,10] In this regard, cystoscopic evaluation with a narrow band imaging system could increase the rate and accuracy of detection of bladder changes including Hunner's lesion in IC/BPS.[138] In the EAU guideline, the treatment is decided by the presence or absence of Hunner's lesion. Transurethral resection is recommended when Hunner's lesion exists. In addition, Nickel et al. proposed the individualized therapeutic strategies based on the phenotype of IC/BPS symptoms, which is classified by the UPOINT categorization (urinary, psychosocial, organ-specific, infection, neurologic or non-bladder, and tenderness of pelvic floor).[139] Taken together, we presume that standardized classification of IC/BPS is important for not only further investigation of etiology, but also the evaluation of efficacy of new treatments.

Declaration of interest

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* **This study shows the classification of IC/BPS symptoms that can be used to develop the tailored approach for IC/BPS treatment**.

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The Journal of Urology

Liposome-based Intravesical Therapy targeting Nerve Growth Factor (NGF) ameliorates Bladder Hypersensitivity in Rats with Experimental Colitis

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Abstract:	Purpose: Pelvic organ cross sensitization is considered to contribute to overlapping symptoms in CPPS. Overexpression of NGF in the bladder is reportedly involved in the symptom development of BPS/IC patients. This study examined whether a reduction of overexpressed NGF in the bladder by intravesical treatment with liposome and OND conjugates ameliorates bladder hypersensitivity in a rat colitis model. Materials and Methods: Adult female rats were divided into; (a) a control group, (b) a colitis-OND group with intracolonic TNBS enema and intravesical liposomal-OND treatments, (c) a colitis-saline group with intracolonic TNBS and intravesical saline treatments, (d) a sham-OND group with intravesical liposomal-OND treatment without colitis and (e) a sham-saline group with intravesical saline treatment without colitis. Liposomes conjugated with NGF antisense OND or saline solution were instilled into the bladder, and 24 hours later, colitis was induced by TNBS enema. Effects of NGF antisense treatment were evaluated by pain behavior, cystometry, molecular analyses and immunohistochemistry 10 days after TNBS treatment. Results: In colitis-saline rats, pain behavior was enhanced compared to other 4 groups including colitis-OND rats, and intercontraction intervals were significantly reduced by acetic acid stimulation as opposed to slight decreases in other groups. NGF expression in the bladder mucosa was higher in the colitis-saline group than in other groups. Conclusions: NGF overexpression in the bladder mucosa and bladder hypersensitivity induced after colitis were reduced by intravesical application of liposomal OND targeting NGF, suggesting that the local anti-NGF therapy could be effective for the treatment of bladder symptoms in CPPS.
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Liposome-based Intravesical Therapy targeting Nerve Growth Factor (NGF) ameliorates Bladder Hypersensitivity in Rats with Experimental Colitis

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Runninghead: Colitis in rat model augmented pain sensation with NGF overexpression in the bladder. Intravesical treatment with liposomal-antisense targeting NGF attenuated bladder hypersensitivity.

Key words: Chronic pelvic pain syndrome, Irritable bowel syndrome, Bladder pain syndrome, interstitial cystitis, Liposome, Nerve growth factor, Antisense

Abstract

Purpose: Pelvic organ cross sensitization is considered to contribute to overlapping symptoms in CPPS. Overexpression of NGF in the bladder is reportedly involved in the symptom development of BPS/IC patients. This study examined whether a reduction of overexpressed NGF in the bladder by intravesical treatment with liposome and OND conjugates ameliorates bladder hypersensitivity in a rat colitis model.

Materials and Methods: Adult female rats were divided into; (a) a control group, (b) a colitis-OND group with intracolonic TNBS enema and intravesical liposomal-OND treatments, (c) a colitis-saline group with intracolonic TNBS and intravesical saline treatments, (d) a sham-OND group with intravesical liposomal-OND treatment without colitis and (e) a sham-saline group with intravesical saline treatment without colitis. Liposomes conjugated with NGF antisense OND or saline solution were instilled into the bladder, and 24 hours later, colitis was induced by TNBS enema. Effects of NGF antisense OND treatment were evaluated by pain behavior, cystometry, molecular analyses and immunohistochemistry 10 days after TNBS treatment.

Results: In colitis-saline rats, pain behavior was enhanced compared to other 4 groups including colitis-OND rats, and intercontraction intervals were significantly reduced by acetic acid stimulation as opposed to slight decreases in other groups. NGF expression in the bladder mucosa was higher in the colitis-saline group than in other groups.

Conclusions: NGF overexpression in the bladder mucosa and bladder hypersensitivity induced after colitis were reduced by intravesical application of liposomal OND targeting NGF, suggesting that the local anti-NGF therapy could be effective for the treatment of bladder symptoms in CPPS.

250 words

Introduction

Chronic pelvic pain syndrome (CPPS) including bladder pain syndrome/interstitial cystitis (BPS/IC) and irritable bowel syndrome (IBS) is defined as a disease entity with painful symptoms in the pelvic region that last for at least six months and presents a major challenge to patients and health care providers [1, 2]. Symptoms of BPS/IC and IBS are often overlapped as one-third of patients diagnosed with BPS/IC exhibit symptoms consistent with IBS, while 25-56% of patients diagnosed with IBS also have symptoms of BPS/IC [3, 4]. In order to explain this complex pathology, pelvic organ "cross sensitization" has been proposed to contribute to the clinically overlapping symptoms of CPPS [5]. We also recently demonstrated that rats with experimental colitis exhibit enhanced pain sensitivity in the bladder as evidenced by enhanced freezing behavior induced by intravesical nociceptive stimulation [6] .

Nerve growth factor (NGF) is known to be a complex regulator of sensory afferent plasticity in response to injury or inflammation [7, 8]. Increased levels of NGF are found in urine obtained from BPS/IC patients [9], and a recent meta-analysis study reported that urinary NGF could be a useful biomarker for the diagnosis of BPS/IC as well as a predictive biomarker to help guide treatments [10]. We also recently reported that instillation of liposomes conjugated with antisense oligonucleotide (OND) targeting

NGF into the bladder suppressed bladder overactivity in a rat model of acute cystitis [11]. However, it is still unknown if NGF contributes to bladder overactivity and enhanced bladder pain sensitivity after colonic inflammation. Therefore, this study investigated whether intravesical liposomal-OND treatment can suppress NGF expression in the bladder and bladder hypersensitivity in a rat model of experimental colitis.

Materials and Methods

Animal model: Experiments were performed in accordance with NIH guidelines, and the protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Adult female Sprague-Dawley rats were used and divided into 5 groups; (1) a control group (no treatment), (2) a colitis-OND group with intracolonic 2,4,6,trinitrobenzen sulfonic acid (TNBS) enema and intravesical liposomal-OND treatments, (3) a colitis-saline group with intracolonic TNBS and intravesical saline treatments, (4) a sham-OND group with intravesical liposomal-OND treatment without colitis and (5) a sham-saline group with intravesical saline treatment without colitis..

Liposome-conjugated NGF antisense instillation: At 24hr before injection of TNBS (day 0), rats were anesthetized with 2% isoflurane (Baxter Inc., IL), and catheterized

through the urethra into the bladder using a 24-gauge angiocatheter. After draining urine from the bladder, 12μM of phosphorothioated NGF antisense OND with the sequence 5'GCCCGAGACGCCTCCCGA 3' complexed with liposomes or saline (vehicle) in a volume of 0.2ml was instilled (n=6 in each group) as we previously described [11]. Rats were then allowed to recover from anesthesia.

Colitis model; Colitis was induced by administration of TNBS solution (50mg/ml), which was prepared by mixing 1ml of TNBS, 1.93ml of H_2O and 2.93ml of ethanol. Rats were fasted for 24 hours before instillation, anesthetized with 2% isoflurane and inserted with a polyethylene catheter attached to a 1-ml syringe into the colon 6 cm proximal to the anus. The lower body of rats was elevated by lifting the tale, and TNBS or vehicle solution in a volume of 0.5ml was injected and kept for 3 minutes (day 1).

Conscious Cystometry: Twenty-seven rats (5 or 6 rats per group) were used for cystometric evaluation. After anesthesia with 2 % isoflurane, laparotomy through a lower abdominal incision was performed and a PE 50 tube (Scientific Commodities Inc., AZ, USA) with the distal end sealed by heat was inserted into the bladder dome as a cystostomy catheter. The catheter was tunneled subcutaneously and placed underneath

111 the back skin 3 days before cystometry (CMG). Thereafter, the abdomen was closed
112 with running sutures.

113 Ten days after TNBS or vehicle treatment, rats were anesthetized with 2 %
114 isoflurane and a distal end of cystostomy tube was exteriorized from the subcutaneous
115 space. After recovery from anesthesia, rats were placed in restraining cages
116 (Yamanaka Chemical Ind., Japan). The cystostomy catheter was connected through a
117 three-way stopcock to a pressure transducer (BLPR2, World Precision Instruments, Inc.,
118 Sarasota, FL, USA) and to a syringe pump (Harvard Apparatus, Holliston, MA, USA).
119 After rats were acclimated in a cage for 1 hour, CMG was performed by filling the
120 bladder with physiological saline (0.04ml/min) to elicit repetitive voiding. The
121 intravesical pressure was recorded using data-acquisition software (sampling rate was
122 400 Hz. Chart, AD Instruments, Colorado Springs, CO, USA) on a computer system
123 (Power Lab, AD Instruments). During CMG, at least 10 reproducible micturition cycles
124 were recorded after an initial stabilization period (15-30min). Intercontraction intervals
125 (ICIs), which are the time between 2 consecutive micturition cycles, were measured 30
126 min after saline infusion or 60 min after acetic acid (AA) infusion and averaged from at
127 least 3 ICIs.

128

129 *Nociceptive behavioral study*: Twenty-four rats (4-5 rats per group) were used for
130 analyses of nociceptive behavior induced by bladder irritation, as previously described
131 [12]. Briefly, rats were acclimated in metabolic cages (Nalgene, Rochester, NY, USA) for
132 3 h. Water (30ml/kg) was then administered orally, and after 15 min, animals placed in
133 a Bollman cage were instilled with resiniferatoxin (RTX; 0.3 μM, 0.3 ml) into the
134 bladder via a temporally inserted urethral catheter (PE-50) for 1 min. Thereafter, the
135 urethral catheter was removed, and rats were placed back to metabolic cages, and
136 licking and freezing behaviors were scored for a period of 15 min with 5-s intervals [12].
137
138 *Quantification of messenger RNA*: Twenty five rats (n=5 in each group) were used to
139 measure mRNA and protein levels of NGF. The bladder was harvested at day 10 after
140 TNBS or vehicle was given. The bladder was separated into mucosal and detrusor
141 layers under a microscope. Total RNA was extracted by using the Rneasy kit
142 (Quagen, Hilden, Germany), and 2 μg of total RNA was reverse-transcribed into
143 complementary DNA using the ThermoScript RT-PCR System (Invitrogen, Carlsbad,
144 CA, USA) according to the manufacturer's instruction. Quantitative polymerase chain
145 reaction (PCR) was performed with an Mx3000P Real-Time PCR System (Stratagene,
146 La Jolla, CA, USA) in a 25 μl volume using SYBR Green PCR Master Mix (QIAGEN,

147 Valencia, CA, USA). Amplification of cDNA was performed using OND primers
148 specific for NGF or β2MG as a control gene. OND primer sequences were as follows:
149 5'-TCCACCCACCCAGTCTTCCA-3' (forward, NGF),
150 5'-GCCTTCCTGCTGAGCACACA-3' (reverse, NGF),
151 5'-GACCGATGTATATGCTTGCAGAGT-3' (forward, β2MG),
152 5'-GGATCTGGAGTTAAACTGGTCCAG -3' (reverse, β2MG). The protocol
153 consisted of 40 replication cycles.
154
155 *Measurement of NGF protein level*: Eighteen rats (3-4 rats in each group) were used.
156 The bladder was divided into mucosal and detrusor layers. Tissues were homogenized in
157 RIPA lysis buffer system (Santa Cruz Biotechnology Inc., USA) in the presence of 1mM
158 Na_3VO_4, 2mM PMSF and 10μL/mL protease inhibitor. Protein concetration was
159 measured by using Pierce BCA protein Assay kit (Thermo Scientific, USA). Lysates of
160 separated mucosa and detrusor tissues were stored at -80°C until assays. The samples
161 were assayed in duplicate using enzyme-linked immunoabsorbent assay (ELISA) kit
162 (Promega, Madison, WI, USA), according to the manufacturer's instruction, and ELISA
163 plates were read at 450 nm wave on an Elx800 microplate reader (Bio-Tek Instruments,
164 Winooski, VT, USA). Tissue NGF values were normalized against protein

concentrations of each sample and expressed as picograms per microgram protein.

Immunohistochemistry: Ten days after TNBS or vehicle treatment, rats were perfused transcardially with cold physiological saline containing heparin sodium (1 unit/ml) followed by cold 4 % paraformaldehyde solution in 0.1M phosphate buffer (PFA). Then the bladder was harvested and post-fixed in 4% PFA at 4 °C for 24 h and soaked in the 20 % sucrose overnight at 4°C. The frozen tissues were then cut at 10 μm thickness (transverse sections) and mounted onto slides. Immunohistochemical staining was performed by using Dako EnVision+ System-HRP Labelled Polumer (Dako Cytomation, Glostrup, Denmark) with anti-NGF antibodies (1:250; sc-548, Santa-Cruz, Heidelberg, Germany). An antigen retrieval was performed in the regent of HistVTone at 70°C (Nacalai, Kyoto, Japan) for 20 min. Background activities were blocked with the blocking agent (Dako, Glostrup, Denmark) at room temperature for 1 h. Reaction products were visualized by Liquid DAB+ Substrate Chromogen System (Dako, Denmark).

Statistical analysis: The data were expressed as mean ± SEM and were analyzed using GraphPad Prism 6.0 statistical software (San Diego, CA). Statistical differences among

groups were determined by one-way ANOVA followed by Turkey's post-hoc test. A statistical comparison of mean values between two groups was performed using Mann-Whitney test with Bonferroni correction. P values less than 0.05 were considered to be statistically significant.

Results

Nociceptive behavior: The number of licking behavior events in the colitis-vehicle group was significantly higher than that in the control group, whereas there was no significant difference in licking behavior score compared to other 3 groups including the colitis-OND group (Figure 1A). In contrast, the number of freezing events in the colitis-vehicle group was higher than that in other groups. These results indicate that the increase of freezing events, but not licking events, after colitis was prevented in the colitis-OND group to the same level as in sham groups (Figure 1B).

Cystometry: There was no significant difference in ICIs among groups before 0.1 % AA infusion (Figure 2 A). The reduction rate of ICIs after AA infusion in the colitis-vehicle group was significantly greater than those in colitis-OND and sham-vehicle groups (one-way ANOVA followed by Turkey's post-hoc test) and those

201 in control and sham-OND groups (Mann-Whitney test) (Figure 2 B).

202

203 **Quantitative mRNA analysis of NGF in the bladder mucosa and detrusor:** The
204 mRNA expression of NGF in the bladder mucosa in the colitis-vehicle group was
205 significantly higher compared to the colitis-OND group (one-way ANOVA followed by
206 Turkey's post-hoc test) (Figure 3A). In addition, there was a tendency of increased
207 NGF mRNA expression in the detrusor of colitis-vehicle rats vs. saline-vehicle rats
208 although the difference was not significant (one-way ANOVA), and no reduction was
209 seen in the detrusor NGF expression of colitis-OND rats compared to colitis-vehicle rats
210 (Figure 3B).

211

212 **NGF protein levels in the bladder mucosa and detrusor:** The protein level of NGF
213 in the bladder mucosa in the colitis-vehicle group was significantly higher than those in
214 control, colitis-OND and sham-OND groups (Figure 4A). There was no significant
215 difference in NGF protein levels of the detrusor among groups (Figure 4B).

216

217 **Immunohistochemistry of NGF expression:** Immunohistochemical staining of the
218 bladder for NGF showed a high level of positive staining in the bladder urothelial layer

of the colitis-vehicle group in contrast to faint staining in control and colitis-OND groups (Figure 5 b, a and c, respectively). The positive NGF staining in the detrusor layer was seen in control, colitis-vehicle and colitis-OND groups at similar levels (Figure 5).

Discussion

The results of our study demonstrated that: (1) rats with TNBS-induced colitis exhibited enhanced bladder pain sensitivity and bladder overactivity in response to nociceptive bladder stimuli using intravesical infusion of RTX and AA, respectively, (2) TNBS-induced colitis increased NGF expression in the bladder mucosa and (3) intravesical instillation of liposomes with antisense OND targeting NGF ameliorated bladder pain behavior and bladder overactivity in association with a reduction of NGF overexpression in the bladder mucosa.

This study confirmed our previous findings that freezing and licking behaviors, which predominantly correspond to bladder and urethral pain induced by activation of bladder and urethral afferent pathways, respectively [12-14], are enhanced after colitis (i.e., enhanced pain behavior in the colitis-saline group vs. the control group) [6]. We also further demonstrated that colitis induces bladder overactivity as evidenced by the

237 significantly larger reduction in ICIs after AA stimulation in the colitis-saline group vs.
238 the control or saline-vehicle group. Because, in our previous study, increased
239 myeloperoxidase activity, which correlates with tissue neutrophil infiltration, was found
240 in the colon, but not in the bladder or urethra, following TNBS-induced colitis [6], it is
241 assumed that increased bladder pain sensitivity in rats with TNBS-induced colitis is
242 produced by the indirect mechanism in the lower urinary tract other than inflammatory
243 changes directly elicited by TNBS administered into the colon.

244 Previous studies reported that pelvic organ cross sensitization is induced by
245 activation of dichotomized afferents innervating different pelvic organs and that
246 activation of nociceptive C-fiber afferents in one organ (e.g., colon) could sensitize
247 afferent pathways in another organ (e.g., bladder) to release neuropeptides such as
248 substance P that trigger neurogenic inflammation and mast cell activation[6, 15, 16]. This
249 study further demonstrated that the colon-to-bladder cross sensitization after colitis also
250 induces overexpression of NGF in the bladder mucosa including the urothelial layer
251 (Figs. 3 & 4) and that urothelially expressed NGF after colitis is a key mediator that
252 increases bladder pain sensitivity because intravesical treatment with liposome and
253 NGF antisense conjugates significantly reduces bladder pain behavior (i.e., freezing
254 behavior) (Fig. 1) and bladder overactivity induced by nociceptive stimuli in the bladder

(Fig. 2). Previous clinical studies showed that urinary NGF could be a useful biomarker for the diagnosis of BPS/IC [9, 10]. NGF has also been highlighted as a chemical mediator in experimental animal models of bladder hypersensitivity [17-19], and chronic administration of NGF into the bladder wall or into the spinal cord induced bladder overactivity and increased excitability of bladder afferent neurons in rats [20-22]. Thus, NGF overexpression in the bladder mucosa induced by the colon-to-bladder cross sensitization following colitis is likely to stimulate bladder afferent pathways to enhance bladder pain sensitivity.

In clinical studies, monoclonal NGF antibody was systemically administered to treat the symptoms in patients with BPS/IC or other chronic pain syndromes [23]. In these studies, systemic adverse events including headache, hyperesthesia, abnormal peripheral sensation and dizziness have been reported as obstacles for the systemic NGF antibody therapy to become a feasible treatment of pain, although some studies showed therapeutic effects on pain symptoms [23]. Thus, the intravesical therapy targeting NGF expressed in the bladder could be an alternate option for the treatment of BPS/IC. We have previously shown that liposomal application is necessary to deliver NGF antisense OND to the bladder urothelium to suppress NGF overexpression and bladder overactivity in a rat model of acute cystitis [11]. In addition, Chuang et al. reported that

intravesical application of empty liposomes significantly decreased painful and urinary urgency symptoms in 24 BPS/IC patients[24]. Recent clinical studies have also demonstrated that the intravesical treatment using liposome-encapsulated onaboturinum toxin-A significantly improved symptoms in patients with overactive bladder without severe adverse events [25, 26]. Therefore, local suppression of NGF in the bladder using intravesical liposome-based delivery techniques could be an attractive approach for the treatment of bladder symptoms, while avoiding systemic side effects, in patients with BPS/IC and IBS.

Conclusion

We showed that intravesical treatment with liposome and NGF antisense conjugates reduced NGF overexpression in the bladder and attenuated bladder hypersensitivity in a rat colitis model. Therefore, the liposome-based antisense treatment targeting NGF in the bladder could be a new, effective modality for the treatment of bladder pain in CPPS patients, in whom the cross-sensitization mechanism is involved in the emergence of overlapping symptoms from different pelvic organs.

Acknowledgement

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377

378 **Figure legends**

379 **Figure1**. The number of nociceptive behavior events, licking (A) and freezing (B),
380 induced by intravesical injection of resiniferatoxin (RTx).

381 (A) The number of licking behavior events in the colitis-saline group was significantly
382 higher than that in the control group, whereas there was no difference in licking score
383 compared to other 3 groups. (B) Freezing behavior events in the colitis-saline group
384 were increased significantly, compared to controls; however, the increase of freezing
385 events was significantly decreased in the colitis-OND group to the same level as in
386 sham-groups. . *: $p < 0.05$, **; $p < 0.01$ (compared to each group; one-way ANOVA
387 followed by Turkey's post-hoc test)

388

389 **Figure2.** Effects of intravesical infusion with acetic acid (AA) on intercontraction
390 intervals (ICIs) in cystometry. (A) ICIs before AA infusion. (B) The reduction ratio of
391 ICIs after AA infusion to ICIs before AA infusion.

392 There was no significant difference in ICIs before AA infusion among groups (A). The
393 ICI reduction rate after AA infusion in the colitis-saline group was significantly higher
394 than that in the colitis-OND group (B). * $P < 0.05$ (one-way ANOVA followed by
395 Turkey's post-hoc test). # $P < 0.05$ (compared to the colitis-vehicle group;

396 Mann-Whitney test).

397

398 **Figure 3.** mRNA expression of NGF in the bladder mucosa (A) and detrusor (B) 10
399 days after intracolonic injection of TNBS or vehicle (50 % ethanol). * $P < 0.05$
400 (one-way ANOVA followed by Turkey's post-hoc test).

401

402 **Figure 4.** Protein expression of NGF in the bladder mucosa (A) and detrusor (B) 10
403 days after intracolonic injection of TNBS or vehicle (50 % ethanol). * $P < 0.05$
404 (compared to each group; one-way ANOVA followed by Turkey's post-hoc test).

405

406 **Figure 5.** Photomicrographs of NGF staining in the rat bladder. There was increased
407 positive staining for NGF in the urothelial layer of the colitis-saline group (B,b), in
408 contrast to faint staining in the mucosal layer of the bladder from the control group
409 (A,a) and the colitis-OND group (C,c). Magnification of pictures (A,B,C) and (a,b,c) is
410 ×100 and ×400, respectively. The magnified areas of picture A, B and C, which are
411 shown in pictures a, b and c, respectively, are indicated by rectangular boxes.

Key of Definitions for Abbreviations (only include abbreviations used 3 times or more in manuscript)

Abbreviations

NGF = nerve growth factor

CPPS = chronic pelvic pain syndrome

OND = oligonucleotide

BPS/IC = bladder pain syndrome/interstitial cystitis

IBS = irritable bowel syndrome

TNBS = intracolonic 2,4,6,trinitrobenzen sulfonic acid

Figure
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Figure 1

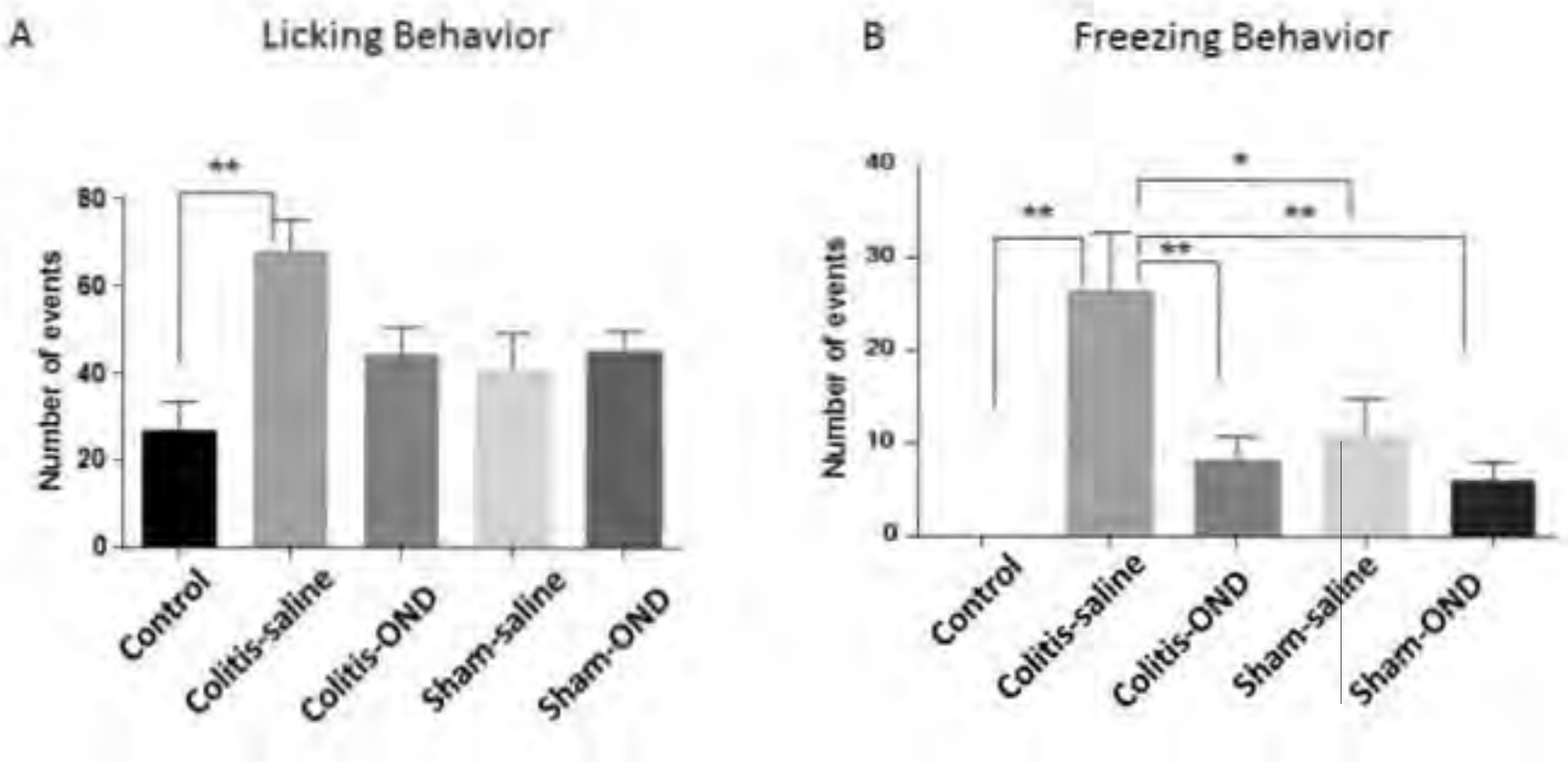

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Figure 2

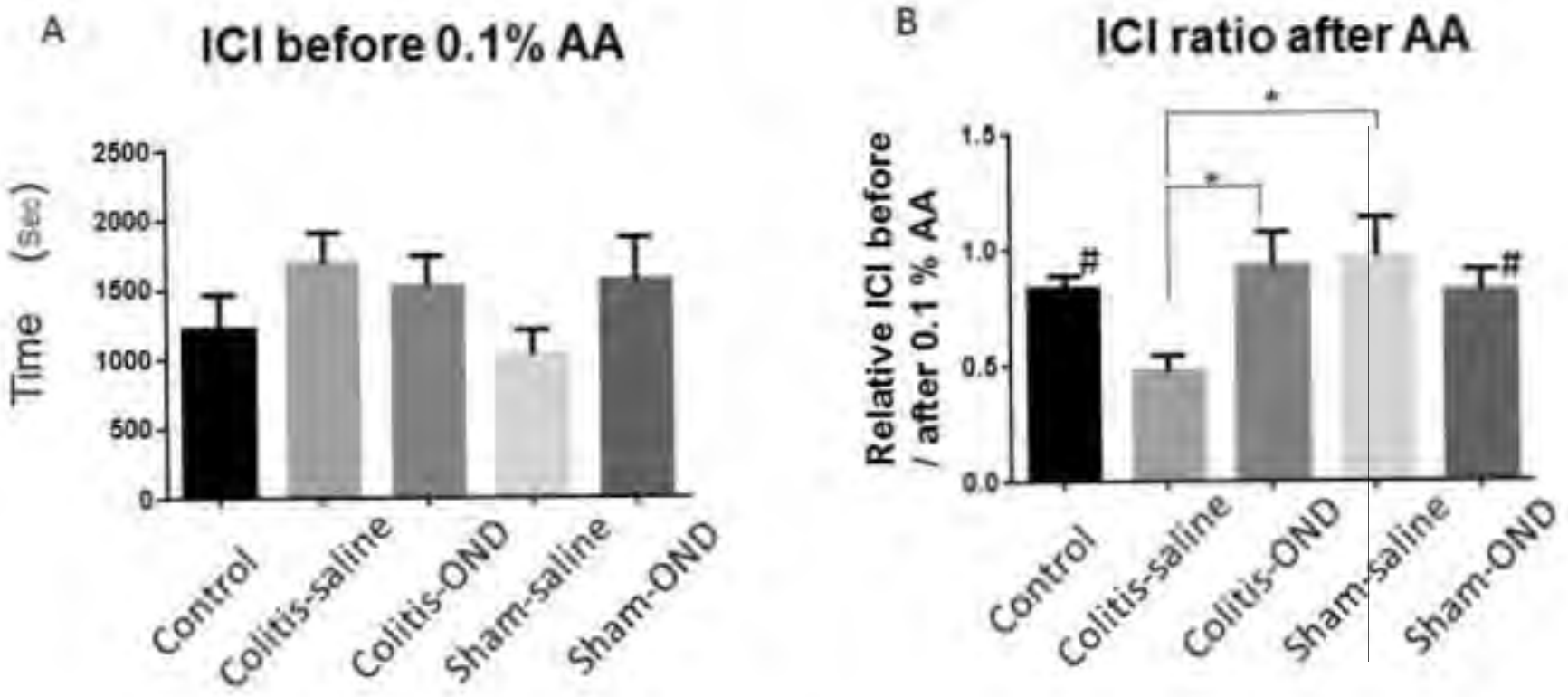

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Figure 3

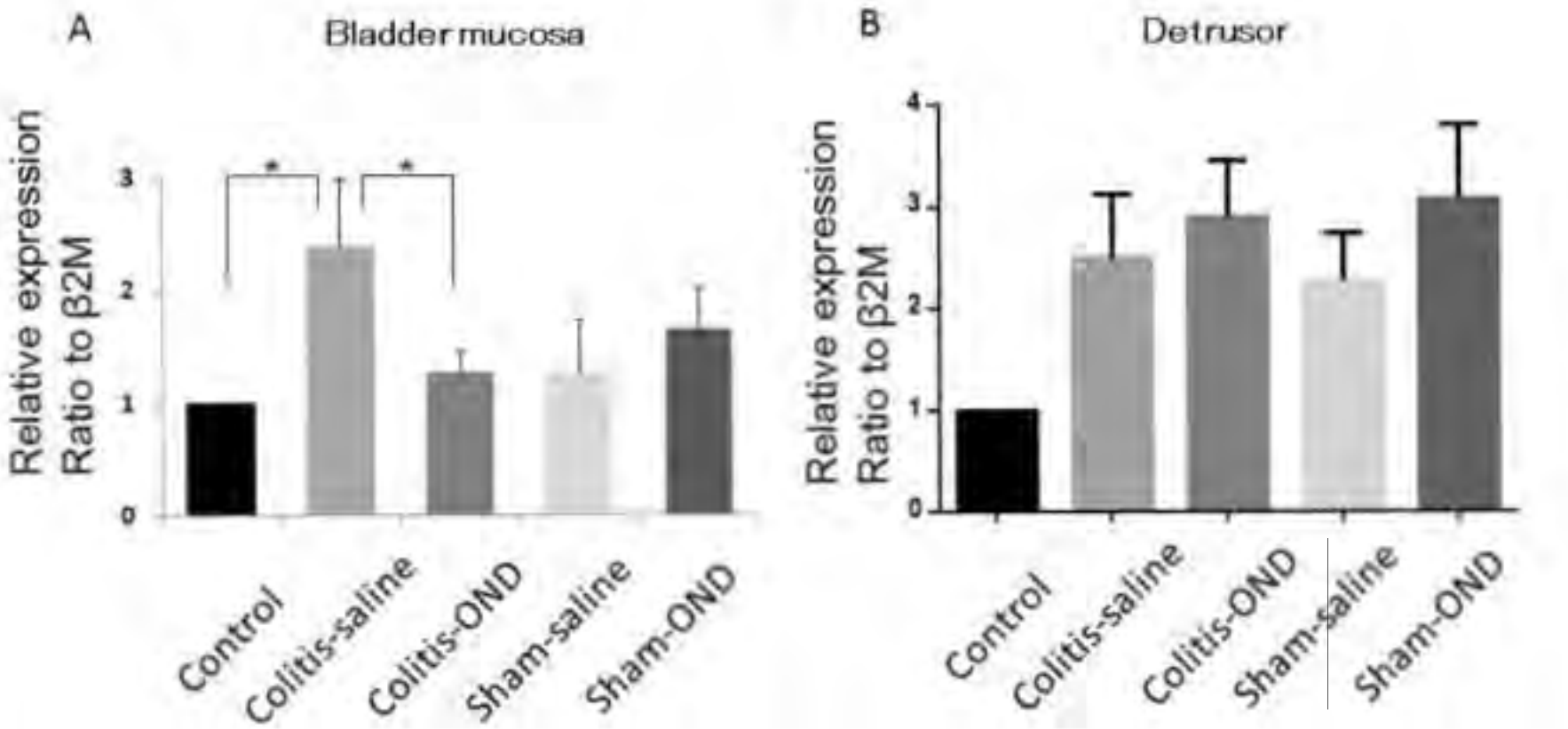

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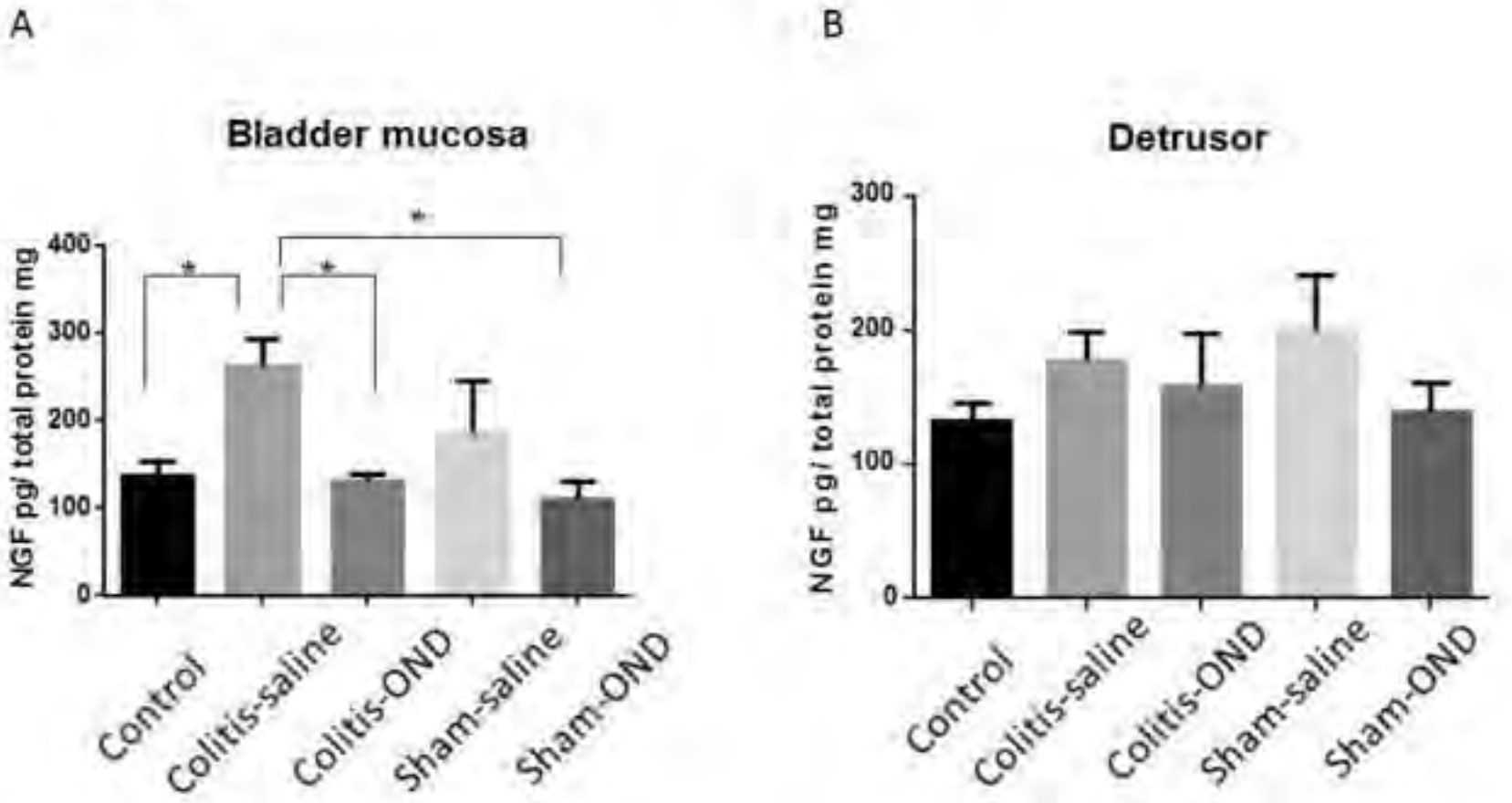
Figure 4



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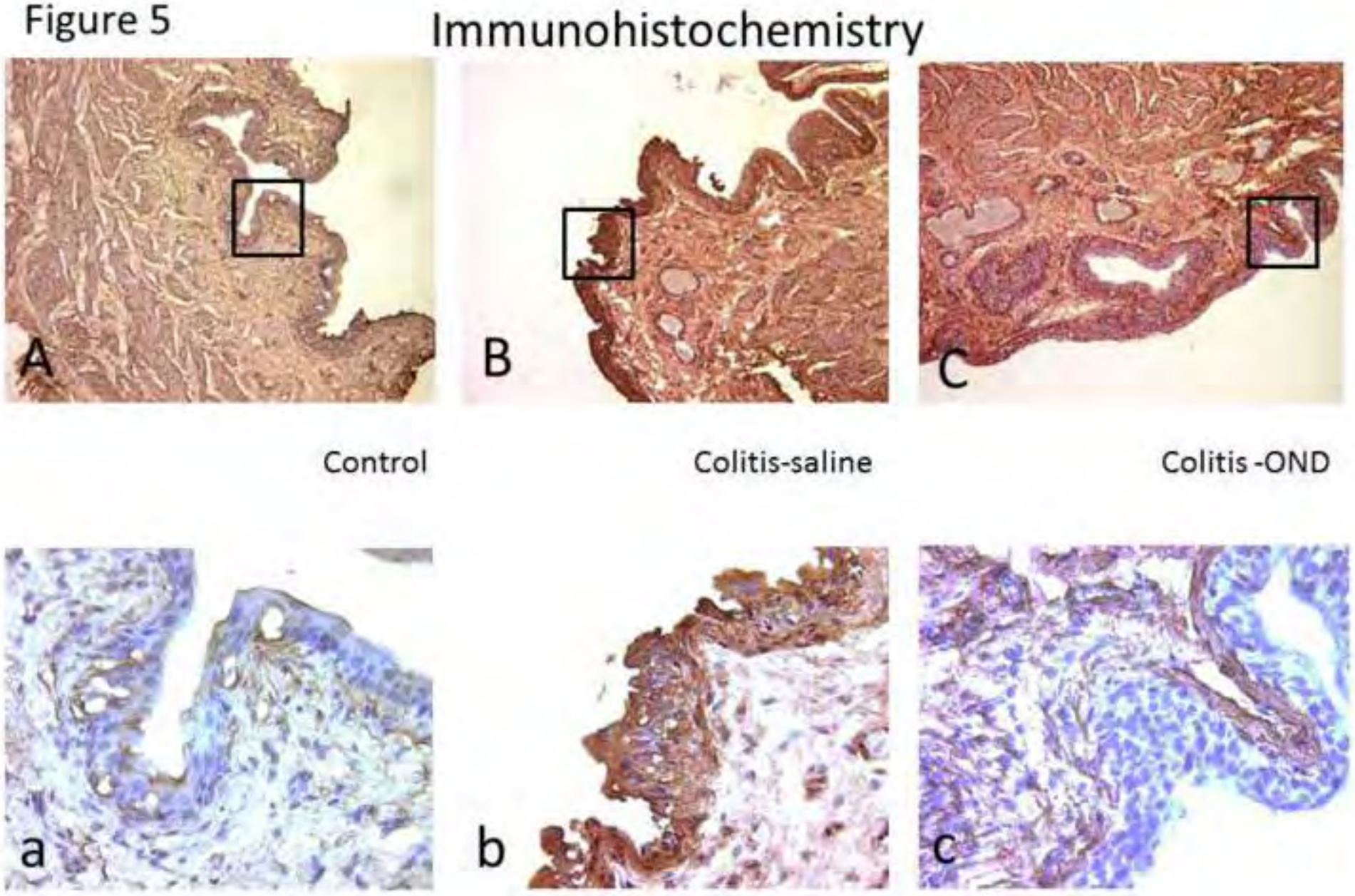

3095

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LIPOSOME-BASED INTRAVESICAL THERAPY TARGETING NERVE GROWTH FACTOR (NGF) AMELIORATES BLADDER HYPERSENSITIVITY IN RATS

Background: It has recently been proposed that pelvic organ "cross sensitization" contributes to the clinically overlapping symptoms in irritable bowel syndrome (IBS) and interstitial cystitis/bladder pain syndrome (IC/BPS). Overexpression of nerve growth factor (NGF) in the bladder is thought to be one of the key factors in the symptom development in IC/BPS patients. We hereby explore whether bladder hypersensitivity induced by experimental colitis and NGF overexpression in the bladder are induced after colitis and whether intravesical administration of NGF antisense-liposome solution (liposomal-OND) treatment can suppress bladder hypersensitivity and NGF expression in a rat model with experimental colitis.

Methods: Adult female SD rats were used; (a) control group, (b) colitis-OND group (intracolonic 2,4,6,trinitrobenzen sulfonic acid [TNBS] enema and intravesical liposomal OND were given) , (c) colitis-saline group (intracolonic TNBS and intravesical saline were given), (d) sham-OND group (intravesical liposomal OND was given without colitis) and (e) sham-saline group (intravesical saline was given without colitis). Intravesical administration of liposomal-OND: Under isoflurane anesthesia, 0.2ml of either liposomal-OND or saline was instilled to the bladder through an inserted urethral catheter. Experimental colitis model: Twenty-four hours after instillation of liposomal-OND or saline and fasting, colitis was induced by the enema of 30mg TNBS dissolved in 50% ethanol through a polyethylene catheter inserted 8 cm proximal to the anus in a head-down position. Ten days after liposomal-OND or saline injection, animals were subjected to either in vivo studies or bladder tissue removal. Assessment included nociceptive behaviour testing in response to 1 -min intravesical administration of resiniferatoxin (RTX), awake cyctometry with saline followed by 0.1% acetic acid (AA) were continuously infused to evaluate changes in intracontraction intervals (ICIs) in conscious rats and immunohistochemistry with NGF antibody and qPCR molecular analysis of NGF.

Results: In the colitis-saline group, the score of freezing behaviour was significantly higher than that of all other groups including the colitis-OND group. The score of licking behavior in the colitis-saline group was significantly higher than in the control group and tended to higher compared to other 3 groups without significant differences. ICIs before intravesical AA stimulation were not different among groups; however, the ICI reduction rate after AA instillation into the bladder was significantly higher in the colitis-saline group than that in the colitis-OND group. There was increased immunoreactivity of NGF in the bladder mucosa in the colitis-saline group, whereas there was only faint staining in the control and colitis-OND groups. The mRNA expression of NGF in the colitis-saline group was significantly increased in the mucosa compared to control and colitis OND groups. In addition the protein level of NGF in the mucosa was also higher in the colitis-saline group compared to other groups.

Conclusion: The liposome-based antisense treatment targeting NGF in the bladder could be a new, effective modality for the treatment of bladder pain and overactivity in chronic pelvic pain syndrome patients including those with IC/BPS, in whom the pelvic organ "cross sensitization" mechanism is involved in overlapping symptoms from different pelvic organs.

234

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THE EFFECT OF INTRAVESICAL LIPOSOME-BASED NGF ANTISENSE THERAPY ON BLADDER OVERACTIVITY AND NOCICEPTION IN A RAT MODEL OF CYSTITIS INDUCED BY HYDROGEN PEROXIDE

Hypothesis / aims of study

Nerve growth factor (NGF) has been proposed to be an important mediator for inducing hyperexcitability of afferent pathways that contributes to pain and storage symptoms of interstitial cystitis/bladder pain syndrome (IC/BPS). A previous clinical study indicated that systemic application of NGF monoclonal antibody significantly reduced pain/urgency in IC/BPS patients, but its systemic adverse events such as paresthesia and hyperesthesia were a critical issue [1]. Therefore, the site-specific reduction of NGF would be desirable to reduce the intrinsic toxicity from systemic blockade of NGF. In this regard, our previous study demonstrated that intravesical liposome-based NGF antisense therapy significantly improved bladder overactivity in a rat model of acute cystitis induced by intravesical application of acetic acid [2]. However, it still remains unclear whether local NGF antisense therapy has a chronic effect. Therefore, we investigated the effect of intravesical liposome-based NGF antisense therapy on bladder overactivity and nociceptive behaviour in a rat model of chronic cystitis induced by hydrogen peroxide (HP).

Study design, materials and methods

Adult Sprague-Dawley female rats were used according to the experimental protocol approved by the Institutional Animal Care and Use Committee.

1) 1.5% HP was administered into the bladder on day 0. Liposomes conjugated with NGF antisense tagged with TYE563 fluorescent protein was given into the bladder on day 2. The expression of TYE563 was observed under a fluorescent microscope on day 3.

2) In another set of experiments, rats were divided into 4 groups: a) saline + vehicle (SV) group、b) saline + liposome-NGF antisense (SN) group, c) 1.5% HP + vehicle (HV) group、d) 1.5% HP + liposome-NGF antisense (HN) group. Saline or 1.5% HP was administered into the bladder on day 0. Each rat was treated with intravesical vehicle or NGF antisense administration on day 2. Continuous cystometry (CMG) was performed in an awake condition on day 7.

3) Nociceptive behaviours induced by 1-min intravesical instillation of resiniferatoxin (TRPV1 agonist, RTx) such as licking behaviour (lower abdominal licking) and freezing behaviour (motionless head-turning towards lower abdomen) was observed on day 7.

4) After rats were intracardially perfused with cold heparinised-saline, followed by 4% paraformaldehyde, the bladder and L6 DRG were removed on day 7. Haematoxylin-Eosin (HE) staining as well as immunofluorescence staining for NGF were performed.

5) Bladder tissue was harvested on day 7. The mRNA expression of NGF, TRPV1 and brain-derived neurotrophic factor (BDNF) was measured by RT-PCR.

Results

1) TYE563 expression was observed in the bladder urothelium 1 day after intravesical application of TYE563- tagged liposome-NGF antisense conjugates.

2) In CMG, the HV group showed significantly (p=0.001) shorter intercontraction intervals (ICI) than the SV group (ICI: 442±64 and 889±73 sec, respectively). The HN group showed significantly (p=0.007) longer ICI than the HV group (ICI: 711±46 and 442±64, respectively). There was no significant difference in ICI between SV and SN groups (p=0.56, Fig.1.)

3) There were no significant differences in licking behaviour among 4 groups. However, the number of freezing events was significantly (p=0.002) higher in the HV group than in the SV group (18±2 and 6±1 events for 15 min after RTx treatment, respectively). The HN group showed the significantly (p=0.04) less number of freezing events than the HV group (9±3 and 18±2 events, respectively.) There was no significant difference in the number of freezing events between the SV and SN group (Fig.2.)

4) HE staining showed that there were substantial infiltration of the inflammatory cells, submucosal bleeding, and detrusor hypertrophy in the bladder wall in the HV group compared with SV group, which were alleviated in the HN group. Immunofluorescence staining indicated that the expression of NGF protein in the mucosa (p=0.02) and L6 DRG (p=0.01) was significantly higher in the HV group than in the SV group. On the other hand, the HN group indicated significantly lower expression of NGF protein in the mucosa (p=0.002) and L6 DRG (p=0.01) than the HV group (Fig.3 and 4.) In the detrusor, there was no significant difference in the NGF expression among 4 groups.

5) In RT-PCR, the HV group showed the significantly higher expression of NGF (p=0.001) and TRPV1 (p=0.03) mRNA in the mucosa than the SV group (p=0.001), whereas the HN group showed the significantly lower expression of NGF (p=0.007) and TRPV1 (p=0.02) than the HV group. There was no significant difference in BDNF expression in the mucosa among 4 groups. In the detrusor, the HV group showed the significantly higher expression of NGF (p=0.03) and BDNF (p=0.02) mRNA than the SV group while HN group did not show significant differences compared to the HV group. There was no significant difference in the expression of TRPV1 in the detrusor among 4 groups.

Fig.1

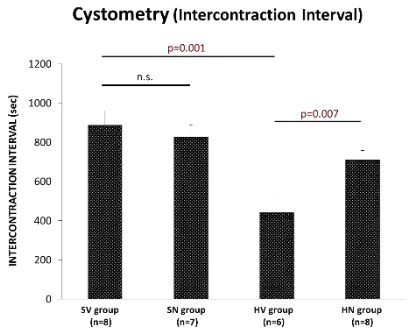


Fig.2

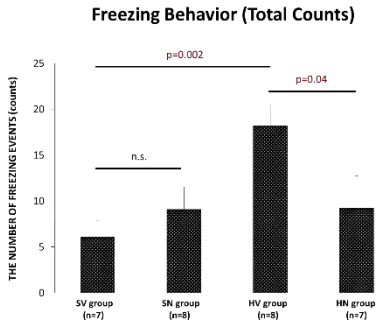


Fig.3

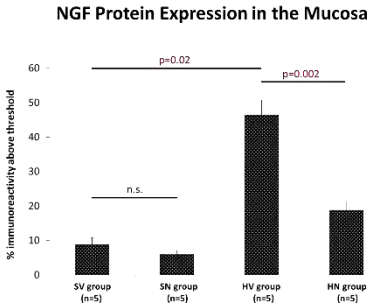
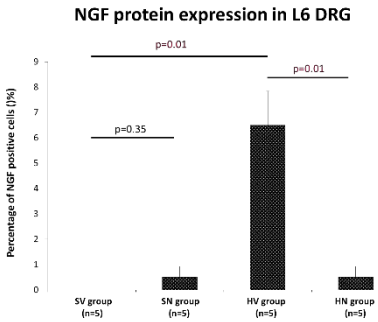


Fig.4



Interpretation of results

These results indicate that; (1) intravesical hydrogen peroxide instillation, which induces bladder inflammation up to 7 days after the instillation, elicit frequent urination shown by reduced ICI, and enhanced bladder pain sensitivity shown by increased freezing behaviour, which are associated with increased expression of NGF mRNA and protein as well as TRPV1 mRNA in the bladder mucosa and bladder afferent pathways and (2) intravesical liposome-based NGF antisense therapy induces a reduction in the NGF mRNA and protein expression in the bladder mucosa and bladder afferent pathways, which results in the improvement of bladder pain behaviour and frequent urination induced by hydrogen peroxide-induced chronic cystitis.

Concluding message

Intravesical liposome-based NGF antisense therapy could be a novel treatment that can avoid systemic adverse events for hypersensitive bladder disorders such as IC/BPS, in which NGF has been implicated as an important mediator for inducing afferent sensitization.

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Disclosures

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