Autonomic dysregulation in a rat model of interstitial cystitis and inhibitory spinal mechanism

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ABSTRACT

Autonomic dysregulation in a rat model of interstitial cystitis and inhibitory spinal mechanism

Background :

Interstitial cystitis/bladder pain syndrome (IC/BPS), a chronic pain disorder affecting the urinary bladder, is characterized by the presence of symptoms such as urinary frequency, urgency, and pain. The etiology of IC/BPS is multifactorial, with changes such as thinning of the urothelium and impaired barrier function being frequently observed. Altered autonomic nervous system (ANS) activity, including elevated urinary norepinephrine (NE) levels and a shift toward sympathetic dominance, has been linked to increased sympathetic activity in patients with IC/BPS. Significant autonomic responses correlated with symptom severity, such as elevated blood pressure and pulse rate, are observed in these patients during hydrodistention (HD). Therefore, the present study aimed to replicate the exaggerated autonomic responses observed in patients with IC/BPS in rat models of acute and chronic IC/BPS to elucidate the underlying mechanisms. In addition, the therapeutic potential of α-1D adrenergic receptor antagonists (naftopidil) and glycine transporter type 2 inhibitors (ALX-1393) to alleviate autonomic dysfunction was also explored to determine their viability as treatment targets for IC/BPS in the future.

Methods :

Two distinct animal models of acute and chronic cystitis were created to model IC/BPS in rats. Cystitis was induced using cyclophosphamide (CYP) in the acute model. The increase in blood pressure during HD was determined according to the CYP dose (50, 100, and 200 mg/kg) and the period post-administration (day 3 and day 7 post-administration). Inflammation was induced over time through the intravesical instillation of protamine sulfate (PS), followed by lipopolysaccharide (LPS), in the chronic model. The changes observed at 1, 3, and 5 weeks post-instillation were examined in different groups. Blood pressure was monitored during HD. Bladder function was assessed using cystometry.

Protein expression analysis were performed using western blotting targeting key inflammatory

markers, such as TNF-α, IL-6, and nerve growth factor (NGF). Histological examinations was performed using immunofluorescence staining of the bladder tissues. C-fiber involvement was assessed through pre-treatment with capsaicin. Intrathecal injections were administered to evaluate the effects of glycine transporter type 2 inhibitors (ALX-1393) and naftopidil, an α-1D/A adrenergic receptor antagonist, in both models. Naftopidil was also administered intravenously.

Results :

Significant changes in blood pressure were observed during HD in the acute and chronic models of IC/BPS. An increase of 19.6 ± 9.5 mmHg, the most pronounced blood pressure change, was observed on the third day post-administration in the 100 mg/kg dosage group in the acute model ($p < 0.001$). No significant changes were observed in the control or other CYP dosage groups (50, 200 mg/kg). An increase in blood pressure during HD was observed at one, three, and five weeks post-treatment in all groups treated with PS and LPS in the chronic model. The mean blood pressure increased from 89.2 ± 16.5 mmHg at baseline to 115.4 ± 18.6 mmHg during HD in the one-week group ($p < 0.001$). Pre-treatment with capsaicin suppressed the increase in blood pressure during HD in both models.

Immunofluorescence analysis revealed a significant increase in VCAM-1 and IL-6 expression in the bladder epithelium, indicating inflammation. Western blotting revealed a dose-dependent increase in the VCAM-1, TNF-α, and IL-6 levels in the bladder tissues following CYP treatment. NGF expression peaked in the bladder three days after the administration of 100 mg/kg of CYP, correlating with spinal c-Fos expression and exaggerated changes in blood pressure. The levels of all inflammatory markers, including NGF, were elevated in the chronic model.

Intrathecal administration of ALX-1393 induced a significant decrease in blood pressure elevation during HD in both models. Intrathecal injection of ALX-1393 significantly prolonged intercontraction interval (ICI), indicating improved bladder function in both acute and chronic models, while saline injection showed no significant effects on any cystometric parameters.

Naftopidil administered intrathecally and intravenously in the chronic model suppressed the increase in blood pressure during HD. Intravenous administration of naftopidil significantly increased the ICI, while no notable changes were observed in the control group.

Conclusion :

The present study demonstrated that the heightened ANS responses observed during HD in patients with IC/BPS were effectively replicated in the acute and chronic animal models. These responses exhibited significant associations with NGF and c-Fos expression in the bladder and spinal cord, respectively. Autonomic dysfunction improved following the intrathecal and intravenous administration of naftopidil. Similarly, intrathecal administration of ALX-1393 mitigated these abnormal responses, leading to enhanced bladder function. These findings confirm the underlying pathological mechanisms of the ANS in patients with IC/BPS, emphasizing its potential as a therapeutic target for future interventions.

Keywords: interstitial cystitis, bladder pain syndrome, autonomic nervous system, glycine, adrenergic receptor

I. INTRODUCTION

Interstitial cystitis/bladder pain syndrome (IC/BPS), a complex chronic pain disorder affecting the urinary bladder, is characterized by the presence of symptoms such as urinary frequency, urgency, and pain. The American Urological Association defines IC/BPS as an unpleasant sensation in the bladder, such as pain, pressure, and discomfort, persisting for >6 weeks that is accompanied by lower urinary tract symptoms in the absence of infection or other identifiable causes.[1] The etiology of IC/BPS is multifactorial. Several findings, such as thinning of the urothelium, abnormal cell-to-cell adhesion, and impaired barrier function, have been observed in the bladders of these patients.[2] Nevertheless, the exact etiology of IC/BPS remains unclear.

The autonomic nervous system (ANS) activity is altered in individuals with IC/BPS.[3,4] A previous study revealed that the urinary norepinephrine (NE) levels in patients with IC/BPS were higher than those observed in the controls; notably, these levels did not decrease with treatment of the bladder mucosa.[5] Another study revealed decreased vagal activity and a shift toward sympathetic nervous system dominance, evaluated through heart rate variability, in patients with IC/BPS.[6] These findings indicate that sympathetic activity is increased in patients with IC/BPS.

A pronounced autonomic response, including elevated blood pressure (BP) and pulse rate (PR), has been observed in patients with IC/BPS during hydrodistention (HD).[7,8] This exaggerated autonomic response during HD is observed in nearly all patients with IC/BPS, regardless of typical cystoscopic findings.[9] It is hypothesized that heightened sympathetic activity may drive this response, with the increase in BP being directly correlated with symptom severity. No changes were noted under spinal anesthesia; thus, these autonomic responses may be mediated through the spinal cord.

Blockade of the spinal α-1D adrenergic receptors resulted in symptom improvement, including alleviation of pain and improvement of bladder function, in an animal model of IC/BPS.[10] These improvements were accompanied by changes in glycine concentration within the spinal cord,[11] indicating a relationship between IC/BPS and the levels of glycine and adrenergic α-1D in the spinal cord. The direct injection of a glycine transporter type 2 inhibitor into the spinal canal of an animal model of IC/BPS led to improvements in both pain response and bladder function in a previous study.[12] These findings indicate that the glycine inhibitory pathway and α-1D adrenergic receptors in the spinal cord are critical targets for therapeutic intervention.

Therefore, this study aimed to evaluate whether the exaggerated autonomic response observed in patients with IC/BPS during HD could be replicated in rat models of acute and chronic IC/BPS. This modeling process sought to elucidate the mechanisms underlying this response. Furthermore, α-1D adrenergic receptor antagonists and glycine transporter type 2 inhibitors, which have emerged as promising therapeutic targets, were used as potential agents to mitigate autonomic nervous system dysfunction to confirm the viability of these agents as therapeutic targets for the treatment of IC/BPS in the future.

2. MATERIALS AND METHODS

Acute and chronic IC/BPS models were established in rats using cyclophosphamide (CYP) and protamine sulfate/lipopolysaccharide (PS-LPS), respectively, during the initial phase of the study. The models were assessed to determine whether the autonomic dysfunction observed during HD in patients with IC/BPS was observed in both models (Figure 1). The concentration of drugs and the duration of treatment required to induce these responses were determined. The histological, molecular, and biological characteristics of the bladder, along with c-Fos expression in the spinal cord, were examined to identify the underlying pathological mechanisms. An inhibitory treatment was commenced to mitigate autonomic dysfunction during HD during the subsequent phase of the study. Intrathecal administration of the GlyT2 inhibitor (ALX-1393) was commenced in the acute model, whereas intrathecal administration of the GlyT2 inhibitor and intrathecal and intravenous administration of the α-1 adrenergic receptor antagonist (naftopidil) were commenced in the chronic model. The ability of these agents to suppress autonomic dysfunction during HD was assessed. Cystometry was performed to ascertain whether treatment protocols that improved autonomic dysfunction can also enhance bladder function.

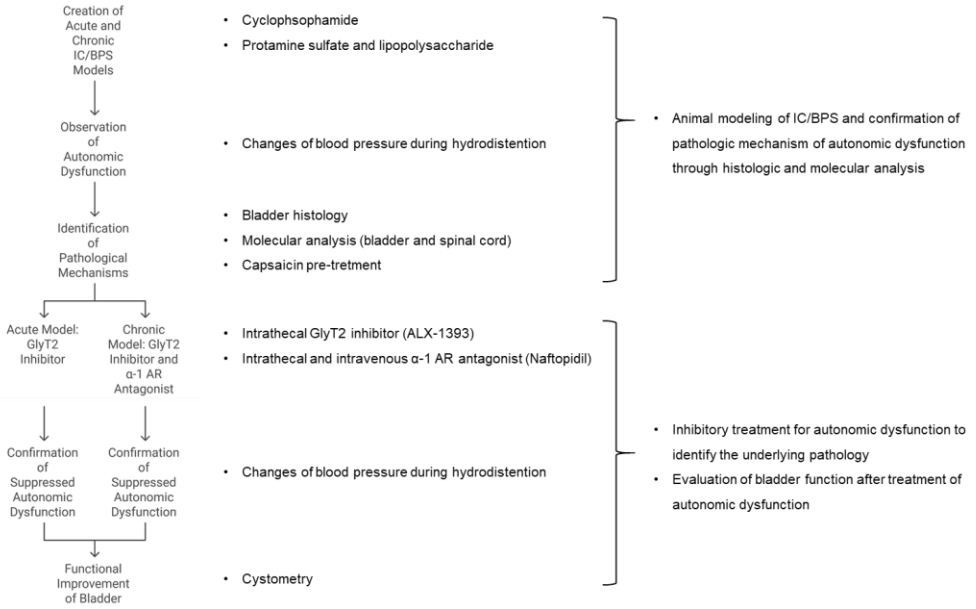

Figure 1. Overall steps and objectives of experiments

2.1 Animal modeling of interstitial cystitis

All animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (Seoul, Korea). All procedures were conducted in accordance with the relevant guidelines and regulations. Six-week-old female Sprague-Dawley rats weighing 200 ± 20 g that were obtained from Orient Bio (Seongnam, Korea) were used to establish two distinct animal models of interstitial cystitis (IC). The rats in the control group did not receive any treatment. A sterile polyethylene catheter (PE-50) was inserted into the bladder of the rats through the urethra for intravesical instillation of protamine sulfate and lipopolysaccharide in the experimental groups.

2.1.1 Acute model of IC/BPS (CYP)

The baseline dose of CYP for inducing cystitis was set as 100 mg/kg based on the findings of previous studies.[13] The rats were divided into the control, three-day, and one-week groups (seven rats per group) to determine the optimal timing post-CYP injection. The changes in BP during HD were most pronounced on the third day post-CYP injection.

Twenty-eight rats were assigned to four groups (control, CYP50, CYP100, and CYP200 mg/kg, with seven rats in each group) after an acclimatization period of one week to determine the appropriate dose of CYP. CYP dissolved in normal saline (40 mg/mL) was also administered intraperitoneally. HD was induced three days post-CYP injection in each group.

2.1.2 Chronic model of IC/BPS (PS and LPS)

PS was administered at a dose of 10 mg/mL to the urinary bladder for 30 minutes. Subsequently, LPS was instilled at a concentration of 750 μg/mL for 45 minutes. The rats were divided into the control, one-week, three-week, and five-week groups to examine the results over time. HD was induced, and the animals were sacrificed one week after the instillation of LPS in the one-week group. The instillation of PS and LPS was performed once a week for three and five weeks in the three-week and five-week groups, respectively (seven rats per group).[14]

2.2. HD and blood pressure measurement

A mixture of Zoletil and Rompun (1 mL/kg) was used to induce anesthesia, and the bladder was surgically exposed. A PE-50 tube containing saline with its distal end flared by heat was inserted into the bladder through the dome. HD was induced for 1 min during each session (totaling three sessions) at an intravesical pressure of 140–150 mmHg following secure catheterization of the bladder. The changes in the BP during HD were measured using intra-arterial catheters placed in the carotid artery.[15]

2.3. Cystometry

A lower midline abdominal incision was made under anesthesia, and a PE-50 tube (Clay Adams, Parsippany, NJ, USA) with its distal end flared by heat was inserted into the bladder through the dome as a cystostomy catheter. The animals were gently restrained in a cage after recovery from anesthesia. The cystostomy catheter was connected to a pressure transducer and syringe pump using a three-way stopcock. The bladder was filled with saline at a rate of 0.04 mL/min to elicit repetitive voiding to record continuous cystometrograms. The intercontraction intervals (ICI), baseline pressure, time to the first non-voiding contraction (NVC), threshold pressure (TP; bladder pressure at the onset of detrusor contraction for micturition), maximal micturition pressure (MP), bladder compliance, and post-void residual pressure (PVR) were determined after rhythmic, stable bladder contractions were achieved post-recovery. PVR was determined with the help of a bladder catheter using gravity, followed by manual compression of the abdominal wall. The entire bladder above the ureteral orifice was dissected post-cystometric evaluation. The bladders of normal rats were harvested as controls. The harvested bladders were frozen and stored at -80℃ until further processing.

2.4. Western blotting

Tissue samples were homogenized and lysed in a protein extraction solution buffer (Intron, Korea). The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine the protein concentration. Equal amounts of protein were separated using 8–12% SDS-PAGE and transferred onto a PVDF membrane. The membranes were incubated overnight at 4℃ with primary antibodies targeting TNF-α, IL-6, ERK, Phospho-ERK, AKT, Phospho-AKT, Vascular Cell Adhesion Protein 1 (VCAM-1), and NGF (Cell Signaling Technology) after blocking the non-specific signals with 5% skim milk. Subsequently, mouse anti-goat-horseradish peroxidase (HRP) and rabbit anti-mouse-HRP secondary antibodies (1:5000) were applied for 60 min at room temperature. The Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) was used for detection. Immunoreactive bands were quantified using Quantity One v4.52 software (Bio-Rad). The actin expression within the same sample was used to determine the relative protein expression levels. The optical density values obtained through densitometric analysis were expressed as arbitrary units. The results were presented as an n-fold increase over the control group values in arbitrary densitometric units. The spinal cord was harvested through dorsal laminectomy, and the L6–S1 segments were isolated. Western blotting of the spinal cord samples was performed to confirm the results obtained by targeting c-Fos.

2.5. Histologic examinations

The bladder tissue samples were fixed in 4% paraformaldehyde for 24 h at 4℃ for fluorescence microscopy. Frozen tissue sections of 12 μm thickness were incubated overnight with antibodies against VCAM-1 and IL-6 at 4℃. The sections were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA, USA) for 2 h at room temperature after rinsing with phosphate-buffered saline (PBS). The signals were visualized, and digital images were acquired using a confocal microscope (FV1000; Olympus, Tokyo, Japan).

2.6. Inhibitory treatment

2.6.1. Capsaicin pre-treatment

A total dose of 125 mg/kg of capsaicin dissolved in a vehicle containing 10% ethanol, 10% Tween 80, and 80% physiological saline at a concentration of 20 mg/mL was administered subcutaneously to seven rats.

2.6.2. Glycine transporter type 2 inhibitor (ALX-1393)

The abdomen was closed with a PE-50 catheter, and a partial laminectomy was performed at the level of the third lumbar vertebra. A PE-10 catheter was inserted into the subarachnoid space through a small opening in the dura mater, and its tip was advanced to the level of the sacral cord. The rats were gently restrained postoperatively and allowed to recover from anesthesia for 1 h.

ALX-1393, purchased from Sigma-Aldrich (St. Louis, MO), was administered intrathecally at a volume of 1 μL. Subsequently, 9 μL of saline was flushed using a polyethylene catheter inserted into the subarachnoid space at the L6–S1 level of the spinal cord.[16]

2.6.3. α-1D/A adrenergic receptor antagonist (naftopidil)

Naftopidil, dissolved in 0.1 M phosphate buffer, was administered intravenously (1 mg, approximately 4 mg/kg) or intrathecally (1 μM).[17]

2.7. Statistical Analysis

Quantitative data are presented as the means ± standard deviation. Inter-group differences were evaluated using unpaired Student's t-test and one-way analysis of variance (ANOVA), followed by Dunnett's T3 multiple comparison post-hoc test. The BP measurements obtained before and after HD were compared using the Paired Student's t-test. Statistical significance was set at $p < 0.05$. All statistical analyses were conducted using GraphPad Prism (version 5.01; GraphPad Inc., La Jolla, CA, USA).

3. RESULTS

3.1. Blood pressure during HD

3.1.1. Acute model of IC/BPS

The changes in BP during HD following the administration of 100 mg of CYP were assessed on the third day and at one week; however, significant changes in BP were observed only in the three-day group (mean change in BP: 19.6 ± 9.5 mmHg, $p < 0.001$) (Table 1). HD was performed on the third day post-CYP injection based on these results in the subsequent experiments aimed at determining the appropriate dosage of CYP.

BP was measured during HD on the third day after the administration of 50, 100, and 200 mg/kg of CYP. A significant increase in the systolic and diastolic BP was observed during HD in the 100 mg CYP group only (Δ SBP: 16.03 ± 6.86 mmHg, Δ DBP: 14.03 ± 4.97 mmHg, $p < 0.001$). No changes in the BP were observed in the control and other groups (Table 2). The previously observed increase in BP was not observed in the 100 mg CYP group following pre-treatment with capsaicin.

Table 1. Autonomic response during hydrodistention in Cyclophosphamide-treated rats over time after modeling

	Control (n=5)	3 days after administering 100 mg of CYP (n=7)	1 week after administering 100 mg of CYP (n=5)	p-value
MBP at Baseline (mmH2O)	73.0±6.2	77.7±28.2	80.7±13.7	0.238
MBP during HD (mmH2O)	74.9±6.3	97.3±35.5	80.9±13.2	0.0694
Δ MBP (mmH2O)	1.8±3.6	19.6±9.5*	0.1±0.7	<0.001

MBP: mean blood pressure; *p<0.05, statistically significant difference between baseline and hydrodistention

Table 2. Autonomic response during hydrodistention in Cyclophosphamide-treated rats according to dosage of Cyclophosphamide and capsaicin pre-treatment

	Control (n=7)	50 mg CYP (n=7)	100 mg CYP (n=7)	200 mg CYP (n=7)	Capsaicin 100 mg CYP (n=7)	p value
SBP at Baseline (mmH2O)	69.20±6.14	104.33±17.20	89.43±42.87	75.20±47.23	104.14±39.68	0.341
SBP during HD (mmH2O)	70.00±7.54	106.86±14.94	105.58±46.80	76.66±48.65	105.13±40.37	0.288
Δ SBP (mmH2O)	0.84±1.68	2.43±3.04	16.03±6.86*	1.31±3.95	0.79±2.41	<0.001
DBP at Baseline (mmH2O)	67.27±6.16	95.40±15.53	83.11±36.53	72.61±45.13	100.74±38.15	0.365
DBP during HD (mmH2O)	68.11±7.36	98.41±13.42	97.15±38.91	73.97±46.59	101.81±39.04	0.330
Δ DBP (mmH2O)	0.84±1.61	3.01±2.53	14.03±4.97*	1.36±3.88	1.06±2.25	<0.001

SBP: systolic blood pressure; DBP: diastolic blood pressure; *p<0.05, statistically significant difference between baseline and hydrodistention.

The acute model that received 100 mg/kg of CYP was confirmed to be the most suitable based on these findings, and the resultant effects were observed after three days. This model was used in further studies.

3.1.2. Chronic model of IC/BPS

A significant increase in BP during HD was observed in all model groups, except in the control group, at weeks 1, 3, and 5 (Table 3). Consequently, the appropriate time frame for the establishment of the PS-LPS model was set as one week and applied in the subsequent experiments. The previously observed increase in BP was not evident in the one-week group following pre-treatment with capsaicin.

Table 3. Autonomic response during hydrodistention in Protamine sulfate-Lipopolysaccharide-treated rats over time after modeling

	Control (n=7)	1 week (n=7)	3 weeks (n=7)	5 weeks (n=7)	Capsaicin-1 week (n=6)	p value
MBP at Baseline (mmH2O)	73.0±6.2	89.2±16.5	125.2±54.2	97.0±27.8	93.2±17.1	0.037
MBP during HD (mmH2O)	74.9±6.3	115.4±18.6	150.6±48.2	131.3±31.6	99.3±12.7	<0.001
Δ MBP (mmH2O)	1.8±3.6	26.2±7.5*	25.4±9.3*	34.3±9.9*	6.1±7.9	<0.001

MBP: mean blood pressure; *p<0.05, statistically significant difference between baseline and hydrodistention

3.2. Western blotting and histologic examinations

3.2.1. Acute model of IC/BPS

Immunofluorescence staining revealed that compared with that observed in the control group, the expression of VCAM-1 and IL-6 in the bladder epithelium was significantly elevated three days post-CYP injection in the experimental groups. The intravesical expression of VCAM-1 and IL-6 was more pronounced in the capsaicin pre-treatment group (Figure 2-A). The most significant increase in the spinal expression of c-Fos was observed in the three-day CYP 100 mg/kg group. Notably, no increase in c-Fos expression was observed in the capsaicin pre-treatment group (Figure 2-B).

Western blot analysis revealed that the levels of VCAM-1, TNF-α, IL-6, and phosphorylated ERK (p-ERK) in the bladder exhibited a dose-dependent increase as the dosage of CYP increased (Figure 2-C). However, the highest expression of NGF in the bladder was observed in the CYP 100 mg/kg group. Evaluation of the expression of these molecules according to the time post-CYP injection confirmed that all molecules, except IL-6, were expressed most abundantly in the bladder three days after the administration of 100 mg/kg of CYP (Figure 2-D).

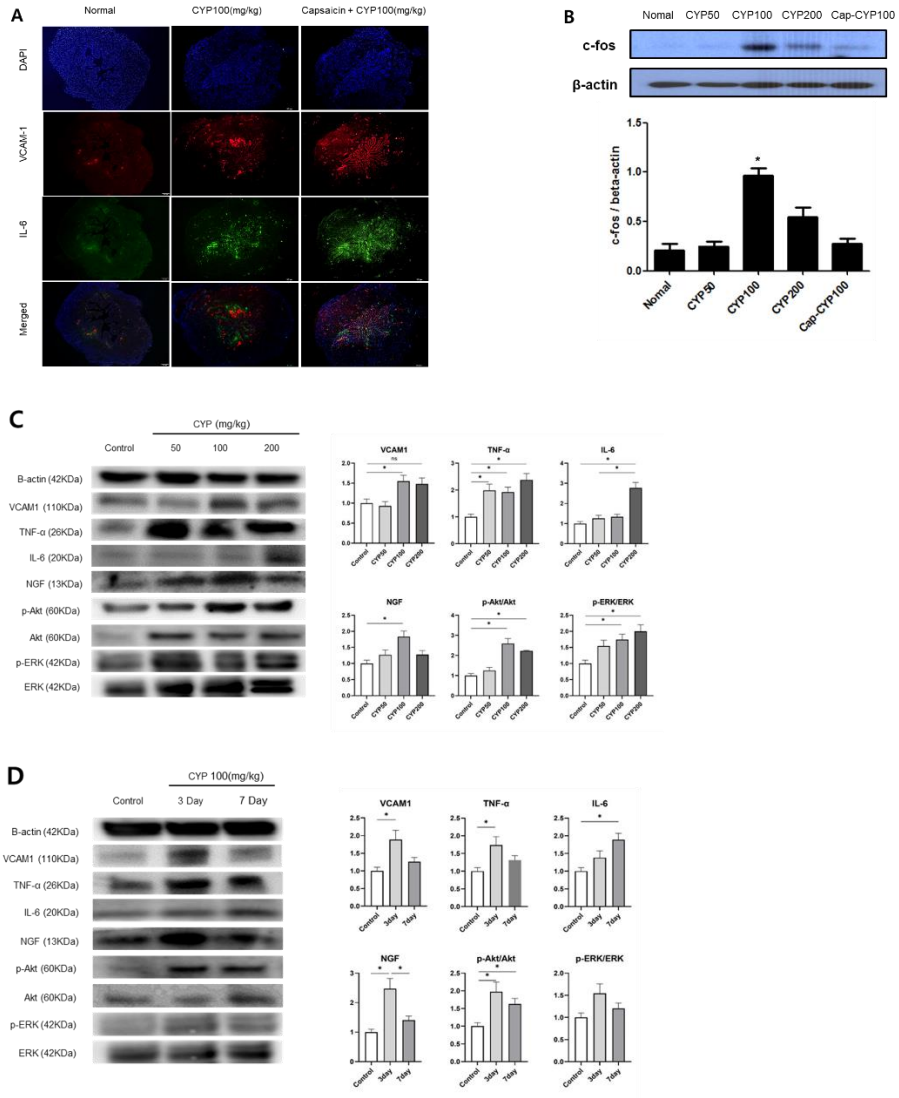

Figure 2. Bladder immunostaining and molecular analysis in bladder and spinal cord in acute model of IC/BPS according to different dosage and timing of cyclophosphamide. (A) VCAM-1(red) and IL-6 (green) immunostaining in bladder tissue control, 3days after 100mg/kg cyclophosphamide and pre-treatment of capsaicin (B) Western blots for c-fos in L6–S1 segments of rat spinal cord 3days after different dosage of cyclophosphamide and quantification by imageJ (C) Western blots of bladder tissue 3days after different dosage of cyclophosphamide (D) Western blots of bladder tissue compared over time after cyclophosphamide 100mg/kg

3.2.2. Chronic model of IC/BPS

Immunofluorescence staining revealed that the PS/LPS group pre-treated with capsaicin exhibited the most prominent expression of VCAM-1 and IL-6 in the bladder. Compared with that observed in the control group, a marked increase in expression was noted in the PS/LPS group (Figure 3-A).

Western blot analysis confirmed that the expression of most molecules in the bladder had increased one week after the administration of PS-LPS (Figure 3-B).

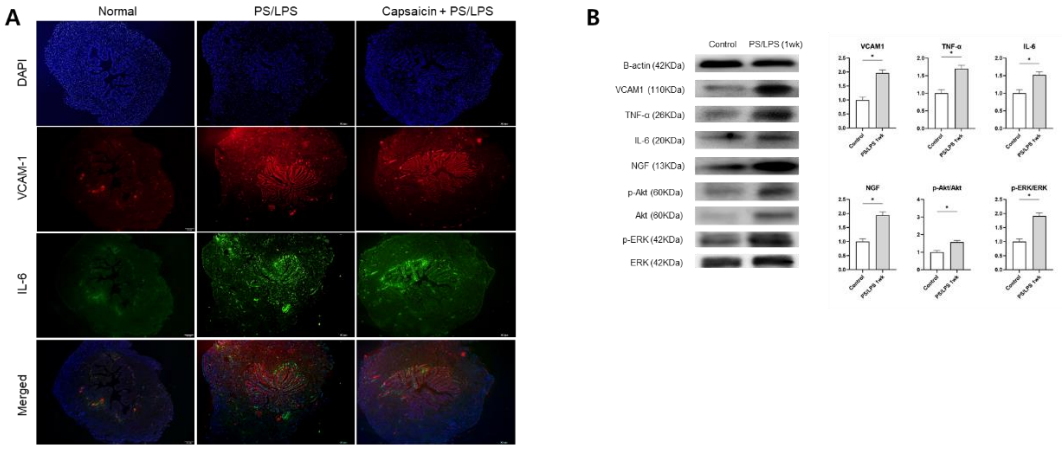

Figure 3. Bladder immunostaining and molecular analysis in bladder in chronic model of IC/BPS according to timing after PS/LPS (A) VCAM-1(red) and IL-6 (green) immunostaining in bladder tissue control, 1week after PS/LPS and pre-treatment of capsaicin (B) Western blots of bladder tissue 1week after PS/LPS

3.3. Inhibitory treatment

3.3.1 Intrathecal administration of Glycine Transporter Type 2 Inhibitor (ALX-1393) in the acute and chronic models

A significant increase in BP was observed during three consecutive sessions of HD in the control group (21.7 ± 10.3, 20.9 ± 7.8, and 22.9 ± 14.6 mmHg) in the acute model with CYP (Figure 4-A). A significant reduction in the increase in BP during HD was observed 5 min after the intrathecal injection of ALX-1393 in the ALX-1393 group (6.3 ± 7.7 mmHg, $p < 0.01$) (Figure 4-B).

The chronic model with LPS also exhibited a similar trend. An increase in BP was consistently recorded before and after saline injection in the control group that received intrathecal saline (32.0 ± 18.6 and 16.7 ± 8.7, 16.0 ± 6.0 mmHg, respectively) (Figure 4-C). This increase in BP was not observed 5 min post-ALX-1393 administration (23.0 ± 7.5 vs. 4.2 ± 9.7 mmHg, $p < 0.01$); however, it was observed again at 30 minutes (15.6 ± 8.6 mmHg) (Figure 4-D).

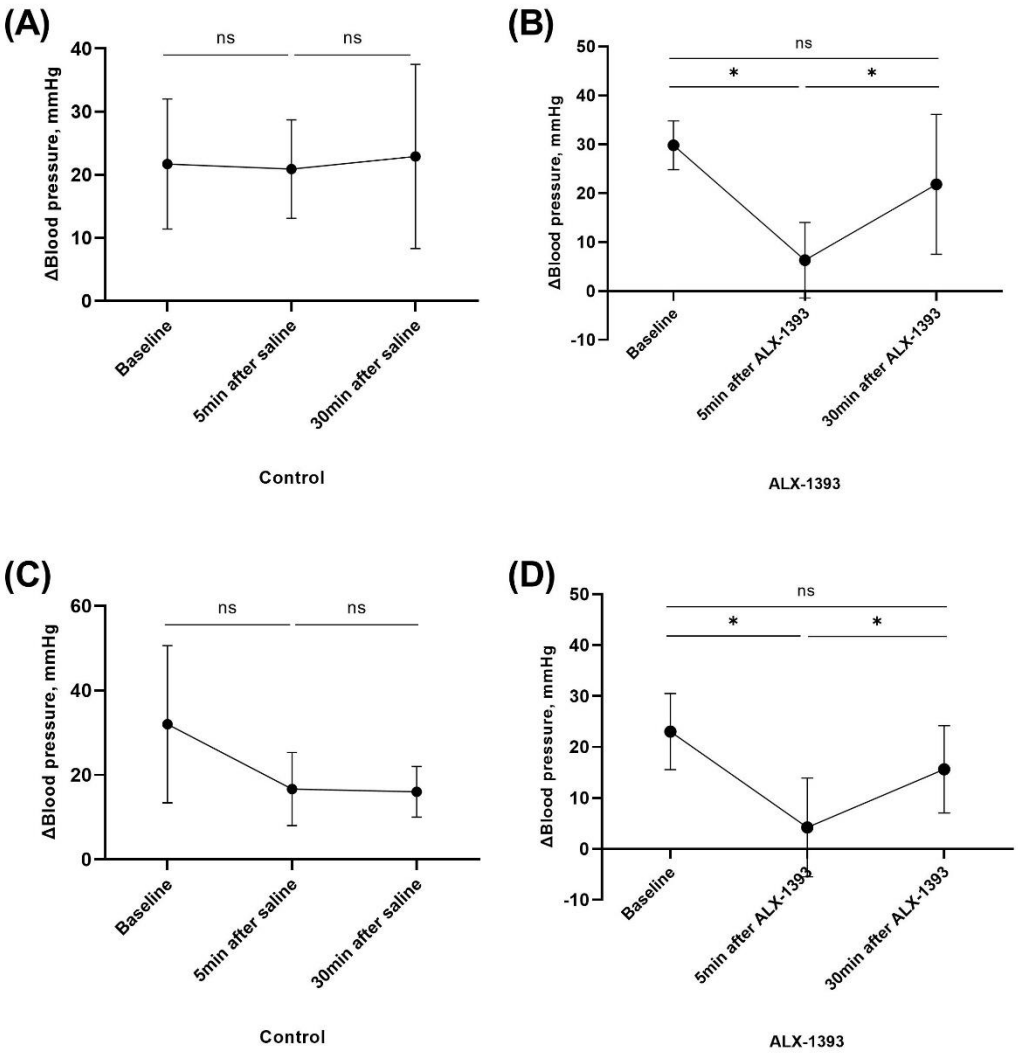

Figure 4. Changes in blood pressure during hydrodistension and the effect of intrathecal administration of ALX-1393 in acute and chronic models of IC/BPS. (A) Control (Acute model) (B) before and after administration of intrathecal ALX-1393 in acute model (C) control (Chronic model) (D) before and after administration of intrathecal ALX-1393 in chronic model

Cystometry revealed that the ICI in the control group exhibited minimal change following the intrathecal injection of saline in the acute model with CYP (8.8 ± 12.3 sec, p = 0.137). In contrast, a significantly prolonged ICI was observed after the administration of ALX-1393 (34.7 ± 25.9 sec, p = 0.022) (Table 4).

Table 4. Comparison of cystometric parameters before and after administration of intrathecal ALX-1393 and control group in acute model with cyclophosphamide

	Control (n=6)				ALX-1393 (n=6)			
	Baseline	Saline	Δ Mean	p value	Baseline	ALX-1393	Δ Mean	p value
ICI (sec)	67.1±31.7	75.9±39.1	8.8±12.3	0.137	55.4±23.9	90.1±32.3	34.7±25.9	0.022*
BP (cmH2O)	19.1±7.2	18.4±7.2	-1.0±2.6	0.525	21.0±7.1	20.0±6.4	-1.1±1.1	0.065
TP (cmH2O)	24.6±7.0	24.8±6.1	-1.6±3.3	0.726	29.6±6.4	25.7±7.1	-4.0±4.0	0.061
MP (cmH2O)	40.2±5.5	39.1±5.4	-0.4±1.6	0.083	42.5±2.3	39.9±1.3	-2.6±2.9	0.079

ICI: intercontractile interval; BP: baseline pressure; TP: threshold pressure; MP: micturition pressure; * $p<0.05$, statistically significant difference between baseline and hydrodistention 5 min after the intrathecal administration of ALX-1393.

Similar results were observed in the chronic model using PS/LPS. No significant differences were observed in other cystometry parameters. However, a significant increase in ICI was observed following the intrathecal injection of ALX-1393. This finding indicates an improvement in bladder function (Table 5). In contrast, no differences were observed in any of the parameters before or after saline injection in the control group.

Table 5. Comparison of cystometric parameters before and after administration of intrathecal ALX-1393 and control group in chronic model with protamine sulfate and lipopolysaccharide

	Control (n=6)				ALX-1393 (n=6)			
	Baseline	Saline	Δ Mean	p value	Baseline	ALX-1393	Δ Mean	p value
ICI (sec)	45.7±22.2	57.8±42.2	12.2±20.6	0.207	55.5±37.2	113.3±61.2	57.8±24.9	0.002*
BP (cmH2O)	16.2±7.8	16.2±7.8	-0.2±3.0	0.907	14.1±8.7	10.9±2.4	-3.2±7.1	0.315
TP (cmH2O)	25.0±10.4	23.8±11.7	-1.2±2.7	0.336	19.4±10.4	17.0±4.2	-2.5±6.9	0.423
MP (cmH2O)	38.4±9.74	39.6±12.8	1.3±3.6	0.419	42.5±2.3	35.8±8.0	-3.2±3.3	0.060

ICI: intercontractile interval; BP: baseline pressure; TP: threshold pressure; MP: micturition pressure; * $p<0.05$, statistically significant difference between baseline and hydrodistention 5 min after the intrathecal administration of ALX-1393.

3.3.2. Intrathecal and intravenous administration of α-1A AR antagonist (naftopidil) in the chronic model

A marked increase in BP during HD (32.1 ±3.5 mmHg) was observed before the intrathecal administration of naftopidil; however, this response was suppressed 5 min after the administration of naftopidil (3.9 ±5.3 mmHg). The increase in BP during HD was observed again 30 min after the administration of naftopidil (31.1 ±20.0 mmHg) (Figure 5). A similar pattern was observed following the intravenous administration of naftopidil, with an increase in BP during HD being observed before and 30 min after the intravenous administration of naftopidil (22.4 ±5.6, 16.4 ±7.6 mmHg, respectively). No such increase in BP was observed 5 min after the administration of naftopidil (5.4 ±4.7 mmHg). This increase in BP was continuously observed before and after the administration of saline injection in the control group.

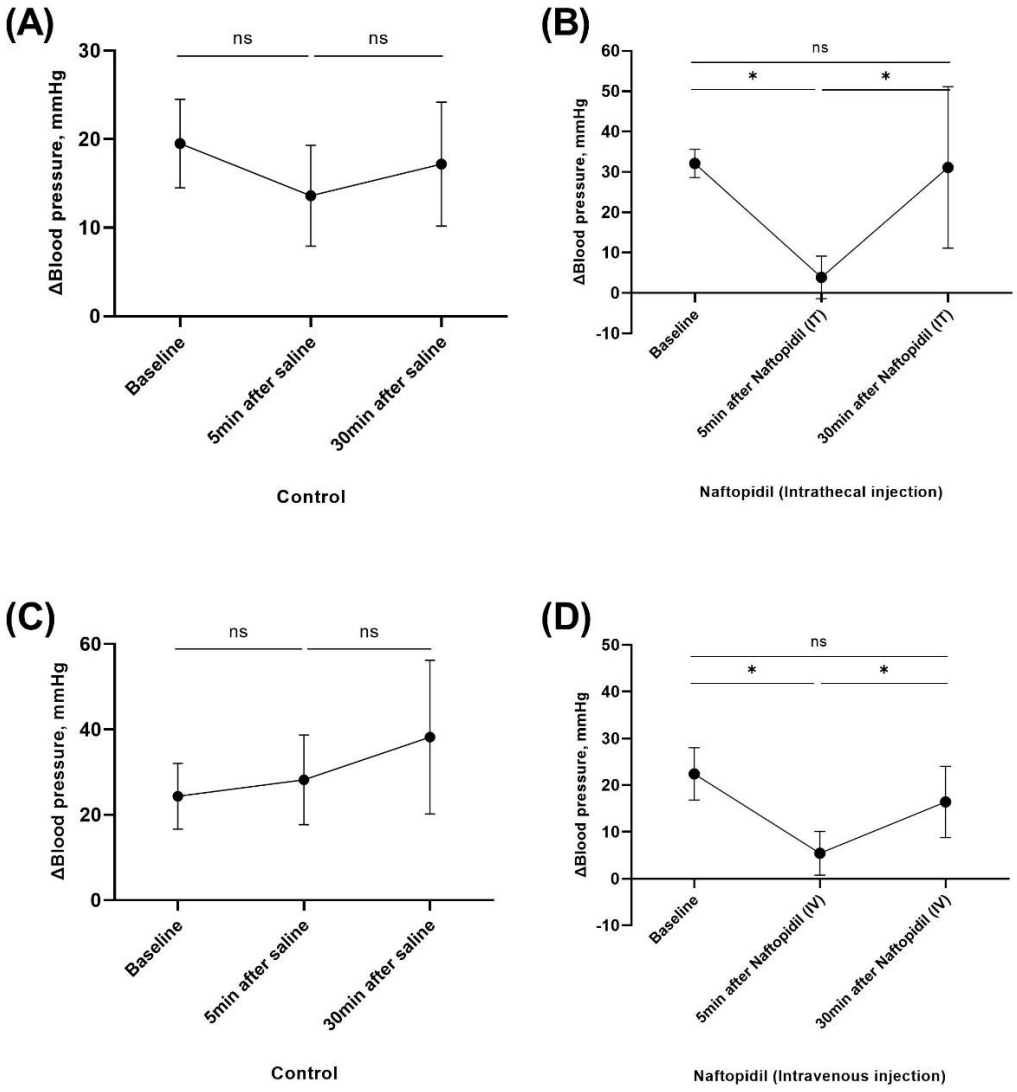

Figure 5. Changes in blood pressure during hydrodistension and the effect of intrathecal and intravenous administration of α-1A adrenergic antagonist (naftopidil) in chronic model of IC/BPS. (A) Control and (B) before and after administration of intrathecal naftopidil in chronic model (C) Control and (D) before and after administration of intravenous naftopidil in chronic model

No notable differences were observed in terms of other cystometric parameters. However, a substantial increase in ICI was observed following the intravenous administration of naftopidil, indicating an improvement in bladder function (Table 6). In contrast, no changes in any parameter were observed before or after saline injection in the control group.

Table 6. Comparison of cystometric parameters before and after administration of intravenous naftopidil and control group in chronic model with protamine sulfate and lipopolysaccharide

	Control (n=6)				Naftopidil (n=6)			
	Baseline	Saline	Δ Mean	p value	Baseline	ALX-1393	Δ Mean	p value
ICI (sec)	91.8±22.2	101.5±35.2	9.7±16.3	0.201	93.4±28.9	173.9±53.6	80.6±56.2	0.017*
BP (cmH2O)	17.1±12.3	15.3±7.7	-1.8±5.5	0.452	16.3±3.5	15.1±3.3	-1.2±1.2	0.056
TP (cmH2O)	23.5±14.2	20.9±8.5	-2.5±8.3	0.492	25.0±3.3	25.5±3.2	0.5±2.8	0.696
MP (cmH2O)	41.0±6.5	38.0±2.8	-3.0±6.1	0.280	39.9±7.1	41.0±7.0	1.1±1.1	0.278

ICI: intercontractile interval; BP: baseline pressure; TP: threshold pressure; MP: micturition pressure; * $p<0.05$, statistical significance between baseline and hydrodistention 5 min after intrathecal administration of naftopidil.

4. DISCUSSION

An exaggerated ANS response characterized by an increase in BP during HD, similar to that observed in patients with IC/BPS, was observed in the acute and chronic animal models of IC/BPS in the present study. Notably, the present study represents the first attempt to reproduce this response in an animal model. Experimental modeling confirmed that this response was correlated with the expression of C-fibers and NGF in the spinal cord and bladder, respectively. Furthermore, the study of the inhibitory mechanisms revealed that this response is mediated by the glycine inhibitory pathway and α-1A adrenergic receptors in the spinal cord. Thus, the excessive ANS response observed during HD is significant as it is closely associated with the pathogenesis of IC/BPS. The degree of this response is correlated with the severity of the disease in patients with IC/BPS.

Evidence that has emerged since the initial report of increased density and number of sympathetic nerves innervating the bladder[18] has indicated an association between dysfunction of the ANS and IC/BPS.[19,20] The heart rate of patients with IC/BPS was higher than that of the controls at baseline and throughout the course of a laboratory mental stress challenge in a previous study.[21] In contrast, chronic adrenergic stimulation increased visceral pain and the frequency of bladder reflex contractions, in addition to inducing changes in the bladder mucosa, in an animal study.[22] Several studies have reported the association of altered balance between sympathetic and parasympathetic nervous activity with chronic painful diseases such as fibromyalgia, chronic widespread pain, and IC/BPS. This alteration contributes to the disease pathology by perpetuating neuro-immune dysregulation and facilitating neurogenic inflammation.[23] The predominant activity of the sympathetic nervous system was observed in the majority of these studies.[24]

The present study revealed that excessive autonomic responses are observed in the acute and chronic models only under certain conditions. Evaluating the conditions of the bladder and spine during the modeling process revealed that ANS dysfunction exhibited a significant correlation with NGF expression in the bladder. Evaluation of the ANS dysfunction and its expression levels in the bladder across various modeling processes revealed that NGF is the only candidate that was consistently and clearly associated with these responses. NGF, a small secreted protein, plays an essential role in the growth and survival of sensory and sympathetic neurons.

Neurotrophin is a key modulator of bladder pathologies of IC/BPS.[25] Clinical and experimental data have revealed a direct association of increased NGF expression in the bladder tissue and urine with painful inflammatory conditions in the lower urinary tract.[26,27] Intravesical instillation of NGF

induces hyperactivity in rats.[28] Bladder inflammation caused by intravesical irritants, as well as that observed in patients with IC/BPS, leads to rapid upregulation and release of endogenous NGF that mediates sensory and reflex changes.[29] An increase in the expression of NGF in the muscle or urothelium initiates signals that are transported along the afferent nerves of the bladder to the dorsal root ganglia or spinal cord. NGF induces functional changes in C-fiber afferents that lead to these relatively unexcitable afferents becoming hyperexcitable. This may contribute to afferent neuroplasticity, leading to the pain symptoms reported by patients with IC/BPS.[30,31] The findings of the present study are consistent with the findings of previous studies, which revealed that the level of NGF expression in the bladder is closely related to the c-Fos expression in the spinal cord. Furthermore, the findings of the present study confirmed that the changes in BP during HD were suppressed by pre-treatment with capsaicin and that this was related to the suppression of c-Fos expression in the spinal cord. Thus, the correlation of this ANS dysfunction with c-fiber activity in the spinal cord could be confirmed indirectly.

NGF may evoke hyperalgesia by triggering the production of α1-adrenoceptors on the peripheral nerve fibers. For instance, exposure to NGF for 24 h led to a two-fold increase in the mRNA levels for the α-1B-adrenoceptor subtype in primary DRG neuron cultures in a previous study, with the receptor protein levels peaking 12 h later.[32] The response of the cultured DRG neurons to noradrenaline depends on the presence of the NGF TrkA receptor. Perfusion of neuronal cultures with noradrenaline can increase the firing rate of TrkA-positive cells following exposure to NGF; the TrkA-negative cells remain unaffected. Thus, NGF may enhance the expression of α1-adrenoceptors and the excitability of DRG neurons in response to noradrenaline. In other animal experiments, it was confirmed that sympathetic axons were already sprouting in the DRG in mice that NGF overexpression is driven by a glial protein (GFAP) promotor, and the sprout density of axon was significantly higher in GFAP-administered mice 2 weeks after chronic constriction injury.[33] These findings indicate that NGF can activate the sympathetic nervous system of DRG and that the activated sympathetic nervous system can induce IC/BPS.

Given the enhanced activity of the sympathetic nervous system observed in IC/BPS, adrenergic α-1 is considered the most relevant adrenergic receptor. Studies have explored the role of adrenergic α-1, especially α-1D, in patients with IC/BPS. The adrenergic α-1D receptor, which is closely related to lower urinary tract symptoms, is most abundant in the human spinal cord.[34] Comparison of the genomic DNA of 55 patients with that of normal control revealed a significant difference in terms

of the prevalence of adrenergic receptor genes α-1D genotype.[35] The factors related to the adrenergic α-1D receptor gene polymorphism may contribute to a predisposition to IC/BPS. Previous studies using naftopidil have demonstrated this relationship between adrenergic α-1D and IC/BPS.

Naftopidil, an α1D/1A-adrenoceptor blocker, improves lower urinary tract symptoms, particularly those of benign prostate hyperplasia. Naftopidil has a stronger affinity for α-1D receptor than other drugs; thus, it is expected to exert a therapeutic effect through a different mechanism in patients with IC/BPS.[34,36] Intrathecal injection of naftopidil abolished rhythmic bladder contraction in vivo in a previous animal study.[37] Similarly, a high concentration of naftopidil inhibited the amplitude of the monosynaptic Aδ-fiber and C-fiber evoked excitatory postsynaptic currents.[38] Naftopidil improved the symptoms of IC/BPS, such as reduced locomotor activity due to pelvic pain and a shortened interval between bladder contractions, in a model of IC/BPS induced by tranilast in a previous study.[10] Consistent with the findings of previous studies, intrathecal and intravenous administration of naftopidil yielded a therapeutic effect by suppressing dysfunction of the autonomic nervous system. This finding indirectly proves that IC/BPS is related to over-expression of the α-1D receptor in the spinal cord.

Suguya et al. reported a decrease in the levels of the inhibitory neurotransmitters glycine and GABA in the lumbosacral cord following the intrathecal injection of noradrenaline; in contrast, intrathecal administration of naftopidil increased the GABA level.[11] Thus, an overactive sympathetic nervous system can suppress the glycine inhibitory pathway in the spinal cord. Glycine, a major transmitter in the spinal cord, mediates postsynaptic inhibition. Notably, compromised glycinergic neurotransmission has been observed in specific regions of the dorsal horn in inflammatory and neuropathic pain.[39] GlyT2 mRNA was expressed at a much higher level (23-fold) in the dorsal spinal cord than GlyT1 mRNA in the spinal cord of rats in a previous study. Furthermore, the mRNA levels of GlyT2 were significantly reduced in the animal model of IC/BPS induced by CYP.[12] The intrathecal administration of ALX-1393 resulted in significant dose-dependent suppression of nociceptive behaviors and improved bladder overactivity. Intrathecal administration of ALX-1393 improved autonomic dysfunction and bladder overactivity in both models in the present study. Thus, GlyT2 plays a major role in the clearance of extracellular glycine in the spinal cord. The inhibition of GlyT2 may lead to increased extracellular glycine levels in the synaptic cleft in the spinal cord and inhibitory effects on bladder overactivity and pain responses induced by nociceptive stimuli in the bladder.

IC/BPC is a progressive condition that transitions from the early to advanced stages of bladder dysfunction. Urothelial barrier disruption occurs as a result of an initial insult to the bladder wall caused by infection, trauma, or excessive distension. This disruption facilitates the entry of irritants such as potassium,[40] leading to suburothelial inflammation, mast cell activation, T cells, and cytokine release. These effects further escalate chronic inflammation.[41,42] The inflammatory response extends along the sensory nerves in the dorsal root ganglion and sacral cord through neurogenic sensitization caused by increased production of NGF, with the signals reaching the cortical gyrus.[43,44]

The findings of the present study suggest that increased intravesical NGF expression following acute and chronic bladder irritation can directly induce the activation of spinal C-fibers. In addition, NGF also induced sprouting of the sympathetic nervous system of DRG, especially, α-1D. The glycine levels in the spinal cord and GlyT2 expression may decrease as an adaptive process. Thus, increased pain sensitivity and allodynia can occur owing to impairment of the glycine inhibitory pathways and activation of the C-fibers in the spinal cord. The symptoms observed in patients with IC/BPS, including autonomic dysfunction, can occur through these processes (Figure 6).

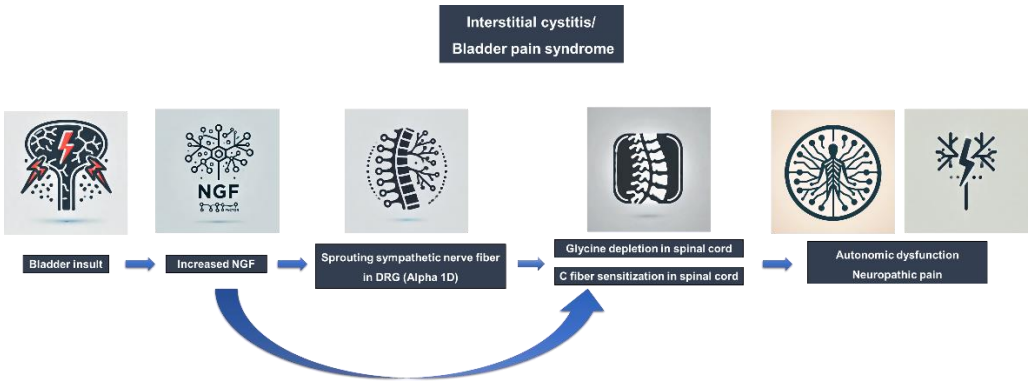

Figure 6. Schematic diagram of a proposed mechanism showing how bladder insult causes autonomic nervous system dysfunction in interstitial cystitis

A model of chronic adrenergic stimulation that mimics most of the signs and symptoms observed in patients with IC/BPS was developed by Charrua et al.[22] Other animal models must also be used to study IC/BPS; however, these models are also associated with some limitation.[45] Models wherein

cystitis is induced through the intravesical application of chemical irritants, such as cyclophosphamide-induced cystitis[46] and lipopolysaccharide-induced cystitis,[47] are the most widely used. In addition, models of bladder irritation induced by intravesical instillation of acetone[48] and other materials have also been used.[49,50] The density of sympathetic fibers is increased in patients with IC/BPS. Thus, studies have been conducted to investigate whether the same phenomenon was observed in the bladders of rats with inflammation.[22] These studies revealed an increase in the density of sympathetic nerve fibers in the body and dome of the muscular and suburothelial layers of the inflamed bladder. Furthermore, they revealed a significant elevation in the urinary noradrenaline levels in animals with cystitis compared with that in the control group. Cats exhibit symptoms consistent with those of IC/BPS; this model is known as the feline interstitial cystitis (FIC) model.[51] Investigation of the expression of tyrosine hydroxylase in the locus coeruleus of six cats with FIC during resting periods and comparison of these levels with those of healthy cats revealed that the cats with FIC exhibited higher levels of tyrosine hydroxylase expression, indicating altered sympathetic nerve activity.[52] Buffington et al. assessed the plasma norepinephrine levels in healthy cats and cats with FIC by analyzing blood samples and revealed that the catecholamine levels in the FIC group were significantly higher.[53] The diverse range of symptoms and underlying causes of IC/BPS and its precise pathophysiology remain undefined; thus, no single model can fully replicate all aspects of this disease in humans. However, based on the available evidence, the animal models used in the present study were considered to be capable of reproducing, to some extent, the ANS dysfunction observed in patients with IC/BPS. This was confirmed in a reproducible manner through this experiment.

This study has certain limitations. First, the α-1D receptor expression and glycine levels in the spinal cord were not evaluated. However, several previous studies have proven the role of impaired glycine inhibitory neurotransmission in chronic pain conditions such as IC/BPS, leading to increased pain sensitivity (hyperalgesia) and the perception of non-painful stimuli as painful (allodynia).[39] Previous studies have demonstrated the relationship between adrenergic α-1D receptor and IC/BPS; thus, the results of the present study strengthen that association. Second, autonomic dysfunction was measured only based on the changes in blood pressure during HD. Methods for measuring the norepinephrine levels in the blood and urine have also been used as indicators reflecting ANS activity in previous studies.[19,22] However, BP is a representative factor reflecting the activity of the sympathetic nervous system.[54] Previous studies have demonstrated that this change in BP during

HD is an objective indicator reflecting the severity of IC/BPS;[9] thus, it can be adapted as an objective parameter of the disease.

5. CONCLUSION

The present study demonstrated that the exaggerated responses of the ANS during HD in patients with IC/BPS were replicated in the acute and chronic animal models of IC/BPS. These responses were closely associated with NGF and c-Fos expression in the bladder and spinal cord, respectively. The intrathecal and intravenous administration of naftopidil improved autonomic dysfunction. Furthermore, the intrathecal administration of ALX-1393 suppressed these abnormal responses, resulting in improved bladder function. Thus, the present study elucidated the pathological mechanisms underlying ANS dysfunction in patients with IC/BPS, emphasizing its potential as a future therapeutic target.

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ABSTRACT(IN KOREAN)

간질성 방광염 백서 모델에서의 자율신경계 조절 이상과 이에 대한 척추의 억제 기전

간질성 방광염/방광 통증 증후군은 만성 통증 질환으로, 발생원인으로는 여러 요인이 복합적으로 작용하는 것으로 알려져 있으며, 방광의 얇아진 상피 및 장벽 기능 저하와 같은 변화가 관찰된다. 이런 환자에서는 특징적으로 자율 신경계 활동성의 변화가 있으며, 이는 높은 요중 노르에피네프린 수치 및 교감신경 우세의 경향이 있다고 알려져 있다. 이러한 환자에서는 수압 확장 동안 혈압 및 맥박수의 유의미한 상승이 나타나며, 이러한 반응은 증상의 중증도와 밀접한 상관관계가 있다. 이에 본 연구는 간질성 방광염/방광 통증 증후군 환자에서 나타나는 과도한 자율 신경 반응을 백서 모델에서 재현하고 기저 병리기전을 확인하고자 하였다. 또한 자율신경계 이상반응이 α-1D 아드레날린 수용체 길항제와 글리신 수송체 2형 억제제에 의해 호전되는 지를 확인하여 이들의 치료적 잠재력을 확인함으로써 새로운 치료방법을 모색하고자 하였다.

재현성을 위하여 급성 및 만성 방광염의 두 가지 동물 모델을 설정하였다. 급성 모델에서는 사이클로포스파미드(cyclophosphamide, CYP)를 투여하여 방광염을 유도하였으며, 동물 모델링 과정에서는 수압확장술동안 혈압 증가 정도를 CYP의 용량(50, 100, 200 mg/kg) 및 투여 후 기간(3일, 7일)에 따라 확인하였다. 만성 모델에서는 프로타민 황산염(protamine sulfate, PS)과 리포폴리사카라이드(lipopolysaccharide, LPS)를 통해 시간의 경과에 따른 염증을 유발하였다. PS/LPS 투여 후 시간 경과에 따른 반응을 보기 위하여 1주, 3주, 5주 후 변화를 평가하였다. 억제기전 확인을 위하여, 캡사이신 전처치, 경막외 글리신 수송체 유형 2 억제제(ALX-1393) 및 나프토피딜 (α-1D/A 아드레날린 수용체 길항제)를 경막외 및 경정맥 투여하였다.
급성 및 만성 모델 모두에서 수압확장술동안 혈압에 유의미한 변화가 나타났다. 급성 모델에서는 CYP 주사 3일 후 특히 100 mg/kg 용량 그룹에서 혈압 변화가 두드러졌으며, 19.6 ± 9.5 mmHg 증가($p < 0.001$)하였고, 대조군 및 다른 CYP 용량 그룹(50, 200 mg/kg)에서는 유의미한 변화가 관찰되지 않았다. 만성 모델에서는 PS 및 LPS로 처리된 모든 그룹이 대조군에 비해 수압확장술 동안 혈압이 상승하여, PS/LPS 처리 1주, 3주, 5주 후에서 모두 이러한 변화가 관찰되었다. 1주 그룹의 평균 혈압은 89.2 ± 16.5 mmHg에서 수압확장술 중 115.4 ± 18.6 mmHg로 증가하였다($p < 0.001$). 캡사이신 전처치를 한 경우 급성 및 만성 모델 모두에서 관찰되었던 수압확장술 중의 혈압 상승 소견이 억제되는 결과가 관찰되었다.

급성 모델에서 면역형광 염색 결과, VCAM-1 및 IL-6의 발현이 방광 상피에서 유의미하게 증가하여 염증을 반영하였다. 웨스턴 블롯 분석 결과, CYP 처치 후 VCAM-1, TNF-α 및 IL-6의 발현 증가가 CYP 용량에 비례하여 증가됨을 확인되었다. NGF의 발현은 CYP 100 mg/kg 주사 3일 후 방광에서 정점에 달하며, 이는 척수의 c-fos 발현 및 과도한 혈압 변화와 밀접한 관련성이 있었다. 만성 모델에서도 NGF를 포함한 모든 염증 표지가 상승하였다. 경막외 글리신 수송체 유형 2 억제제(ALX-1393)의 주사는 급성 및 만성 모델 모두에서 수압확장술동안 혈압 상승을 유의미하게 감소시켰으며 방광의 수축 간격을 연장시키는 치료 효과가 있었다. 나프토피딜은 만성 모델에서 경막외 및 정맥 주사를 통해 투여되었으며, 두가지 방법 모두에서 수압확장술동안 혈압 상승을 억제하였다. 만성 모델에서 나프토피딜의 정맥 주사는 방광의 수축 간격을 유의하게 연장하는 효과를 보였다.

본 연구에서는 수압확장술 동안 관찰된 자율 신경계 반응이 급성 및 만성 동물 모델에서 효과적으로 재현되었음을 보여주었다. 또한 이러한 반응은 방광의 NGF 발현 및 척수의 c-fos 발현과 유의미하게 연관되어 있었다. 자율 신경 기능 장애는 나프토피딜의 경막외 및 정맥 주사 후 개선되었다. 또한 ALX-1393의 경막외 주사는 이러한 비정상 반응을 완화시켜 방광 기능을 향상시켰다. 이러한 결과는 간질성 방광염/방광 통증 환자의 자율 신경계의 기저 병리 기전을 확인하며, 이를 통한 미래 치료 표적으로서의 가능성을 확인할 수 있었다.

핵심되는 말 : 간질성 방광염, 방광 통증 증후군, 자율신경계, 글리신, 아드레날린 수용체