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Effect of Stress on the Expression of Rho-Kinase and Collagen in Rat Bladder Tissue

Hana Yoon, Donghyun Lee, Kyemin Chun, Hyunsuk Yoon, Jaeyeong Yoo

Department of Urology, Medical Research Center, Ewha Womans University School of Medicine, Seoul, Korea

Purpose: We examined the effect of stress on the pathophysiology of bladder stability in terms of enzyme levels, Rho-kinase, and bladder relaxation.
Materials and Methods: A total of 48 female Sprague-Dawley rats were studied in scheduled stress environments for 7, 14, and 28 days; 24 rats were in the control group and 24 rats were in the test (stressed) group.
Results: Estrogen decreased significantly whereas testosterone and dopamine increased significantly in the stress group ($p<0.05$). Rho-kinase was significantly increased in the rats exposed to stress stimuli for 14 days ($p<0.05$). Collagen types I and III in the bladder tissue were significantly higher in rats exposed to stress for 14 days and 28 days (collagen type I in the 14-day group, $p<0.01$; collagen type I in the 28-day group, $p<0.05$; collagen type III in the 14-day and 28-day groups, $p<0.05$). Voiding frequency increased significantly as the duration of stress exposure was prolonged, in addition to a significant decrease in volume per voiding ($p<0.05$).
Conclusions: The changes observed in micturition pattern, factors that contribute to smooth muscle contraction, and relaxation in the female rat bladder support the hypothesis that stress affects bladder stability.

Key Words: *Physiology; Urinary bladder*

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Corresponding Author:
Hana Yoon
Department of Urology, Medical Research Center, Ewha Womans University School of Medicine, 911-1, Mok-dong, Yangcheon-gu, Seoul 158-710, Korea
TEL: +82-2-2650-5046
FAX: +82-2-2654-3682
E-mail: wowhana@ewha.ac.kr

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INTRODUCTION

Stress is a physical response that results from danger or other changes that cause a physical reaction. The hypothalamo-pituitary-adrenal axis is stimulated if the stress continues and cortisol is released from the adrenal cortex. Typical in clinical settings is that prolonged stress impairs immune function and increases the risk of acquiring certain diseases [1].

Over 280 different diseases currently indicate stress as a primary risk factor. Several urological symptoms or diseases show a relationship with stress that suggests stress as one of the major aggravating factors or triggers for disease recurrence or progression; examples include painful bladder syndrome, interstitial cystitis, acute or chronic cystitis, and lower-urinary-tract symptoms (LUTS) [2]. Typically, the symptoms of urgency, frequency, vesical tenesmus, a weaker urinary stream, and urinary incontinence are aggravated by stress [1-3].

Stress is an important aggravating factor of the symptoms of interstitial cystitis. In addition, autonomic nervous system overactivity is suggested as one of the pathogenic factors in the genesis of interstitial cystitis and LUTS with benign prostatic hyperplasia (BPH) [4]. Meanwhile, estrogen, which is well known to be effective on female bladder contractility and stability, is also affected by stress.

Studies have shown that many patients who experience significant LUTS also complain of somatization disorders, hysteria, anxiety, and depression [5]. The importance of managing these disorders in women who are at high risk of chronic stress is imperative considering that stress mitigates or aggravates the symptoms of such disorders.

Rho-kinase is one of the major mediators for muscle contraction and relaxation responses. Adequate collagen contents and their ratio in the bladder interstitium are important for good bladder function.

This study examined the effect of stress on the pathophysiology of bladder stability by observing changes in enzymes, Rho-kinase, and two types of collagen, which are required for proper muscle activity and stability within the bladder. We hypothesized that stress acts on the human body not only as a breaker of psychological stability but also

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as a physiological inducer of tissue changes that lead to certain symptomatic manifestations.

METERIALS AND METHODS

1. Animals and stress conditioning

Animal testing was conducted in two separate experiments. For the first experiment, 48 female Sprague-Dawley rats each weighing 230-270 g were used, with 24 rats in the control group and 24 rats in the test group. For the second experiment, which used a metabolic cage, voiding frequency was measured in the test group before stress was applied and for the following 27 days. The control rats were kept in cages measuring 260x420x180 mm with four rats per cage. The control rats were allowed a comfortable general environment with a general diet and free access to water. Rats in the test group were under scheduled stress conditions. Stress conditions consisted of starvation, low temperatures (4°C), immobilization, and changes in the diurnal rhythm. The cages used for immobilization were 200x200x100 mm in size, and eight rats were kept in each cage on the scheduled days. Rats in the test group were kept in cages identical in size to those used for the control group on unscheduled days. Rats in each group (8 rats per group) were sacrificed on the 7th day, 14th day, and 28th day of conditioning. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

2. Tissue preparation and measurement of serum hormones

Specimens for blood and tissue sampling were obtained after sacrifice (by decapitation) in each group. Bladder tissues were removed, snap-frozen in liquid nitrogen, and stored at −70°C until tested. Serum levels of dopamine and norepinephrine were measured to confirm the adequacy and effectiveness of the stress treatment. Serum estrogen and testosterone were also measured to assess changes in these hormones according to stress. Blood was sampled from the inferior vena cava, and serum estrogen (17-β-stradiol), testosterone, dopamine, and norepinephrine levels were assayed by radioimmunoassay (RIA).

3. Observation of Rho-kinase and collagen subtype expression in bladder tissue

1) Immunohistochemical staining: Frozen bladder specimens were sectioned to 5 μm thickness and then stored at −70°C until processed for staining. Immunohistochemical localization was performed by using specific polyclonal and monoclonal antibodies to types I and III collagen (Novus Biologicals, USA) and Rho-kinase (ROKα; BD Transduction Laboratories, USA). The antibody dilution used was 1:250 for the collagens and ROKα. The bound antibody was subsequently detected by using an avidin-biotin peroxidase system (Dako Cytomation, USA) and diaminobenzidine (DAB) as a substrate chromogen. Ten regions were randomly selected for each specimen, and the percentage of DAB positive area per unit area (μm^2) was measured and analyzed with an image analyzer program (Analysis, Soft Imaging system GmbH, Lakewood, USA).

2) Western blotting assay: For the Western blot analysis, frozen bladders were pulverized, resuspended in 1 ml of lysis buffer (5 mmol/l glycerophosphate, 2 mmol/l $MgCl_2$, 1 mmol/l EGTA, 0.5% Triton X-100, 0.5% NP-40, 1 mmol/l DTT, 100 ml protease inhibitor cocktail), and sonicated. The protein concentration was determined by the Bradford method (Biorad, USA). The protein (30 μg) was separated on denaturing sodium dodecyl sulfate 10% polyacrylamide gels by electrophoresis and was then blotted on a nitrocellulose membrane by wet electroblotting for 90 minutes. The membranes were blocked with 2% skim milk in TBS containing 0.05% Tween 20 (TBST) for 2 hours at room temperature. The blots were then incubated with primary antibodies [ROKα antibody (BD Transduction Laboratories, USA), type I collagen antibody (Novus Biologicals, USA), and type III collagen antibody (Novus Biologicals, USA)] for 2 hours at 4°C. After washing with TBST three times for 15 minutes, the blots were incubated with horseradish peroxidase-labeled secondary antibody for 1 hour at room temperature. After additional washes, the blots were detected by enhanced chemiluminescence by using an ECL detection kit according to the instructions of the manufacturer. The expressions were observed at 180 kDa for ROKα, at 95 kDa for type I collagen, and at 142 kDa for type III collagen. Blot density was measured with an imaging analysis program (BioRad, USA).

4. Observation of voiding pattern

The voiding pattern was observed by measuring the voided volume and voiding frequency. For this purpose, animals were kept in metabolic cages and the total amount of voided urine for 3 hours was measured every 5 days from day 1 to day 27. In addition, one examiner performed video monitoring to check the voiding frequency of the 3 hour metabolic cage observation.

5. Statistical analysis

Data analysis was performed by using Student's *t*-test and ANOVA. All data were compared between the control group and test groups throughout the 3-day period of the experiment (7-day stress, 14-day stress, and 28-day stress). A p-value of $p<0.05$ was considered to be significant.

TABLE 1. Changes in body weight in the control and stress groups

Date	Control groups, gram (n=24)	Stress group, gram (n=24)
1 day	223±6	213±8
7 days	247±4	228±12
14 days	253±9	244±8
28 days	267±6	249±8

The body weight of each group was significantly different between the control and stress group for each experimental day ($p<0.05$).

Table 2. Result of changes in sex hormones and neurotransmitters in the three groups

	Estrogen (pg/ml)	Testosterone (ng/ml)	Dopamine (pg/ml)	Norepinephrine (pg/ml)
Baseline control	23.43±10.01	0.03±0.01	37.60±1.56	321.56±12.06
Stress 7 days	21.05±9.65	0.04±0.02	49.52±3.81	452.02±16.52
Stress 14 days	18.40±10.36[a]	0.06±0.05[a]	75.20±7.66[a]	647.99±44.82[a]
Stress 28 days	16.85±10.11[a]	0.07±0.04[a]	82.56±5.24[a]	752.95±52.16[a]
Control 7 days	22.11±8.05	0.02±0.01	38.25±2.02	342.98±11.52
Control 14 days	20.40±10.36	0.03±0.02	40.15±5.12	362.01±42.15
Control 28 days	21.85±10.11	0.03±0.02	42.05±6.51	386.25±32.15

[a]: analysis of the significance was compared with control ([a]: $p < 0.05$).

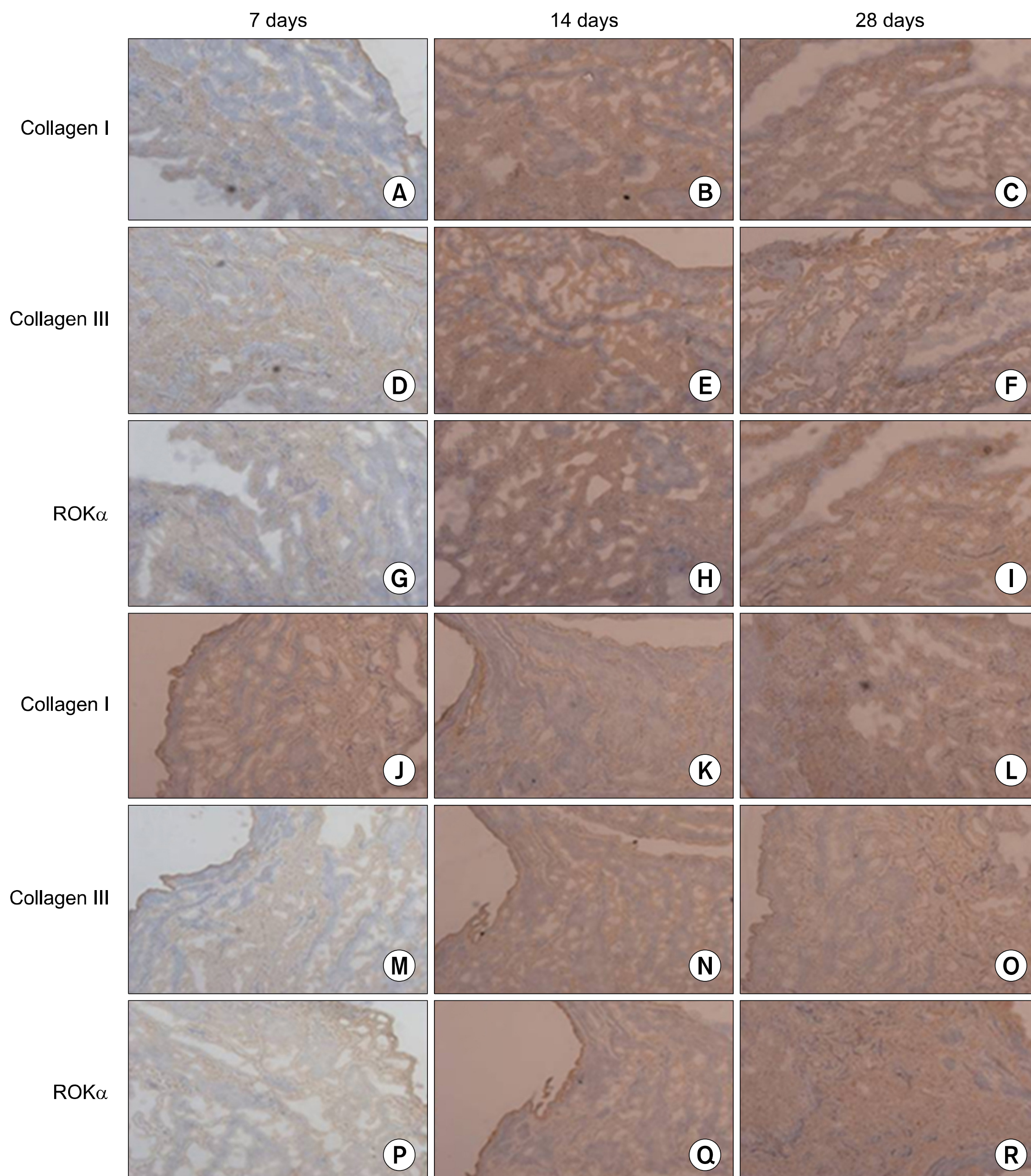

Fig. 1. Immunoreactivity pattern for type I collagen, type III collagen, and Rho-kinase (ROKα) in the rat bladder after immunohistochemical staining (x100) (Stress group: A-I; Control group: J-R).

RESULTS

1. Changes in body weight and serum hormone levels

Rats in the control and stress groups gained weight during testing, but the stress group was underweight relative to the control group, although the baseline weights of the rats were similar in both groups. After 28 days of stress, the average weight of the stressed rats was significantly lower than that of the control group ($p < 0.05$) (Table 1).

Changes in serum sex hormone levels and neurotransmitter levels are shown in Table 2. Serum estrogen levels significantly decreased, whereas testosterone levels significantly increased in the stress groups compared with the control group ($p < 0.05$). Dopamine and norephinephrine levels were significantly elevated in the stress group ($p < 0.05$).

2. Immunohistochemical staining

After immunological staining, DAB-positive areas were observed in the urothelium, submucosa, and muscularis mucosa under the optical microscope at x100 and x400 magnification (Fig. 1).

A significant change was observed in the expression of ROKα. The immunohistochemical expression of ROKα increased in the stress groups compared with the control groups. These changes were significantly increased according to the duration (days) of stress exposure ($p < 0.05$). The DAB percentages for ROKα were 7.8±3.7% in the 7-day stress group, 8.9±2.3% in the 14-day stress group, and 18.6±2.9% in the 28-day stress group.

The analysis of DAB percentage for collagen type I showed that the level of DAB staining of the 14-day (10.8±2.7%) and the 28-day (19.8±4.0%) stress groups was significantly higher than in the control group. Although the expression of collagen type I in the 7-day stress group did not significantly increase compared with that of the control group, such expression significantly increased as the duration of stress exposure increased to 28 days.

The level of collagen type III also significantly increased in the 14-day and the 28-day stress groups. The percentages of immunostained cells were 2.79±1.68%, 3.73±2.05%, and 10.54±4.29% in the control animals and 3.82±

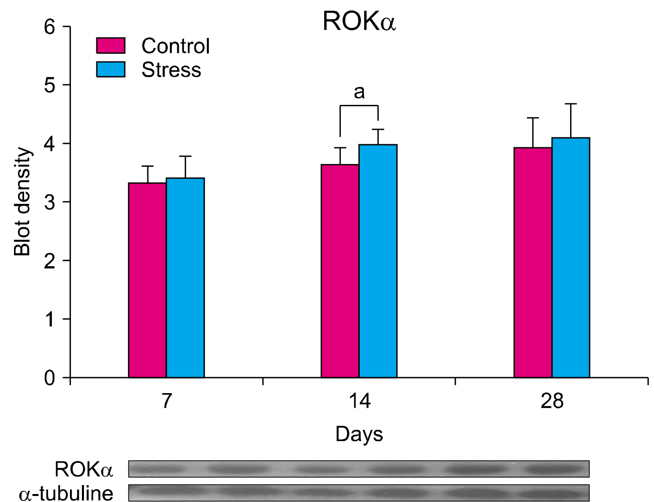

FIG. 2. Western blot analysis of the protein expression of Rho-kinase (ROKα). Control group: female rats in a comfortable general environment (n=24). Stress group: female rats under stressful condition (n=24). Analysis of significance was performed between the stress and control groups, with a significant difference in the 14-day stress group, [a]: p=0.015.

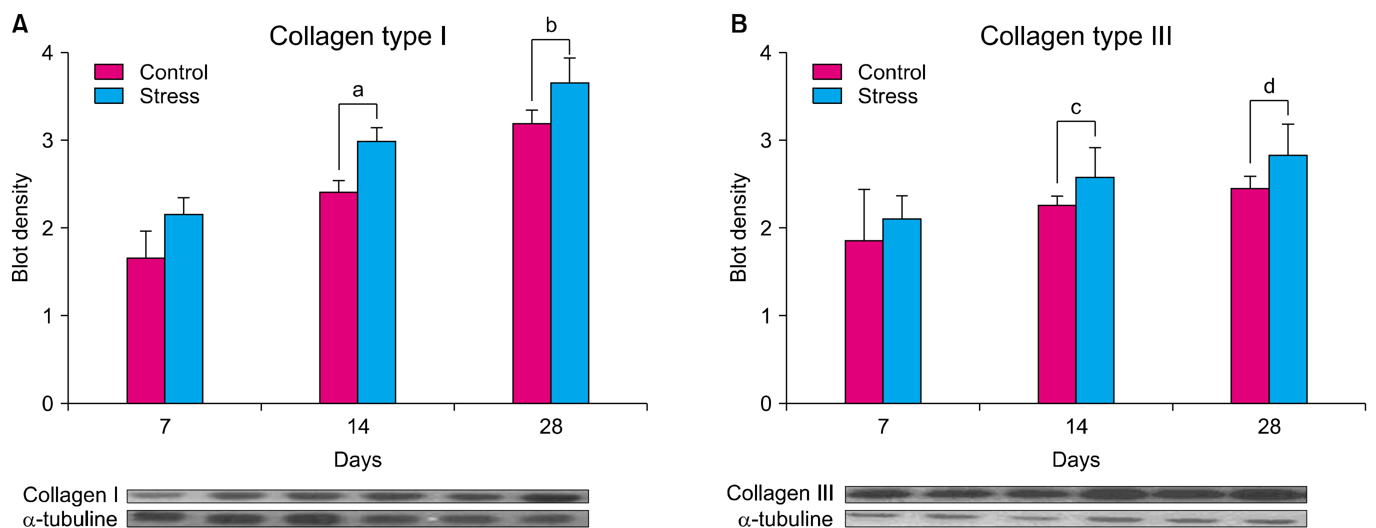

FIG. 3. Western blot analysis of the protein expression of Rho-kinase (ROKα). Control group: female rats in a comfortable general environment (n=24). Stress group: female rats under stressful condition (n=24). (A) Expression of collagen type I. Significant differences were noted in the 14-day and 28-day stress groups, ([a]: p=0.003, [b]: p=0.047), respectively. (B) Expression of collagen type III. Significant differences were noted in the 7-day and 28-day stress groups ([c]: p=0.015, [d]: p=0.021).

2.00%, 6.49±3.30%, and 11.27±2.37% in animals exposed to stress for 7, 14, or 28 days, respectively. The expression of collagen type III also significantly increased according to the number of days of stress exposure.

3. Western blot assays: ROKα, collagen types I and III

The protein expression of ROKα and of collagen types I and III in the bladder tissue was measured by Western blot analysis. The ROKα expression level was significantly higher in animals exposed to stress for 14 days but not in the 7-day or 28-day stress groups ($p<0.05$) (Fig. 2).

The expression level of collagen type I was 1.64±0.8, 2.41±0.30, 3.16±0.39 in control animals and 2.16±0.51, 3.02±0.37, and 3.69±0.83 in animals exposed to stress for 7, 14, and 28 days, respectively (Fig. 3A). Comparing the expression of collagen type I among the control and the three stress groups, a significant difference was noted in the 14-day and 28-day stress groups, $p=0.003$, $p=0.047$, respectively. In the expression of collagen type III, there were significant differences in the 7-day and 28-day stress groups, with p-values of $p=0.015$ and $p=0.021$, respectively (Fig. 3B).

4. Changes in micturition behavior

Voiding frequency was higher in the stress groups (Fig. 4) and increased significantly over time when compared with that of the control group. The mean voiding frequency in the control group was 3.0±0.82, 3.5±0.58, 4±1.15, and 4±1.15 on days 2, 7, 17, and 27, respectively. The mean voiding frequency in the stress group was 3.5±0.58, 2.5±0.82, 5.5±0.58, and 6.5±0.58 on days 2, 7, 17, and 27 of stress exposure, respectively. The difference between the control and the stress group was statistically significant ($p<0.05$).

The amount of urine per void decreased as the number of days of stress exposure increased, and the difference was significant when comparing the control and stress groups ($p<0.05$). The mean voided volume per void was 261.9±99.9 μl, 196.6±93.3 μl, 535.3±92.6 μl, and 344.8±115.3 μl on days 2, 7, 17, and 27 in the controls, respectively. The mean voided volume per void in the stress group was 361.5±132.1 μl, 295.4±108.4 μl, 296.5±122.8 μl, and 266±73.1 μl on days 2, 7, 17, and 27 of stress, respectively.

DISCUSSION

Stress is a contributing factor to the initiation and progression of several diseases. Chronic stress can cause physical and mental fatigue, disruption of the immune system and hormonal homeostasis, and stimulation of the autonomic nervous system [1]. Stress is also known to trigger aggravation of symptoms of interstitial cystitis, painful bladder syndrome, and chronic pelvic pain syndrome [1,6-8]. Clinically, patients often complain of aggravation of LUTS related to stressful events or conditions. These phenomena suggest that stress affects bladder stability. The results of the present study confirmed this hypothesis; we observed histopathologic changes in enzyme expression that resulted in detrusor muscular relaxation and contraction.

Sprague-Dawley rats were chosen as an animal model because the Ca-ATPase activity of these rats is five times that of rabbits, they have high energy consumption, and they have a hyperactive bladder that is very easily examined for neurological disorders caused by stress [9]. In addition, the main mechanism for human bladder contraction is cholinergic with an insignificant proportion of purinergic contraction [10]. Rats possess a bladder contraction mechanism similar to that of humans and were viewed as the most suitable model for the experiment.

Rho-kinase is one of the major mediators in muscle contraction and relaxation responses, including in the detrusor. RhoA activates the enzyme Rho-kinase, which is involved in the phosphorylation process. Rho-kinase induces smooth muscle contraction by phosphorylating the

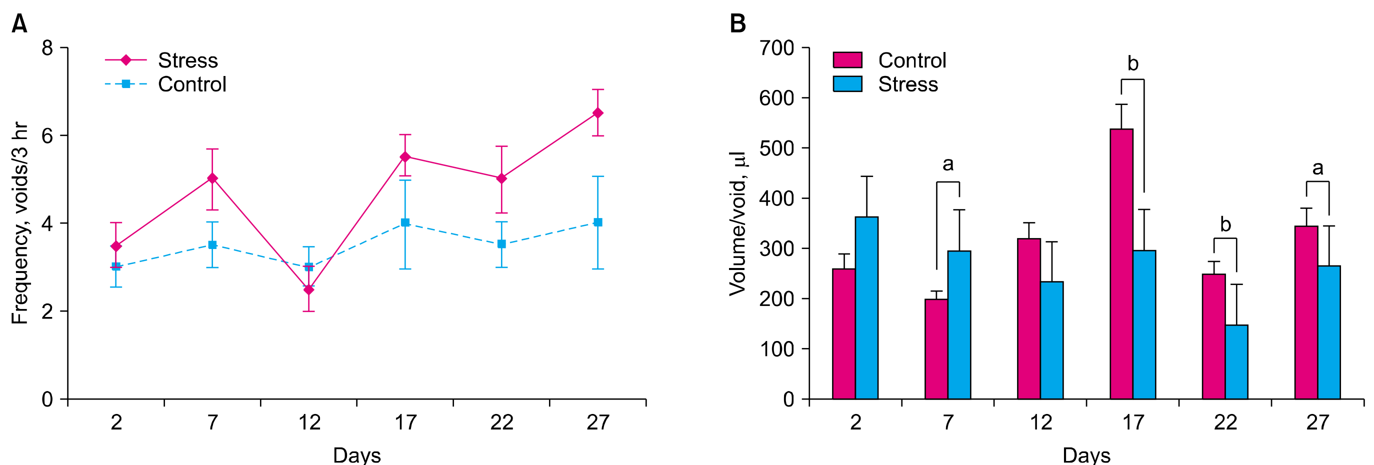

FIG. 4. Micturition behavior of the rats, with and without stress. (A) Frequency of voiding during 3 observational hours ($p<0.05$). (B) Volume (μl)/void in the stress and control groups with cumulative experimental days ([a]: $p<0.05$, [b]: $p<0.01$).

binding region of the MLCP [11]. Rho-kinase is present in the bladder muscle and is involved in contractions [12]. We hypothesized that certain prolonged stressful conditions may lead to bladder overactivity, and that Rho-kinase may participate in this change. Therefore, we investigated changes in Rho-kinase expression after exposure to stress.

We examined changes in the expression of ROKα in rat bladder tissue. ROKα is an indirect marker of detrusor muscle contractile response, because it is the mediator to the synthesis of Rho-kinase. We observed significantly higher ROKα expression in the 14-day stress group. It is possible to presume an increase in rat detrusor contraction by the observed increase in ROKα expression. This is also supported by the voiding behavior of the rats, which showed a significant frequency in the stress-exposed rats. The lack of significant changes in ROKα expression in the 7-day or 28-day stress groups suggests that physiologic compensation and adaptation occurred. When ROKα expression was compared between day 0 and day 7 in the stress group, a significant increase in expression was shown. Therefore, as the duration of stress exposure increased, the expression of ROKα also increased compared with that of the control group. The blunted changes or decreased expression in the 28-day stress group suggests a compensation and adaptation response to stress stimuli over a relatively long period.

Bladder fibrosis leads to functional changes, such as decreased contractibility and stability due to changes in the detrusor muscle and the extracellular matrix [13]. The extracellular matrix in the bladder is made up primarily of types I and III collagen. Type I collagen helps the tissue maintain tension, binding to smooth muscle and decreasing contractibility and compliance. Type III collagen is known to be involved in maintaining the elasticity and contractility of smooth muscle and is also involved with increased contractility of the bladder [14-16].

This study showed a significant increase in types I and III collagen in bladder tissue in the 14-day and 28-day stress groups compared with the control group. This result also supports that stress induces pathologic changes in bladder stability. Changes in collagen content could be related to bladder contractility or stability.

Additionally, we also observed significant changes in estrogen and testosterone according to stress exposure. It is well known that estrogen acts to maintain adequate bladder function, contractility, and stability, in particular. Therefore, these results support that stress may not only alter hormonal homeostasis, but also induce pathophysiologic changes leading to functional alterations.

Although levels of testosterone also changed after stress exposure, the meaning of these changes is uncertain. Increased serum levels of dopamine and norepinephrine support that the stress conditioning was good enough to fulfill the aim of our study, and those results also showed that elevated dopamine and norepinephrine can alter bladder stability. In addition, according to our previous experiences [17], testosterone may participate in some steps required to adapt and modulate the stress response.

The micturition behavioral changes observed in this study included increased voiding frequency and decreased volume per voiding in the stress group and showed the effect of stress on bladder function.

CONCLUSIONS

There are limitations in directly applying the findings of this study to human detrusor function; however, this study clearly shows that stress is a factor in bladder stability-overactivity. It is clinically recommended to monitor and control stress in treating overactive bladder patients or managing the symptoms of LUTS.

Conflicts of Interest
The authors have nothing to disclose.

REFERENCES

1. Reiser MF. Psychophysiology of stress and its sequelae. In: Reiser MF. Mind, brain, body: toward a convergence of psychoanalysis and neurobiology. New York: Basic Books; 1984;161-85.
2. McVary KT, Rademaker A, Lloyd GL, Gann P. Autonomic nervous system overactivity in men with lower urinary tract symptoms secondary to benign prostatic hyperplasia. J Urol 2005;174:1327-433.
3. Gordon D, Groutz A. Evaluation of female lower urinary tract symptoms: overview and update. Curr Opin Obstet Gynecol 2001;13:521-7.
4. van Os-Bossagh P, Pols T, Hop WC, Bohnen AM, Vierhout ME, Drogendijk AC. Voiding symptoms in chronic pelvic pain (CPP). Eur J Obstet Gynecol Reprod Biol 2003;107:185-90.
5. Morrison LM, Eadie AS, McAlister A, Glen ES, Taylor J, Rowan D. Personality testing in 226 patients with urinary incontinence. Br J Urol 1986;58:387-9.
6. Walters MD, Taylor S, Schoenfeld LS. Psychosexual study of women with detrusor instability. Obstet Gynecol 1990;75:22-6.
7. Engström G, Henningsohn L, Steineck G, Leppert J. Self-assessed health, sadness and happiness in relation to the total burden of symptoms from the lower urinary tract. BJU Int 2003; 95:810-5.
8. Gerwin RD. A review of myofascial pain and fibromyalgia--factors that promote their persistence. Acupunct Med 2005;23:121-34.
9. Damaser MS, Whitbeck C, Barreto M, Horan P, Benno H, O'Connor LJ, et al. Comparative physiology and biochemistry of rat and rabbit urinary bladder. BJU Int 2000;85:519-25.
10. Wang P, Luthin GR, Ruggieri MR. Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. J Pharmacol Exp Ther 1995;273: 959-66.
11. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 1996;273:245-8.
12. Abrams P, Andersson KE. Muscarinic receptor antagonists for overactive bladder. BJU Int 2007;100:987-1006.
13. Lluel P, Palea S, Barras M, Grandadam F, Heudes D, Bruneval P, et al. Functional and morphological modifications of the urinary bladder in aging female rats. Am J Physiol Regul Integr Comp Physiol 2000;278:R964-72.

14. Inaba M, Ukimura O, Yaoi T, Kawauchi A, Fushiki S, Miki T. Upregulation of heme oxygenase and collagen type III in the rat bladder after partial bladder outlet obstruction. Urol Int 2007;78:270-7.
15. Stevenson K, Kucich U, Whitbeck C, Levin RM, Howard PS. Functional changes in bladder tissue from type III collagen-deficient mice. Mol Cell Biochem 2006;283:107-14.
16. Deveaud CM, Macarak EJ, Kucich U, Ewalt DH, Abrams WR, Howard PS. Molecular analysis of collagens in bladder fibrosis. J Urol 1998;160:1518-27.
17. Yoon H, Chung WS, Park YY, Cho IH. Effects of stress on female rat sexual function. Int J Impot Res 2005;17:33-8.