Changes in Apoptosis-Related Proteins in The Urothelium of Rat Bladder Following Partial Bladder Outlet Obstruction and Subsequent Relief

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Purpose: To compose a comprehensible and fluent Persian translation of the National Institute of Health Chronic Prostatitis Symptom Index (NIH-CPSI), and to determine its linguistic validity in a Persian sample population.

Methods: The standard double-back translation method, provided by the previous studies were utilized by three professional linguists to translate the English version of the NIH-CPSI to Persian, and a group of 10 urologists further reviewed and translated questionnaire. The questionnaire was then presented to the sample study, comprised of 60 men with CP/CPPS and 60 controls with adverse urological history, and the collected data was analyzed through IBM-SPSS software to test its validity, evaluative, and discriminatory power, psychometric qualities and internal consistency.

Results: A total of 80 subjects (42 CP/CPPS patients and 38 healthy controls) were considered eligible for this study. The total Persian NIH-CPSI scores and each subdomain showed significant difference ($P < 0.001$) between the two study groups, indicating a satisfactory discriminant validity for the index. Psychometric analysis established the index to benefit from a high internal consistency. The translation was also considered by both the subjects and the physicians to be easily comprehensible.

Conclusion: The Persian NIH-CPSI is a reliable and valid instrument for evaluating CP/CPPS symptoms in general population, while also benefitting from high discriminatory power, and can be utilized with ease in both clinical practice and laboratory studies.

Keywords: asymptomatic inflammatory prostatitis; asymptomatic inflammatory prostatitides; chronic prostatitis with chronic pelvic pain syndrome; national institute of health chronic prostatitis symptom index; prostatitis; prostatitides

INTRODUCTION

Partial bladder outlet obstruction (PBOO) is a common urinary tract disorder caused by a variety of urologic diseases, such as benign prostatic hyperplasia (BPH), bladder neck contracture, and urethral stricture. Sustained bladder overdistension caused by PBOO decreases the blood flow and worsens the denervation of the bladder, resulting in functional change and oxidative damage of the urothelium[1,2]. Various animal studies have reported that repeated ischemic injury due to PBOO leads to chronic damage to the urothelium[3]. Currently, research indicates that chronic ischemia/reperfusion (I/R) may develop due to pathological PBOO-induced oxidative stress[4]. Although various mediators are reportedly related to changes in the bladder[5], the early stages of PBOO-induced changes of the urothelium and related molecular signal pathways have not been well elucidated.

Hypoxia, due to I/R injury plays an essential role in the generation of various chronic diseases by increasing the levels of reactive oxygen species (ROS)[6]. Thus far, many studies have described a significant association between ROS and the mechanism of I/R[7]. Increased ROS produce oxidative stress in the bladder and changes in the bladder occur via alterations in the blood vessels, nerves, and cellular fibrosis[8]. If this oxidative damage exceeds the compensatory capacity of the bladder, the bladder proceeds to the decompensatory phase, resulting in apoptosis of the bladder tissue[5]. To date,

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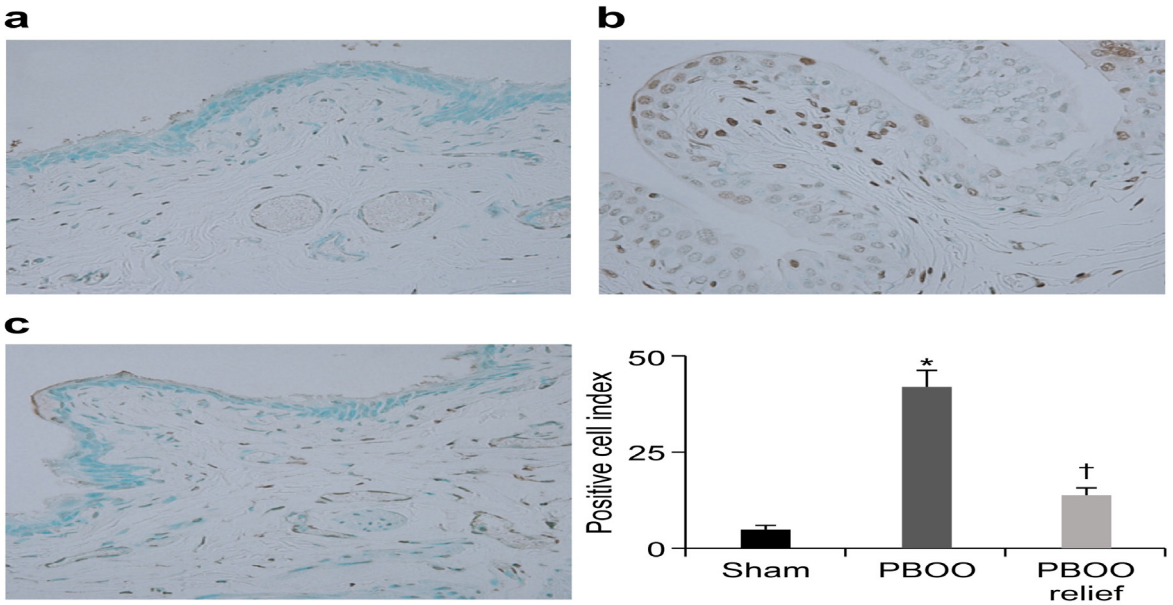


Figure 1. Detection of apoptosis. Representative micrographs (magnification x400) show TUNEL-positive cells presenting brown or black color in the urothelium of the rat bladder. Bar graphs show the quantitative analysis. The apoptosis index represents the fraction of apoptosis cells in the field. The sham-operated group **(a)**; the PBOO only group **(b)**; and the PBOO plus subsequent relief group **(c)**; *indicates that the PBOO group is significantly different from the sham-operated group ($P < 0.001$); † indicates that the PBOO plus subsequent relief group is significantly different from the PBOO group ($P < 0.001$).

it has reported that apoptosis is significantly associated with cellular death following I/R[9]. However, there are few studies describing the molecular mechanisms related to the restoration of bladder dysfunction in its early stages followed by I/R-induced apoptosis.

Survivin, known as a member of the apoptosis inhibitor family, inhibits apoptosis through intracellular expression[10]. Several studies have described survivin as a tumor marker for the diagnosis of urinary cancer[11]. In addition, survivin revealed a protective effect on oxidative stress caused by I/R in kidney, testis, and cerebra [12,13]. At present, no study has described the role of survivin concerning the recovery of I/R-induced apoptosis and cellular alteration in the urothelium.

In the present study, we investigated changes of apoptosis-related protein after PBOO following subsequent relief and expression of survivin associated with apoptosis in the urothelium of a rat bladder.

MATERIALS AND METHODS

Animal model and experimental groups

The experimental animals used in this study were female Sprague-Dawley white rats with a bodyweight of 180-220mg. We purchased Sprague-Dawley rats from Damul Science (Daejeon, Korea). All animals were fed normal chow and were exposed to a 12 h day/night light cycle. All processes were handled following the guidelines of the ethics committee of Chungnam National University and the Institutional Animal Care and Use Committee (IRB No. CNU-099). Sixty rats were subdivided into three groups: sham-operated (n = 20), PBOO only (n = 20), and PBOO plus subsequent relief (n = 20).

All rats were anesthetized using by intramuscular injection of ketamine and xylazine before a lower midline incision was made. To expose the bladder and urethra, the pre-vesical fats were retracted. In the PBOO group, the urethra was ligated to a 1 mm steel rod using a 3-0 nylon ligature. The steel rod was then removed, and the incision was sutured layer by layer. We followed a modified surgical method[14] to induce relief in the partial PBOO group. A 4-0 stay suture was done to pull up the vaginal epithelium to make it easy to make an incision in the vaginal epithelium. The 3–0 nylon ligature was inserted through the paraurethral small incision, passed through the vagina. The nylon ligature was tied gently around the urethra with the vaginal epithelium in the presence of a 1 mm steel rod placed along the urethra. The steel rod was removed after suturing. The ends of knots were pulled down through the paraurethral incision, the knot was located in the vaginal space to easily remove the knots. Sham surgery was performed as described above, and a 3–0 nylon ligature was tied around the urethra and vaginal epithelium without adding tension. The end of the knots was also placed in the vaginal space. In the sham-operated group, the loose knot was removed through the vagina. The de-obstruction period lasted 2 weeks, and then the bladder was harvested from the sham-operated group and PBOO relief group (Supplement Figure 1). We sacrificed the rat with CO2 gas after study following the IRB guidelines for the euthanasia of animals.

Detection of apoptosis

For the detection of apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method was used. Briefly, slides containing 5 μm sections of bladder tissue were de-paraffinized and rehydrated in a graded series of xylene and methanol. Slides were then treated with 20 μg/ml proteinase K and quenched in 3% hydrogen peroxidase in PBS for 20 min. After equilibration, all tissue sections were incubated for 1 h with a terminal deoxynu-

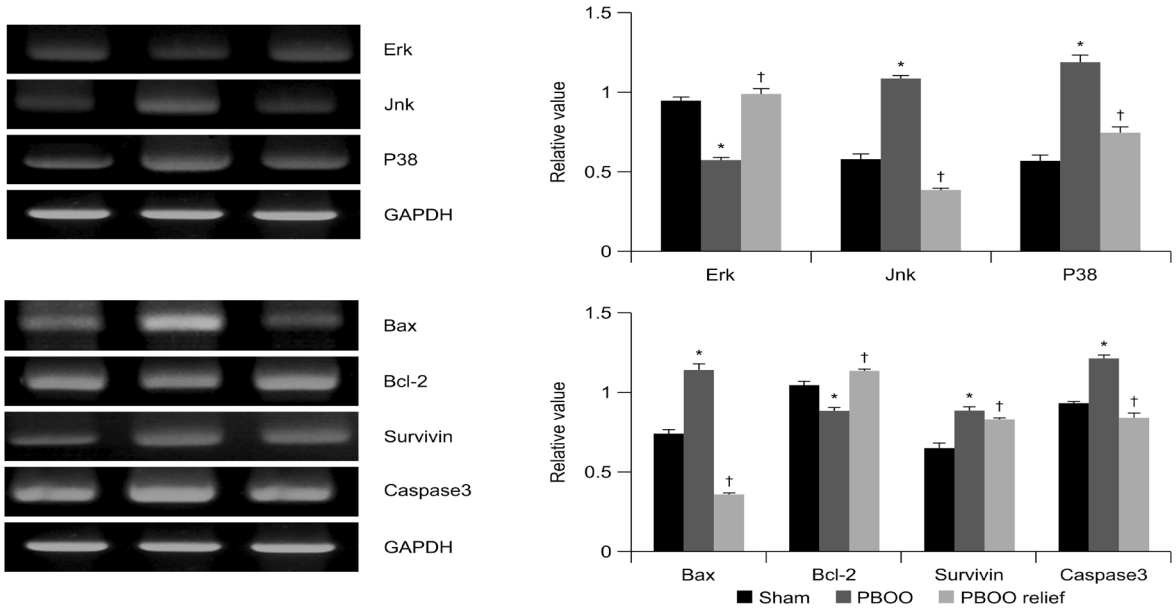


Figure 2. Representation of the quantitative PCR analysis. Erk, JNK, p38 MAPKs, Bax, Bcl-2, Caspase-3, and surviving mRNAs from the sham-operated, PBOO only, and PBOO plus subsequent relief groups in the rat urothelium. Each bar data shows the mean ± SEM. The expression of each mRNA was normalized to GAPDH. *$P < 0.001$, compared with the sham-operated group; † $P < 0.001$, compared with the PBOO only group.

cleotidyl transferase enzyme and an anti-BrdU-biotin monoclonal antibody in a dark humidified chamber at 37^{0}C. The slides were incubated with an anti-digoxigenin-peroxidase conjugate for 30 min and stained with 0.05% diaminobenzidine and then analyzed using a microscope. When dark brown apoptotic bodies were detected, slides were counterstained in 0.5% methyl green for 10 min. The slides were then washed with 100% N-butanol (ethanol and xylene) and mounted. Three high-power (×400) fields were randomly selected for each slide.

Quantitative PCR assay of urothelium

RNA extraction and generation of cDNA were conducted according to the protocols as previously described[15]. Reverse transcription was performed at 50°C for 50 min, followed by 70°C for 10 min. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 25 s; 54.5°C for 25 s; and 72°C for 25 s; and then a final extension step of 72°C for 5 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels. GAPDH was used as a housekeeping gene. The primer sequences were as follows: ERK (515 bp), 5'-GCCTTGCCCGGATTGCTGAC-3' (forward) and 5'-AGGCCGGACACTGGGAA-CACTAA-3' (reverse); JNK (354 bp), 5'-CACAGTCCTAAAACGATACC-3' (forward) and 5'-CCACACAGCATTTGATAGAG-3' (reverse); p38 (468 bp), 5'-GTGCCCGAGCGATAC-CAGAAC-3' (forward) and 5'-AGTGTGCCGAGCCAGCCCAAAATC-3' (reverse); Bax (246 bp), 5'-ACTGGGGCCGGGTGGTTG -3' (forward) and 5'-AGATGGTGAGTGAGGC-AGTGAGGA-3' (reverse); Bcl-2 (411 bp), 5'-TGCCAAGGGGGAAACACCAGAATC-3' (forward) and 5'-GCGACAAGGGGCCGTAGAGG-3' (reverse); Caspase-3 (282 bp), 5'-ACGGTACGCGAAGAAAAGTGAC-3' (forward) and 5'-TCCTGACTTCGTATTTCAGG-GC-3' (reverse); Survivin (460 bp), 5'-TGCGCCTTCCTTACAGTCAA-3' (forward) and 5'-CCCCCTCCCCACCCATAG-3' (reverse); and GAPDH (189 bp), 5'-CACGGCAAGTTCA-ACGGCAC-3' (forward) and 5'-AGCGGAAGGGGCGGAGATGA-3' (reverse).

Western blot assay of urothelium

The urothelium tissues were homogenized at 4°C in 1 × radio-immunoprecipitation assay buffer (Sigma-Aldrich), whole tissue homogenates were centrifuged at 13,000 ×g for 20min and the supernatants were collected and stored at –70°C. Western blot were performed according to the protocols as previously described (16). The membrane was incubated overnight at 4°C with an antibody targeting ERK1 (1:500), JNK1 (1:1000), p38 mitogen-activated protein kinase (MAPK) (1:1000), Bax (1:200), Bcl-2 (1:200), caspase-3 (1:1000), or survivin (1:200). All antibodies were purchased from Santa Cruz Biotechnology (CA, USA) or Cell Signaling Technology (MA, USA). Immunoreactive proteins were visualized and detected using chemiluminescence reagent (Dogen, Korea) and the scanned films were quantified using a documentation system (VIVID, Korea).

Statistical analysis

All data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS), version 18.0 (SPSS Inc., Chicago, IL, USA). Three groups were compared by Kruskal-Wallis test and the p-value was adjusted with the Bonferroni correction, and comparison between the two groups and other variables were made using a Mann–Whitney U tests. $P < 0.05$ was considered statistically significant in each case.

RESULTS

TUNEL findings

TUNEL staining was performed to detect apoptotic

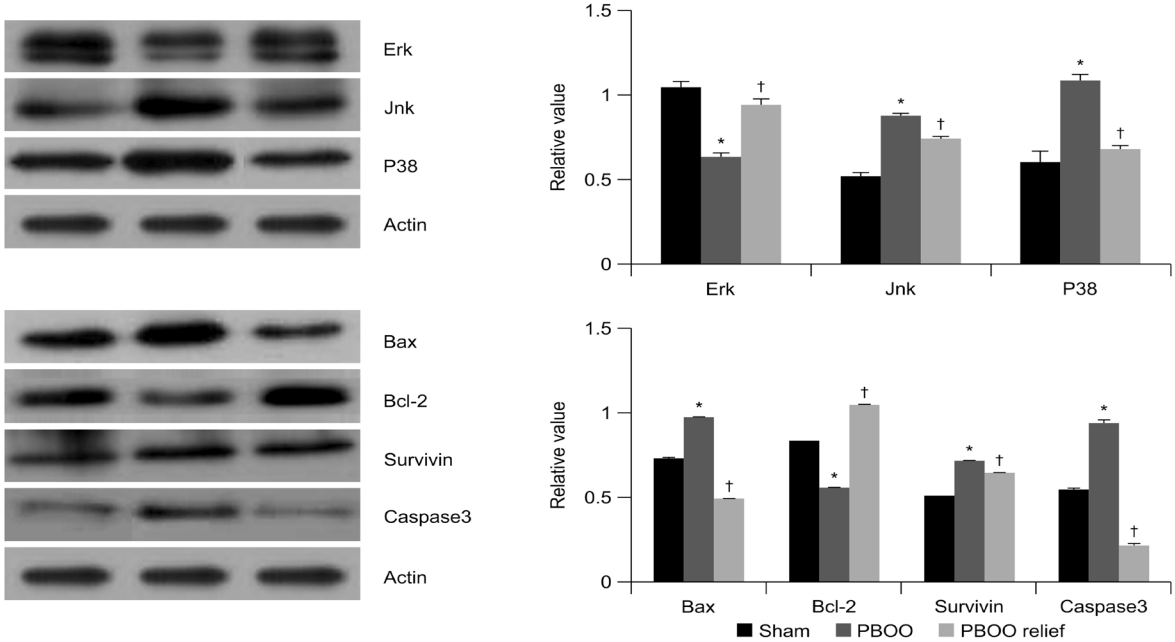


Figure. 3. Representation of the immunoblots. Erk, JNK, p38 MAPKs, Bax, Bcl-2, Caspase-3, and Survivin protein levels of the sham-operated, PBOO only, and PBOO plus subsequent relief group in the rat urothelium. Expression levels of each protein were normalized to β-actin. Data show mean ± SEM. * $P < 0.001$, compared with the sham-operated group; † $P < 0.001$, compared with the PBOO only group.

cells in the urothelium of the rat bladder (Figure 1). Compared with the sham-operated group, the PBOO group exhibited a significant increase in the number of TUNEL positive cells at 2 weeks post-PBOO, as well as a significant decrease in the number of these positive cells 2 weeks after relief. There was no statistically significant difference between the numbers of TUNEL-positive cells in the sham-operated and PBOO plus relief groups ($P < 0.001$).

mRNA expression in the urothelium

We investigated the mRNA expression levels of Erk (515 bp), JNK (354 bp), p38 (468 bp) MAPKs, Bcl-2 (411 bp), Bax (246 bp), caspase-3 (282 bp), and survivin (460 bp) in the urothelium of the bladders from sham-operated, PBOO only, and PBOO relief groups using quantitative PCR (Figure 2). Compared with the sham-operated group, mRNA expression levels of Bax, caspase-3, JNK, and p38 significantly increased in the PBOO only group. A significant decrease in expression levels of these kinases was also observed after PBOO relief ($P < 0.001$). Conversely, mRNA expression levels of Erk and Bcl-2 were significantly decreased in the PBOO group as compared to the sham-operated group ($P < 0.001$). A significant increase in the expression of Erk and Bcl-2 after PBOO relief of the obstruction was also observed ($P < 0.001$). mRNA expression levels of survivin were significantly higher in both the PBOO only, and PBOO relief groups as compared to the sham-operated group ($P < 0.001$). In addition, the expression levels of survivin were significantly different between the PBOO only and PBOO relief groups ($P < 0.001$).

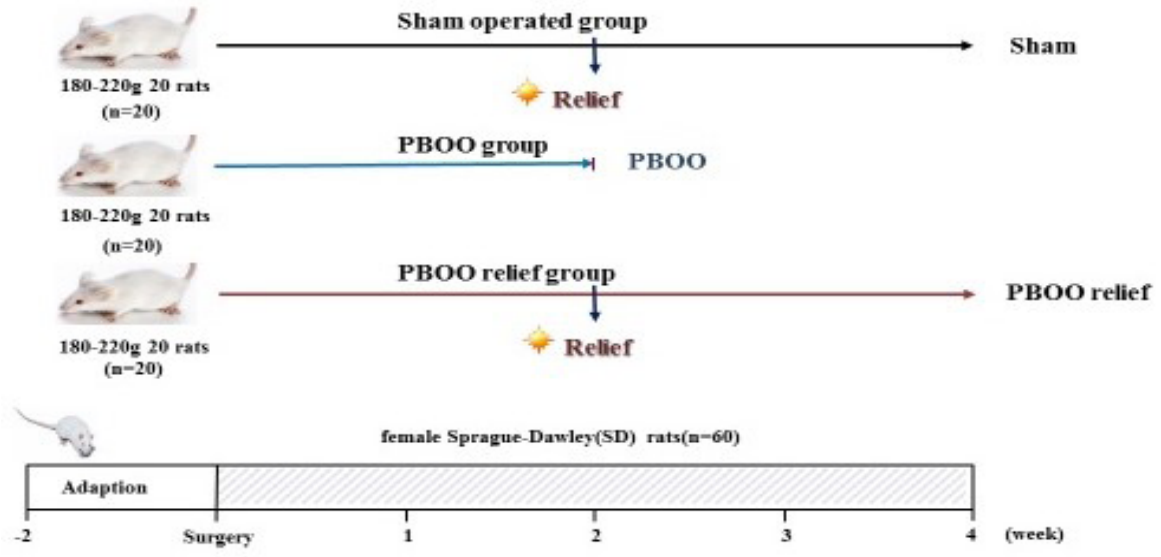
Western blot assay

We evaluated the expressions levels of MAPKs (Erk, JNK, P38), Bcl-2, Bax, caspase-3 and survivin in the urothelium of rat bladders from sham-operated, PBOO only and PBOO relief groups used by western blot analysis (**Figure 3**). In the western blot assay, these proteins showed similar patterns as those obtained from the quantitative PCR results. Western blotting revealed that the protein levels of JNK and p38 MAPK were higher while protein expression levels of Erk were lower in the urothelium sample from the PBOO rats when compared with those of the control rats ($P < 0.001$). Conversely, relief of the obstruction significantly reduced the I/R-related increase in JNK and p38 expression, while simultaneously increasing Erk protein expression levels ($P < 0.001$). When compared with the control group, the protein levels of Bax and caspase-3 increased significantly in the PBOO group. A significant decrease in expression levels of these kinases was also observed after PBOO relief ($P < 0.001$). In contrast, the reduced expression of Bcl-2 as a pro-survival protein in PBOO rats was recovered after PBOO relief ($P < 0.001$). Survivin protein expression levels were significantly increased in both the PBOO and PBOO relief rats as compared to the control rats, and the expression of survivin was significantly different between these two groups ($P < 0.001$).

DISCUSSION

In the present study, we determined an experimental model for analyzing I/R injury in rat urothelium caused by PBOO and subsequent relief. Through the detection of proteins and measurement of mRNA expression of Erk, Jnk, p38 MAPKs, Bax, Bcl-2, caspase-3, and survivin along with the detection of apoptosis, we analyzed the molecular mechanisms associated with I/R in the urothelium of the rat bladder.

Apoptosis has a potent role in maintaining structural integrity and homeostasis in many organisms. In addi-



Supplement 1. Experimental design. 60 rats were divided randomly into three groups: the sham-operated group (Sham; n = 20), the PBOO group (PBOO; n = 20), and the PBOO relief group (PBOO + relief; n = 20). Two weeks after the bladder outlet partial obstruction surgery, The PBOO group was sacrificed, the PBOO relief group and Sham group were removed the knot around the urethra. At 2 weeks after removal of the obstruction, the Sham and PBOO relief groups were sacrificed.

tion, apoptosis also as acts as a major mechanism of cellular destruction in I/R injury of rat urothelium [17]. I/R-induced apoptosis is more prominent in the mucosal layer rather than the detrusor muscle of the bladder[18]. Despite many experimental studies, the mechanisms underlying the pathologic changes in the urothelium caused by PBOO following subsequent relief, along with an analysis of the changes occurring in apoptosis-related proteins have not been demonstrated. Here, we demonstrated, in a rat model, that the number of apoptotic cells significantly increased 2 weeks after the induction of PBOO; moreover, the levels decreased 2 weeks after relief of the obstruction in the rat urothelium took place. In addition, apoptosis induced by I/R after PBOO and subsequent relief was associated with the activation of MAPK pathways and an imbalance of pro-apoptotic and anti-apoptotic proteins in the rat urothelium.

PBOO is a common clinical urologic disease that causes voiding problems, acute urinary retention, and detrusor overactivity, leading to structural instability and dysfunction of the bladder[2]. Human and animal studies have reported that over-distension of the bladder secondary to PBOO causes hypoxia by decreasing blood flow to the bladder[19]. Repeat I/R caused by PBOO can lead to oxidative damage of tissue, inflammatory changes, and cell apoptosis in the urothelium[20]. Ischemic cellular damage is closely related to the activation of MAPKs, including JNK and p38[21]. Conversely, pro-survival kinases such as Erk may also be activated to restore tissue against I/R[22]. Eventually, the relative expressions of pro- and anti-apoptotic kinases may be involved in the determination of the cell survival or death. In the present study, expression levels of JNK and p38 were significantly higher in the PBOO group as compared to the sham operated group; moreover, they reduced significantly after PBOO relief. By contrast, ERK showed the opposite pattern of expression following PBOO and subsequent relief. The relatively higher activity of ERK, compared with p38 and JNK, may favor cell survival during ischemic insult.

Bax and Bcl-2 are considered important apoptosis modulators, and their relative values are used to judge cellular state. It has been reported that Bcl-2 can prevent apoptosis and prolong cell survival, while Bax is known as the apoptotic antagonist of the Bcl-2 protein[23]. Relative activity of Bax and Bcl-2 has been reported in several ischemic animal models, particularly in renal ischemic injury[24]. In this regard, upregulation of Erk and MAPK reportedly prevents apoptosis by blocking translocation to mitochondria[25,26]. However, there are limited data describing the changes of apoptosis-related proteins after PBOO and subsequent relief in the urothelium of rat bladder. In this study, expression of Bax paralleled bladder apoptosis in the PBOO group. In addition, increased expression levels of Bcl-2 and ERK along with decreased expression levels of Bax and caspase-3 were observed after relief of the obstruction. These results suggest that upregulation of ERK and an imbalance of Bcl-2 family proteins could play a critical role in the development and restoration after bladder apoptosis following PBOO and subsequent relief.

Survivin is a member of the inhibitor of apoptosis protein family, and upregulation of survivin is known to inhibit apoptosis in cells[27]. One mechanism through which occurs involves inhibition of caspase-3 and caspase-7, which are downstream effector proteins in the cascade of proteolytic caspase family enzymes. Survivin and Bcl-2 may have common mechanisms guiding transcription and activation that work synergistically to exert anti-apoptotic effects[28]. Several studies reported that the expression of the survivin protein increases after cerebral I/R damage, which affects amelioration of the tissues[29,30]. In the present study, ischemic bladder injury induced the expression of survivin in the PBOO groups. There was statistically significant difference

between the expressions of survivin in the PBOO and PBOO plus relief groups. However, expression of Bcl-2 does not parallel that of expression of survivin following relief of the obstruction. Our results suggest that the effect of survivin on tissue survival after I/R damage by PBOO and subsequent relief in the urothelium of the rat bladder is unclear.

CONCLUSIONS

The results of this study demonstrate that PBOO-induced bladder apoptosis is associated with an imbalance in the MAPK pathways and that the pro-survival ERK signaling cascade is activated in response to I/R damage and associated with restoration of urothelium after relief of the obstruction. Our results enhance current knowledge regarding apoptosis-related molecular changes involved in urothelial ischemic damage and cell survival after PBOO plus subsequent relief. Our study suggests that the role of survivin for cellular recovery against I/R injury after PBOO relief in rat urothelium remains unclear. However, large scale studies must be done to investigate the effect of survivin against I/R in the urothelium and determine its driving mechanisms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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