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ORIGINAL ARTICLE

Decreased expression of zonula occludens-1 and occludin in the bladder urothelium of patients with interstitial cystitis/painful bladder syndrome

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Background/Purpose: Unique barrier properties of the urothelial surface membrane permit urine storage without contents leak into the bloodstream. Previous reports suggested that the bladder urothelial barrier might be compromised in interstitial cystitis/painful bladder syndrome (IC/PBS). We examined the changes of tight junction proteins (zonula occludens-1 (ZO-1) and occludin) in IC/PBS patients.
Methods: Bladder samples were derived from of 32 patients with IC/PBS and eight controls. We detected the tight junction proteins of ZO-1 and occludin expression by immunoblotting, immunohistochemical (IHC) staining and double immunofluorescent (IF) staining with confocal microscopy. Data were analyzed using the Mann-Whitney U-test.
Results: Expression of ZO-1 and occludin in the IC/PBS group was reduced compared to the control group by immunoblotting and IHC staining. Also, the thinning and denudation of urothelium were demonstrated in the IC/PBS group by histological study. IF staining showed the interruption of bladder urothelium in IC/PBS patients under confocal microscopy.
Conclusion: Our data showed that decreased expression of tight junction proteins (ZO-1 and occludin) and interruption of bladder urothelium in IC/PBS patients. Treatment to repair the discontinuous urothelium may be useful to relieve some clinical symptoms of patients with IC/PBS.

The authors have no conflicts of interest relevant to this article.

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Introduction

In interstitial cystitis/painful bladder syndrome (IC/PBS), current theories of pathogenesis include a chronic or subclinical infection, autoimmunity, neurogenic inflammation or bladder urothelial defects.[1] The discontinuous or disruption of bladder urothelium with leaky tight junctions leads to the migration of urinary solutes, in particular potassium, that depolarizes nerve and muscles and cause tissue injury then resulting in patients with bladder pain, urgency, and frequency in urination.[1,2] The molecular mechanisms underlying this disease are not well known.

In mammals, the umbrella cells have the tight junctions that in epithelial cells regulate the movement of ions and solutes between adjacent cells and prevent molecules cannot leak freely across the urothelium. The urothelial barrier function is supported by the structural integrity of tight junction.[2] The tight junction-associated proteins zonula occludens-1 (ZO-1) and occludin to make up a high-resistance barrier in the bladder epithelium.[1–3]

So we examined the tight junction proteins (ZO-1 and occludin) involved with bladder impermeability in biopsies from patients with IC/PBS and controls by immunoblotting, immunohistochemical (IHC) staining and immunofluorescent (IF) staining. The present study may provide the treatment mechanisms for mucosal repair and decreasing the urothelial permeability to improve patient's clinical symptoms.

Materials and methods

Patients and tissue samples

Bladder specimens were obtained from 32 patients (26 women and six men) with IC/PBS who were undergoing cystoscopy under anesthesia for diagnosis or therapeutic bladder distention. Patients met the cystoscopic criteria established by the National Institute of Diabetes, Digestive and Kidney Diseases,[4] including moderate to severe disease symptoms of greater than 6 months duration, and had an average age of 39.4 years (range: 20–55 years). In the study group, a cold-cup biopsy was taken after bladder hydrodistention. Control specimens were obtained from 8 patients (median age: 43.7 years, range: 38–47 years; six women and two men) undergoing a bladder neck suspension procedure for stress urinary incontinence, who showed no evidence of IC/PBS or bladder mucosal disease. All tissues were stored at −80°C for immunoblotting or fixed in formalin for immunostaining. All specimens were removed only after obtaining informed written consent from the patients. This study was approved by the Institutional Review Board of Tri-Service General Hospital.

Antibodies

Five primary antibodies were used in the present study: (1,2) ZO-1: a rabbit polyclonal antibody for immunoblotting and IHC staining (sc-10804, Santa Cruz, CA, USA; dilution 1:400) and a mouse monoclonal antibody for whole mount IF staining (#33-9100, Invitrogen, Camarillo, CA, USA; dilution 1:100); (3) occludin: a rabbit polyclonal antibody for immunoblotting, IHC and whole mount IF staining (#71-1500, Invitrogen; dilution 1: 500 and 1:100 for immunoblotting and immunostaining, respectively); (4) CK7 (cytokeratin 7): a mouse monoclonal antibody (NCL-L-CK7-OVTL, Novocastra, UK; dilution 1:500) for IHC staining; and (5) β-actin: a mouse monoclonal antibody (#8226, Abcam, Cambridge, MA, USA; dilution 1:8000) for immunoblotting.

The secondary antibodies for immunoblotting were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (#0031430, Pierce, Hercules, CA, USA) or goat anti-rabbit IgG (#0031460, Pierce). The secondary antibodies for immunostaining were the commercial kit (PicTure; Zymed, South San Francisco, CA, USA) for IHC staining and the Alexa-Fluor 488 conjugated goat anti-rabbit (Molecular Probes, Eugene, OR, USA; dilution 1:50) or the Alexa Fluor 546 conjugated goat anti-mouse antibodies (Molecular Probes; dilution 1:200) for whole mount IF staining. Preliminary experiments of negative controls (samples stained with only primary or secondary antibodies) demonstrated that either non-specific staining or over-staining of the background was not found (data not shown) in whole mount IF staining.

Immunoblotting

The method used was modified from our previous studies.[5,6] After homogenizing and centrifuging, the supernatants were used for determination of protein concentrations or immunoblotting. Protein concentrations were identified by reagents from BCA Protein Assay Kit (#23225, Pierce, Hercules, CA, U.S.A.), using bovine serum albumin (BSA) as a standard (#23209, Pierce).

For immunoblotting, the antibodies of ZO-1, occludin and β-actin (loading control) revealed molecular weights of about 220, 65 and 42 kDa, respectively. The blots were cut into upper and lower portions at feasible sites for incubation, incubated at 4°C overnight with the diluted primary antibodies, and then incubated with diluted secondary antibody for 1 hour. Finally, the immunoreactive bands were analyzed by using MCID software version 7.0 (Imaging Research, Ontario, Canada). The results were converted to numerical values in order to compare the relative protein abundance of the immunoreactive bands.

Immunohistochemistry

The method of staining and microscopy were modified from our previous studies.[5,7] In brief, the formalin-fixed and paraffin-embedded sections (4 μm) of samples were dewaxed, and then they were rinsed by phosphate buffered saline. The sections were stained with the primary antibody (CK7, ZO-1, or occludin) before being analyzed with the commercial kit. Negative control experiments, in which phosphate buffered saline was used instead of the primary antibody, were conducted to confirm the positive results of CK7, ZO-1, or occludin (data not shown). Finally, the sections were counterstained with hematoxylin (Cat. No.1.05175.0500, Merck, Darmstadt, Germany) and rinsed by tap water. Sections were observed using a light

microscope (BX50, Olympus, Tokyo, Japan) and the micrographs were pictured.

Whole mount double immunofluorescent staining and confocal microscopy

The method used for IF staining and microscopy was a modified version of that used in our previous studies.[6,7] The whole samples were fixed overnight with formalin, and then they were washed by washing buffer (phosphate buffered saline). The samples were postfixed and permeabilized with 100% methanol at −20°C overnight. Samples were then incubated in PBST (phosphate buffered saline with 0.05% Triton) for 10 minutes. After washing, the samples were incubated in 2 mg/ml proteinase K for 10 minutes at 37°C. The samples were then incubated for 30 minutes in blocking buffer (phosphate buffered saline containing 5% (wt/vol; BSA) to minimize nonspecific binding. The samples were then incubated at 4°C overnight with the diluted primary antibodies (ZO-1 or occludin). Following incubation, the samples were washed, and they were then exposed to the respective secondary antibody for 1 hour. Finally, the sections were covered by a slip with mounting solution (Zymed, Carlton Ct., South San Francisco, California, USA) before being viewed by confocal laser scanning microscopy.

To determine and compare the localization of specific proteins, IF-stained samples were observed with a confocal laser-scanning microscope (Leica Lasertechnik, Heidelberg, Germany). The 488-nm argon−ion laser and the 543-nm helium−neon laser were used for observing the fluorescent staining of Alexa Fluor 488 and Alexa Fluor 546, respectively, to give the appropriate excitation wavelengths. The micrographs taken from each photomultiplier were subsequently merged so that the different-colored labels could be simultaneously visualized.

Statistical analysis

The difference between groups was analyzed by the Mann-Whitney U-test using the SPSS 12.0 software (SPSS, Chicago, IL, USA), and significance was established when $p < 0.05$. Values are expressed as the means ± standard error (SE).

Results

The decreased expressions of ZO-1 and occludin in the IC/PBS group compared to the control group by immunoblotting and IHC staining. The relative intensities of the ZO-1 and occludin were higher approximately 1.8- and 1.7-fold in the control group than in the study group, respectively (93.79 ± 3.75 vs. 52. ± 4.25 and 103.23 ± 7.51 vs. 60.20 ± 6.10) These results are shown in Figs. 1 and 2. We use the CK7 stain to identify the thickness of bladder urothelial layer that revealed thicker and intact urothelium of the control group, conversely, the bladder exhibited thinning and denudation of urothelium in the study group (Fig. 3A and D). IHC staining for ZO-1 and occludin showed stronger immunoreactivity in the control group than in the study group (Fig. 3B,C,E and F). By confocal microscopic findings, the results of longitudinal micrographs showed

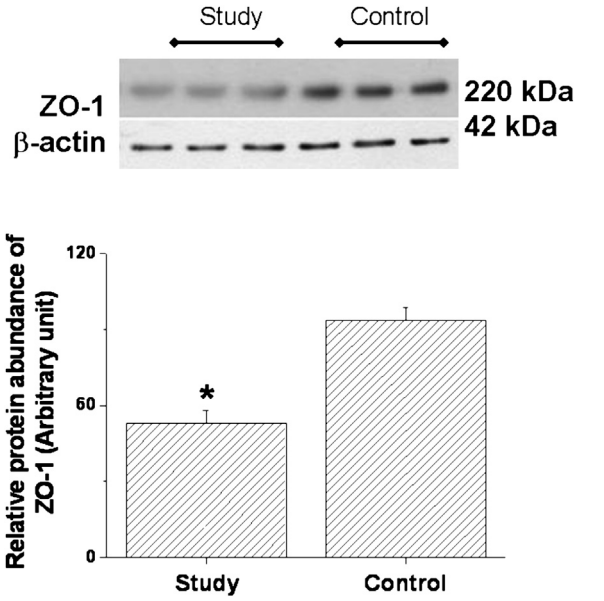

Figure 1 Representative immunoblot and relative intensities of ZO-1 protein in study and control groups. β-actin was used as loading control. The asterisk indicates a significant difference ($p < 0.05$). ZO-1 = zonula occludens-1.

that the expression of ZO-1 and occludin were discontinuous distribution in bladder urothelium of the IC/PBS group (Fig. 4). Moreover, the decreased tight junction proteins expression causing thinning cell junction and interrupted urothelial layer of the study group (Fig. 5A and B) compared with the control group (Fig. 5D and E). The merged images showed colocalization of ZO-1 and occludin in both groups (Fig. 5C and F).

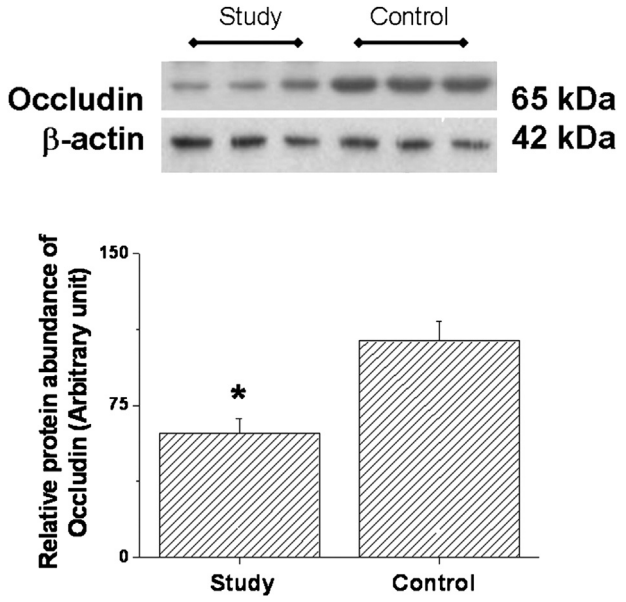

Figure 2 Representative immunoblot and relative intensities of occludin protein in study and control groups. β-actin was used as loading control. The asterisk indicates a significant difference ($p < 0.05$).

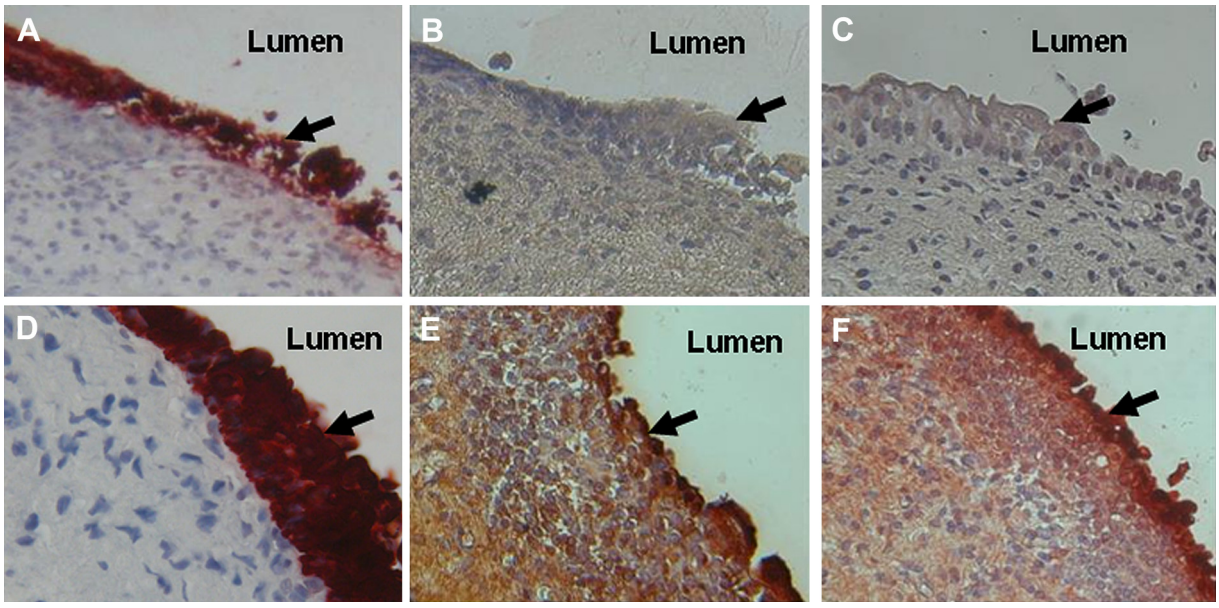

Figure 3 (A—C) Micrographs of representative sections of study; and (D—F) and control groups after CK7 (A and D), ZO-1 immunostaining (B and E), or occludin immunostaining (C and F). Black arrow: urothelium. Magnification: 400×. ZO-1 = zonula occludens-1.

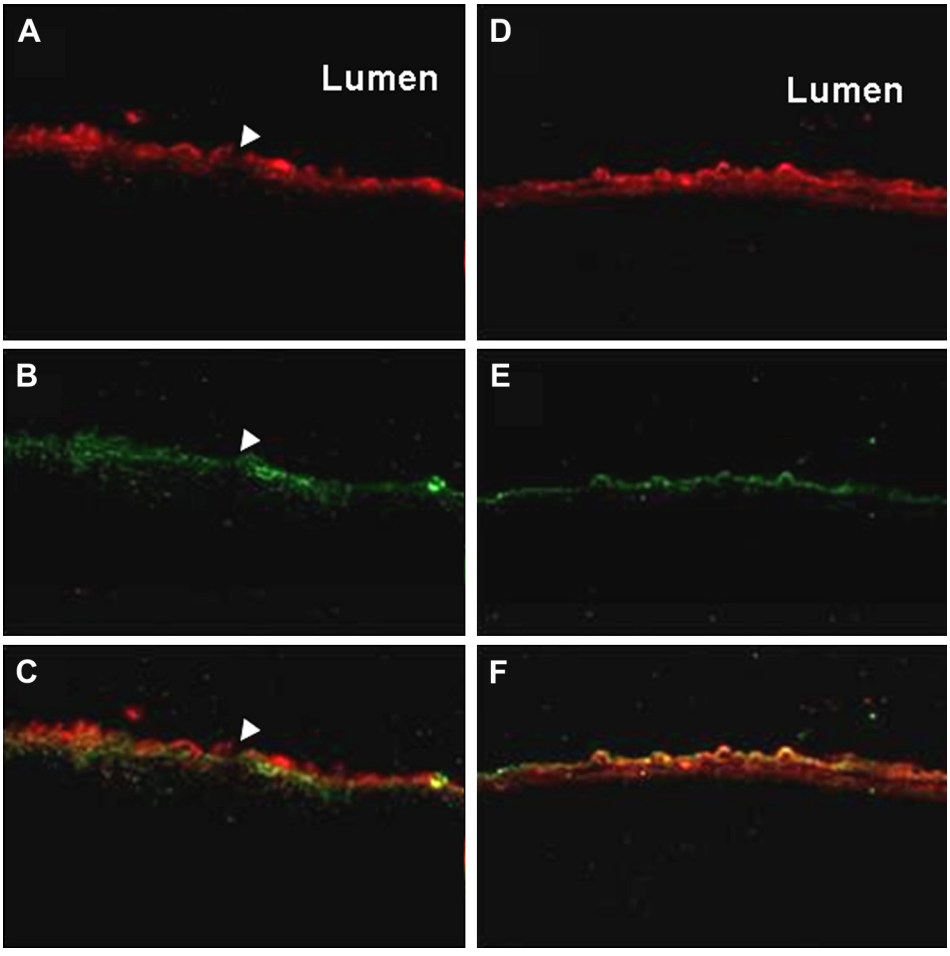

Figure 4 (A—C) Representative longitudinal micrographs of patients with study; and (D—F) control groups after double immunofluorescent staining and confocal laser scanning microscopy for ZO-1 (red; A and D), occludin (green; B and E) and merged image (C and F). Arrowhead: discontinuous. Magnification: 200×. ZO-1 = zonula occludens-1.

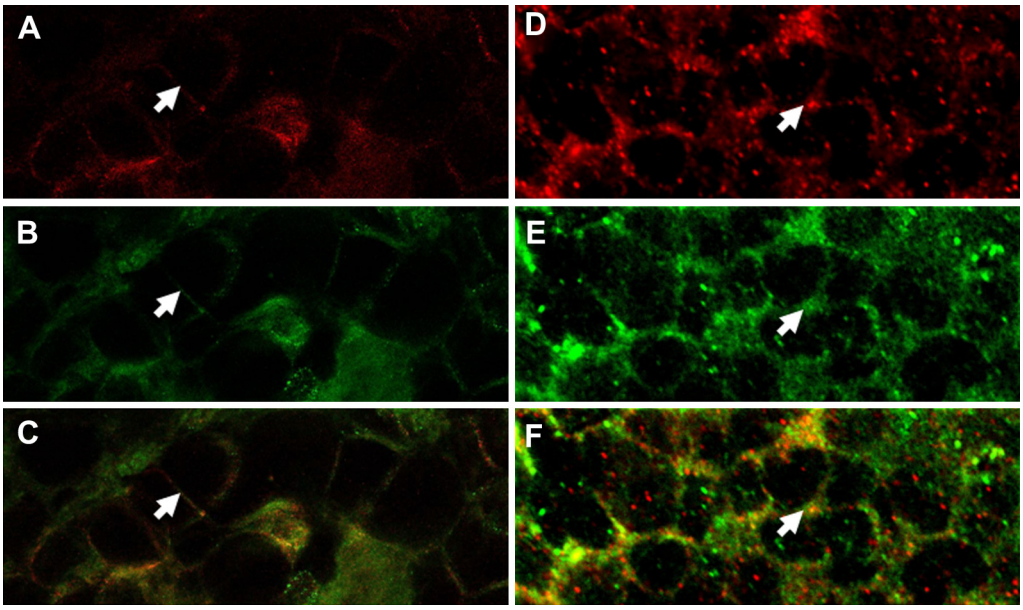

Figure 5 (A—C) Representative horizontal micrographs of patients with study; and (D—F) control groups after double immunofluorescent staining and confocal microscopy for ZO-1 (red; A and D), occludin (green; B and E) and merged images showed colocalization of ZO-1 and occludin (C and F). Arrows: cell junction. Magnification: 1600×. ZO-1 = zonula occludens-1.

Discussion

The role of tight junctions is to allow urothelium to serve as a barrier between urine and its solutes and the underlying bladder.[8,9] In IC/PBS, it has long been recognized as a disease of the urothelial barrier dysfunction.[8,10] The IC/PBS bladder loses its normal impermeability (leaky epithelium) combined with potassium diffusion into the bladder wall that causes depolarization of sensory nerves and muscles and injures tissue which lead the pelvic pain, urgency, and frequency of urination.[8,11,12] The urothelial barrier function is supported by the structural integrity of tight junctions. Tight junctions are the most apical junction complexes, and they were recently shown to include ZO-1 and occludin (transmembrane protein) in the mammalian bladder.[13] The decreased expression of tight junction proteins causing an increase of bladder urothelial permeability that results in passage of small ions across the blood—urine barrier in patients with IC/PBS.

It has been hypothesized that potassium fulfills this role because its levels in urine are quite high, ranging from 30—120 mEq/L.[8,10] Conversely, the potassium concentration is only 3.5—4.5 mEq/L of serum in human. Such levels not only depolarize nerves and muscles and cause tissue injury,[8,10] these may be also the reasons to lead clinical symptoms when potassium leakage in IC/PBS patients. Furthermore, it could increase potassium leakage due to the combination of osmotic and increased hydrostatic pressure.[10] Simultaneously, to decrease blood perfusion and exacerbates the urothelial defect causing more significant symptoms when bladder distention in IC/PBS patients.

Previous studies have reported that antiproliferative factor is a small glycoprotein could inhibit cell growth then decrease the expression of the tight junction proteins zonula occludens-1 and occludin causing an increase of paracellular permeability in IC urothelium.[13] Hauser et al. have also reported that the loss of barrier function markers in the IC urothelium.[11] The loss of the glycosaminoglycan layer was associated with abnormal expression of proteoglycan core proteins biglycan and perlecan on the luminal layer. And the defects in the expression of perlecan (located on the basal laminae) or syndecan (located on the cell surface) in IC urothelium, may be reduced of critical growth factors such as fibroblast growth factor-7 which regulates the growth of urothelial cells or to affect downstream signaling by the receptor.[11] Recent findings suggest that the higher levels of cell apoptosis and abnormal E-cadherin expression in the bladder urothelium of patients with IC/PBS.[14] These above mechanisms might decrease the thickness of bladder urothelium as our findings then causing an increase of urothelial permeability and associated with patient's clinical symptoms.

The establishment and maintenance of tight junctions function are crucial to the bladder urothelium because its defective integrity plays a role in the pathogenesis of IC/PBS. Currently, some symptoms of IC/PBS patients would be relieved by the oral pentosan polysulfate (Elmiron, Ivax Pharmaceuticals, Miami, FL, U.S.A) or intravesical instillation of hyaluronic acid and liposome.[9,15—18] Our findings might provide the treatment mechanisms to repair the discontinuous urothelium and to decrease the urothelial permeability of patients following the above treatments.

Taken together, our data showed that decreased expression of tight junction proteins (ZO-1 and occludin) and interruption of bladder urothelium in IC/PBS patients under confocal microscopy. These findings might provide the basis of treatment mechanisms to repair the discontinuous urothelium for relieving some clinical symptoms of patients with IC/PBS.

Acknowledgments

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References

1. Keay S. Cell signaling in interstitial cystitis/painful bladder syndrome. *Cell Signal* 2008;**20**:2174—9.
2. Acharya P, Beckel J, Ruiz WG, Wang E, Rojas R, Birder L, et al. Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium. *Am J Physiol Renal Physiol* 2004;**287**:F305—18.
3. Slobodov G, Feloney M, Gran C, Kyker KD, Hurst RE, Culkin DJ. Abnormal expression of molecular markers for bladder impermeability and differentiation in the urothelium of patients with interstitial cystitis. *J Urol* 2004;**171**:1554—8.
4. Gillenwater JY, Wein AJ. Summary of the National Institute of arthritis, diabetes, digestive and kidney diseases workshop on interstitial cystitis, National Institutes of Health, Bethesda, Maryland, August 28-29, 1987. *J Urol* 1988;**140**:203—6.
5. Lee JD, Lee TH, Cheng WH, Jeng SY. Involved intrinsic apoptotic pathway of testicular tissues in varicocele-induced rats. *World J Urol* 2009;**27**:527—32.
6. Jeng SY, Wu SM, Lee JD. Cadmium accumulation and metallothionein overexpression in internal spermatic vein of patients with varicocele. *Urology* 2009;**73**:1231—5.
7. Lee JD, Lee MH. Increased expression of hypoxia-inducible factor-1α and vascular endothelial growth factor associated with glomerulation formation in patients with interstitial cystitis. *Urology* 2011;**78**. 971:11—5.
8. Parsons CL. The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis. *Urology* 2007;**69**:9—16.
9. Dell JR, Mokrzycki ML, Jayne CJ. Differentiating interstitial cystitis from similar conditions commonly seen in gynecologic practice. *Eur J Obstet Gynecol Reprod Biol* 2009;**144**:105—9.
10. Parsons CL. The role of a leaky epithelium and potassium in the generation of bladder symptoms in interstitial cystitis/overactive bladder, urethral syndrome, prostatitis and gynaecological chronic pelvic pain. *BJU Int* 2011;**107**:370—5.
11. Hauser PJ, Dozmorov MG, Bane BL, Slobodov G, Culkin DJ, Hurst RE. Abnormal expression of differentiation related proteins and proteoglycan core proteins in the urothelium of patients with interstitial cystitis. *J Urol* 2008;**179**:764—9.
12. Erickson DR, Schwarze SR, Dixon JK, Clark CJ, Hersh MA. Differentiation associated changes in gene expression profiles of interstitial cystitis and control urothelial cells. *J Urol* 2008; **180**:2681—7.
13. Zhang CO, Wang JY, Koch KR, Keay S. Regulation of tight junction proteins and bladder epithelial paracellular permeability by an antiproliferative factor from patients with interstitial cystitis. *J Urol* 2005;**174**:2382—7.
14. Shie JH, Kuo HC. Higher levels of cell apoptosis and abnormal E-cadherin expression in the urothelium are associated with inflammation in patients with interstitial cystitis/painful bladder syndrome. *BJU Int* 2011;**108**:E136—41.
15. Shao Y, Shen ZJ, Rui WB, Zhou WL. Intravesical instillation of hyaluronic acid prolonged the effect of bladder hydrodistention in patients with severe interstitial cystitis. *Urology* 2010;**75**:547—50.
16. Parsons CL. Interstitial cystitis and lower urinary tract symptoms in males and females-the combined role of potassium and epithelial dysfunction. *Rev Urol* 2002;**4**(Suppl. 1):S49—55.
17. Van Agt S, Gobet F, Sibert L, Leroi AM, Grise P. Treatment of interstitial cystitis by intravesical instillation of hyaluronic acid: A prospective study on 31 patients. *Prog Urol* 2011;**21**:218—25.
18. Chuang YC, Lee WC, Lee WC, Chiang PH. Intravesical liposome versus oral pentosan polysulfate for interstitial cystitis/painful bladder syndrome. *J Urol* 2009;**182**:1393—400.