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Urinary Bladder Mucosal Responses to Ischemia

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Abstract

Purpose—The objectives of this study were to examine the expression of various cellular proteins within the urothelium (UT) and lamina propria (LP) following chronic bladder ischemia in the rat urinary bladder.

Materials and Methods—Urinary bladders were removed from adult Sprague-Dawley rats 8 weeks after creation of bladder ischemia, and from sham controls. Immunocytochemistry was used to examine distribution of LP-vimentin-immunoreactive (IR) cells and connexins (Cx26; Cx43); and Western immunoblotting or ELISA for proteins involved in UT barrier and sensory functions.

Results—Ischemia was associated with a significant increase in LP-vimentin-IR cells and increased expression of the gap-junction proteins Cx26 and Cx43 within the bladder urothelium as compared to sham control. Ischemia also resulted in an increased ($p<0.05$) expression level of the junctional marker (ZO-1), and non-significantly increased expressions of the trophic factor nerve growth factor (NGF) as well as norepinephrine (NE).

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Authors Contribution:
Sunagawa: Project development, data collection, manuscript writing
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Conclusions—Our findings reveal that chronic ischemia alters a number of proteins within the urothelium and underlying lamina propria. These proteins are involved in barrier function, remodeling, repair as well as intercellular communication. The increased expression of LP-vimentin-IR cells suggests that changes in cell-cell interactions could play a role in ischemia-induced changes in bladder activity.

Keywords

Urothelium; lamina propria; sensory; barrier; blood flow

Introduction

The prevalence of bladder dysfunctions, such as lower urinary tract symptoms (LUTS) including the overactive bladder (OAB) syndrome, increases due to normal aging and in various disorders such as hypertension and diabetes, which are reported to be associated with reduction in urinary bladder blood flow (chronic bladder ischemia) [1]. There are also reports of decreased bladder perfusion in patients with LUTS, and that the administration of α_1-adrenoceptor (AR) antagonists significantly improves blood flow and reduces patient symptoms [2].

A number of animal models (such as partial outlet obstruction or atherosclerosis-induced bladder ischemia in rodents and rabbits) have been used to study mechanisms underlying ischemia induced bladder dysfunction [3–5]. These studies have reported increased bladder activity and elevated cytokines as well as markers for oxidative stress. A reduction in oxygen tension in the bladder may be linked with changes in bladder nerves and markers for apoptosis. Urinary bladders from patients with bladder overactivity (and periodic ischemic episodes) have been shown to exhibit a patchy innervation that has been linked with increased activity [6].

The urinary bladder urothelium is a highly efficient barrier and thus plays a very important role in maintenance of normal bladder function [7,8]. Though the urothelium maintains a tight barrier to ion and solute flux, a number of factors including mechanical or chemical trauma, hormonal changes as well as hypoxia and ischemia are able to modulate the barrier function. For example, short-term (1/2–2 hours) induction of experimental ischemia in rodents has been shown to result in increased permeability of the blood-urine barrier with associated urothelial damage [9]. However, there is little information as to how chronic hypoxia or ischemia alters the properties of the urothelium and lamina propria. In this pilot study we utilized our previously described model of chronic bladder ischemia in the rat [4], and characterized the expression of some cellular proteins that may be involved in maintaining urothelial barrier (and sensory) functions. In addition, we examined the distribution of vimentin-immunoreactive (IR) cells within the lamina propria, which may constitute a link between the urothelium and underlying bladder nerves.

MATERIALS AND METHODS

Animals

Bladder samples from the lateral wall were obtained from adult (16-week old) Sprague-Dawley male rats that had been subjected to arterial injury for characterization of a chronic ischemia model [4,10]. In brief, rats underwent mechanical endothelial injury of the iliac arteries: an air-filled balloon catheter was inserted into and subsequently withdrawn from the common iliac artery via the femoral artery. The procedure was repeated 10 times on each side. The animals also received a 2% cholesterol diet for 8 weeks. These rats (termed ischemia rats, n = 10) developed neo-intimal hyperplasia and vascular occlusion of the common iliac arteries, reduced bladder blood flow, morphological changes in the bladder wall and functional changes, as described previously [4,10]. The controls (n = 10) were incised bilaterally in the inguinal region without balloon endothelial injury and received a regular diet for the same period.

Immunohistochemistry

Excised urinary bladders were embedded in optimum cutting temperature compound (Tissue-Tek OCT, Sakura Finetek, Torrance, CA), snap frozen and stored at −80°C. Serial cryosections (6μm) were mounted onto microscope slides (Fisher Scientific, Pittsburgh, PA), fixed (4% paraformaldehyde) and washed in phosphate-buffered saline (PBS). The slides were incubated with permeabilizing/blocking solution (0.5% Triton X-100 and 10% goat or donkey serum in PBS) prior to incubation with primary antibodies. These include incubation with mouse monoclonal vimentin (Sigma-Aldrich, St. Louis, MO; 1:500), goat polyclonal Cx26 (Abcam; Cambridge, MA, 1:1000) or mouse monoclonal Cx43 (Millipore, Billerica, MA; 1:1000) and control experiments were done by eliminating the primary antibody. Co-localization studies were conducted using rabbit polyclonal cytokeratin 17 (CK17, Abcam, Cambridge, UK; 1:2000). The tissue was washed with PBS and incubated with the fluorophore-tagged secondary antibodies (2h): donkey anti-mouse Alexa Fluor 555 (1:1000) for vimentin and Cx43, a donkey anti-goat Alexa Fluor 555 (1:1000) for Cx26 and a donkey anti-rabbit Alexa Fluor 488 (1:2000) (Life Technologies, Carlsbad, CA) for CK17. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Life Technologies; 1:2500). The slides were mounted in medium ProLong Gold Antifade Reagent (Life Technologies) and imaged on a BX-62 Olympus upright fluorescent microscope using CImaging software (Hamamatsu Photonics, Bridgewater, NJ). Background immunofluorescence was assessed in the absence of primary and secondary antibodies.

We assessed the number of vimentin-IR cells that were also DAPI-positive within a fixed area (100μm × 50μm) in the suburothelium. The expression of connexins (Cx26; Cx43) was analyzed and quantified within a demarcated region (50μm × 20μm) within the urothelium/suburothelium using ImageJ software (http://rsb.info.nih.gov) whereby the tissue sections were converted into black-and-white bitmap after color-filtration to render images for the Cx alone. The relative area of Cx labeling (red) was measured in relation to the number of nuclei (DAPI, blue); all values were reported as an average of at least twenty micrographs per bladder.

Western immunoblotting and ELISA

The urinary bladder mucosa was dissected from the underlying smooth muscle tissues and homogenized in HBSS (5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHO_3$, 0.3 mM Na_2HCO_3, 0.3 mM Na_2HPO_4, 5.6 mM glucose and 10 mM Hepes, pH 7.4) containing complete protease inhibitor cocktail (1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, 1:100). The homogenate was centrifuged (13,000rpm; 15min) and the membrane protein fraction was prepared by suspending the membrane pellets in lysis buffer (0.3 M NaCl, 50 mM Tris-HCl, 0.5% Triton X-100) and the same concentration of protease inhibitors as above. The suspensions were incubated on ice and centrifuged (13,000 rpm; 15 min) and the protein concentrations of the combined supernatants were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL).

After denaturation (100°C for 5min), lysate from each sample was separated on an SDS-PAGE gel using a standard Western protocol [11]. Proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% Milk TBS-T (1h), rinsed in TBS-T, and incubated (overnight at 4°C) with primary antibodies diluted in 5% Milk TBS-T. The antibodies used were CK17 (48kDa, Abcam; 1:2000), zona occludens-1 (ZO-1) (220 kDa, Millipore; 1:2000), uroplakin III (UP3) (47 kDa, American Research Products, Waltham, MA; 1:500) and vascular endothelial growth factor (VEGF, 43 kDa, Abcam; 1:1000). The membranes were then incubated with HRP-secondary antibodies (Santa Cruz, Dallas, TX) for 1hr, developed with WesternBright (Advansta, Menlo Park, CA), and exposed to X-film. The volume of each band was determined using a Personal Densitometer SI (Molecular Probes, Carlsbad, CA). The membranes were stripped using a membrane recycling kit (Alpha Diagnostic International, San Antonio, TX) and re-probed overnight with rabbit anti-β-actin (47 kDa, Abcam; 1:200,000) as a loading control. Single immunoreactive bands were observed for the targets aforementioned.

The levels of nerve growth factor (NGF) and norepinephrine (NE) were measured using ELISA. Equal amounts of whole cell lysates for NGF ELISA were acidified (1 N HCl, pH 2.0–3.0) after dilution with 4 volumes of D-PBS (0.02% KCl, 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 0.0133% $CaCl_2 \cdot 2H_2O$ and 0.01% $MgCl_2 \cdot 6H_2O$, pH 7.35, Sigma). The samples were assayed using ELISA commercial kits (NGF: Promega, Madison, WI) (NE: LDN, Nordhorn, Germany) according to manufacturer instructions. Plates were read at 450 nm using a SpectraFluor Plus (Tecan, Maennedorf, Switzerland).

Statistical analysis

Values are shown as the means ± SEM and were analyzed using Student's *t*-test and $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of ischemia on Cx distribution within the urothelium and suburothelium

A number of studies have demonstrated alterations in connexin expression in bladder pathologies such as spinal cord injury that may contribute to altered bladder activity [12].

We measured expression of Cx26, associated with the urothelium, and also Cx43 that has been associated with suburothelial interstitial cells. The relative area of Cx labeling (red, see figures 1A–D) was measured in relation to the number of nuclei (DAPI-blue) within the same field to record any relative changes in Cx within the bladder relative to cell number. Cx26 was expressed within the urothelial layers, with increased intensity of staining in the ischemic bladder as compared to control (Figure 1A–B). The data for both Cx26 and Cx43 were examined and quantified by analyzing a specific area (example indicated within the white dotted line) and recording percent area of Cx labeling normalized to the number of DAPI-labeled nuclei to account for variability in cell density. Table 1 represents the mean change in Cx26 expression within the urothelium in ischemia versus control groups. Cx43 label was observed in both the urothelium as well as underlying suburothelium. Figure 1C–D is representative of Cx43 staining in both normal and ischemic conditions. Similar to Cx26, the density of Cx43 labeling (percent area per DAPI-labeling particle) was significantly increased in the ischemic bladder versus that of control (Table 1).

Effect of ischemia on lamina propria vimentin-immunoreactivity (IR)

The distribution of vimentin-IR cells was examined within the lamina propria in both control and ischemic bladders. Vimentin-IR cells had a typical morphology (Figure 1), many with several branches or processes that were more prevalent in ischemia. A typical ‘stellate’ shape in ischemia is indicated in Figure 1F, which may be indicative of lamina propria interstitial cells (LP-IC) as described by others [13]. Omission of primary antibodies from the incubation buffer completely attenuated secondary antibody labeling (data not shown). In addition, the number of vimentin-positive cells for each field of view was counted and expressed per $100\mu m^2$. The average number of vimentin-positive cells was significantly increased in ischemia compared with control (Table 1).

Effect of ischemia on expression of mucosal proteins

Because a number of chronic bladder conditions have been linked with alterations in the bladder barrier, we examined various structural and differentiation markers expressed within the mucosa. In chronic ischemia, the terminal differentiation marker uroplakin III (UP-III) remained unchanged relative to control (Table 1). In contrast, the tight junction protein ZO-1 or zonula occludens was significantly elevated in ischemia, which may suggest a reorganization that occurs following ischemic insult.

We also examined expression of the trophic factors nerve growth factor (NGF) which has been shown to be altered in pathology and may contribute to changes in bladder function with ischemic injury. We found a trend (though not significant) toward increase in NGF expression within the ischemic bladder mucosa (Table 1). Similarly, we also found a trend toward decreased expression of norepinephrine (NE) in the ischemic bladder mucosa (Table 1).

Discussion

The urothelium is distinguished from most other mammalian epithelia by having an extremely slow turnover, estimated at one cell division per cell per year [14]. However,

regeneration of the urothelium is rapid and effective in order to maintain a barrier to urine following tissue injury [15]. Ischemia can rapidly mediate a breakdown of the mucosal layer as a permeability barrier [9], probably by an effect on the on the cellular metabolism. The mucosa seems to have a higher sensitivity to ischemic insults that the rest of the bladder wall since the rate of glucose metabolism to lactic acid of the mucosa was more than three-fold that of the smooth muscle [16].

The induction of a chronic bladder ischemia in the rat by endothelial injury of the iliac arteries (combined with a 2% cholesterol diet) results in high levels of oxidative stress markers and pro-inflammatory cytokines [10]. Although we did not directly measure the reduction of bladder blood flow in all animals, the degree of neo-intimal hyperplasia of the common iliac arteries was quantified and similar in the individual animals [4, 10]. This was taken as indicator ensuring that a relevant chronic ischemia had been created.

The current study revealed a number of changes within the urothelium and lamina propria, which could play a role in the ischemia-associated bladder dysfunction. Though urothelial damage has been reported in response to acute (1/2 – 2 hours) experimental ischemia [9], less is known about effects of chronic ischemic or hypoxic effects on the urothelium. We found that chronic ischemia may lead to an increase in zonula occludens (ZO) protein-1, a component of both tight and adherens junctions, which is also expressed at sites of cell-cell contact. Increased ZO-1 expression has been reported in human melanoma [17] and during remodeling of cardiac gap junctions [18]. In addition, we also found significant increases in the gap junction proteins, connexin 26 and 43, within the urothelium following chronic ischemia. Changes in expression levels of connexins in both (animal and human) smooth muscle as well as urothelium have been reported in pathologies such as in the neurogenic or obstructed bladder and may be linked with detrusor overactivity [19]. Increased expression of these gap junction proteins may mediate changes in permeability and could provide a more efficient means for cell-to-cell communication within and between the urothelium and underlying structures.

The urothelium and underlying lamina propria (LP), which is composed of a number of cell types, are thought to function together as a signaling system [7,20]. Within the LP there exists a layer of spindle shaped cells (often termed LP-interstitial cells or LP-ICs) that typically label positive for the intermediate filament marker vimentin, and have close contacts with bladder nerves. Because these cells may constitute a structural and functional link between the urothelium and underlying sensory nerves, there has been speculation that the LP-IC cells may be involved in pathophysiology of various urinary tract disorders [7,20]. In the present study, we find that chronic ischemia also affects the distribution and structure (increased cellular processes) of vimentin-immunoreactive (IR) cells within the suburothelium. It is also possible that the phenotype of these vimentin-IR cells may be altered in stressful conditions such as ischemia similar to that described in the neurogenic bladder [21]. Changes in the expression or number of these cells after an ischemic insult could also change the transduction of sensory signals in the bladder wall as well as in the maintenance of bladder and urothelial homeostasis. Taken together, it is likely that ischemia alters factors that can eventually result in a remodeling of structural proteins and altered

synthesis and/or release of mediators within the bladder mucosa that impact the barrier and sensory functions of the bladder.

Studies have shown that neurotrophins such as nerve growth factor (NGF) can facilitate gap junction communication by phosphorylation of connexin hemichannels [22]. Liang et al (2010) found decreased NGF immunofluorescence and elevated NGF mRNA in the bladder 1–4 weeks after bilateral ligation of the vesical arteries of female rats [23] We found no significant change in the expression of NGF in the ischemic bladder mucosa after 8 weeks of ischemia (even if there was a numeric increase), but it is possible that temporal changes in NGF levels could have been missed.

Sustained ischemia (4–6 h) has been shown to result in excessive release of NE, likely from a variety of sources [24]. We detected a non-significant decrease of the *expression* of NE within the bladder mucosa of chronic ischemic rats, and whether or not this may be of importance for the release NE from sympathetic terminals and possibly from other cells (such as the urothelium) can only be speculated on. Most probably, ischemic changes related to NE release may be decreased over time.

Conclusions

The present pilot study demonstrated that a reduction in urinary bladder blood flow (using our rat model for chronic ischemia) alters a number of proteins within the urothelium and underlying lamina propria. Many of these proteins are involved in cellular communication and remodeling/repair mechanisms. Thus the increased voiding frequency previously demonstrated using this same rat model for chronic bladder ischemia may, in part, involve altered expression of proteins involved in both sensory and barrier functions. This is likely to be important in a number of bladder conditions, including normal aging, whereby reduction in blood flow and oxygen tension may be an underlying factor associated with development of LUTS. To confirm and expand these observations a larger study is needed. Such a study including further vascular damage by the addition of the NOS inhibitor L-NAME is underway.

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References

1. Ponholzer A, Temmi C, Wehrberger C, et al. The association between vascular risk factors and lower urinary tract symptoms in both sexes. Eur Urol. 2006; 50:581–6. [PubMed: 16519993]
2. Pinggera GM, Mitterberger M, Pallwein L, et al. Alpha-blockers improve chronic ischaemia of the lower urinary tract in patients with lower urinary tract symptoms. BJU Int. 2008; 101:319–24. [PubMed: 18005199]
3. Gosling JA, Kung LS, Dixon JS, et al. Correlation between the structure and function of the rabbit urinary bladder following partial outlet obstruction. J Urol. 2000; 163:1349–56. [PubMed: 10737542]
4. Nomiya M, Yamaguchi O, Andersson KE, et al. The effect of atherosclerosis-induced chronic bladder ischemia on bladder function in the rat. Neurourol Urodyn. 2012a; 31:195–200. [PubMed: 21905085]

5. Azadzoi KM, Tarcan T, Siroky MB, et al. Atherosclerosis-induced chronic ischemia causes bladder fibrosis and non-compliance in the rabbit. J Urol. 1999; 161:1626–35. [PubMed: 10210430]
6. Brading A, Pessina F, Esposito L, et al. Effects of metabolic stress and ischaemia on the bladder, and the relationship with bladder overactivity. Scand J Urol Nephrol Supp. 2004; 215:84–92.
7. Birder LA, Andersson KE. Urothelial Signaling. Physiol Reviews. 2013; 93:653–80.
8. Khanderwal P, Abraham SN, Apodaca G. Cell biology and physiology of the uroepithelium. Am J Physiol Renal Physiol. 2009; 297:F1477–501. [PubMed: 19587142]
9. Korosec P, Jezernik K. Early cellular and ultrastructural response of the mouse urinary bladder urothelium to ischemia. Virchows Arch. 2000; 436:377–83. [PubMed: 10834542]
10. Nomiya M, Sagawa K, Yazaki J, et al. Increased bladder activity is associated with elevated oxidative stress markers and proinflammatory cytokines in a rat model of atherosclerosis-induced chronic bladder ischemia. Neurourol Urodyn. 2012b; 31:185–9. [PubMed: 21953769]
11. Bolt MW, Mohoney PA. High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide, gel electrophoresis. Anal Biochem. 1997; 247:185–92. [PubMed: 9177676]
12. Ikeda Y, Fry C, Hayashi F, et al. Role of gap junctions in spontaneous activity of the rat bladder. Am J Physiol Renal Physiol. 1997; 293:F1018–25. [PubMed: 17581924]
13. McCloskey DK. Bladder interstitial cells: an updated review of current knowledge. Acta Physiol. 2013; 207:7–15.
14. Limas C. Proliferative state of the urothelium with benign and atypical changes. Correlation with transferrin and epidermal growth factor receptors and blood group antigens. J Pathol. 1993; 171:39–47. [PubMed: 8229455]
15. Baskin RS, Sutherland AA, Thomson HT, et al. Growth factors in bladder wound healing. J Urol. 1997; 157:2388–2395. [PubMed: 9146676]
16. Hypolite JA, Longhurst PA, Gong C, et al. Metabolic studies on rabbit smooth muscle and mucosa. Mol Cell Biochem. 1993; 125:35–42. [PubMed: 8264570]
17. Smalley KS, Brafford P, Haass NK, et al. Up-regulated expression of zonula occludens protein-1 in human melanoma associates with N-cadherin and contributes to invasion and adhesion. Am J Pathol. 2005; 166:1541–54. [PubMed: 15855653]
18. Barker RJ, Price RL, Gourdie RG. Increased association of ZO01 with connexin 43 during remodeling of cardiac gap junctions. Circ Res. 2002; 90:317–24. [PubMed: 11861421]
19. Kim SJ, Park EY, Hwang TK, et al. Therapeutic effects of connexin inhibitors on detrusor overactivity induced by bladder outlet obstruction in rats. Urology. 2011; 78:31–7.
20. Andersson KE, McCloskey K. Lamina propria:The functional center of the bladder? Neurourol Urodyn. 2013 Jul 11. Epub ahead of print. 10.1002/nau.22465
21. Gevaert T, De Vos R, Everaerts W, et al. Characterization of upper lamina propria interstitial cells in bladders from patients with neurogenic detrusor overactivity and bladder pain syndrome. J Cell Mol Med. 2011; 15:2586–93. [PubMed: 21251216]
22. Cushing P, Bhalia R, Johnson AM, et al. Nerve growth factor increases connexin43 phosphorylation and gap junctional intercellular communication. J Neurosci Res. 2005; 82:788–801. [PubMed: 16302187]
23. Liang CC, Tseng LH, Ko YS, et al. Expression of nerve growth factor immunoreactivity and messenger RNA in ischemic urinary bladder. Neurourol Urodyn. 2010; 29:512–6. [PubMed: 19618449]
24. Kuroko Y, Tokunaga N, Yamazaki T, et al. Effect of sustained limb ischemia on norepinephrine release from skeletal muscle sympathetic nerve endings. Neurochem Int. 2006; 49:448–53. [PubMed: 16632086]

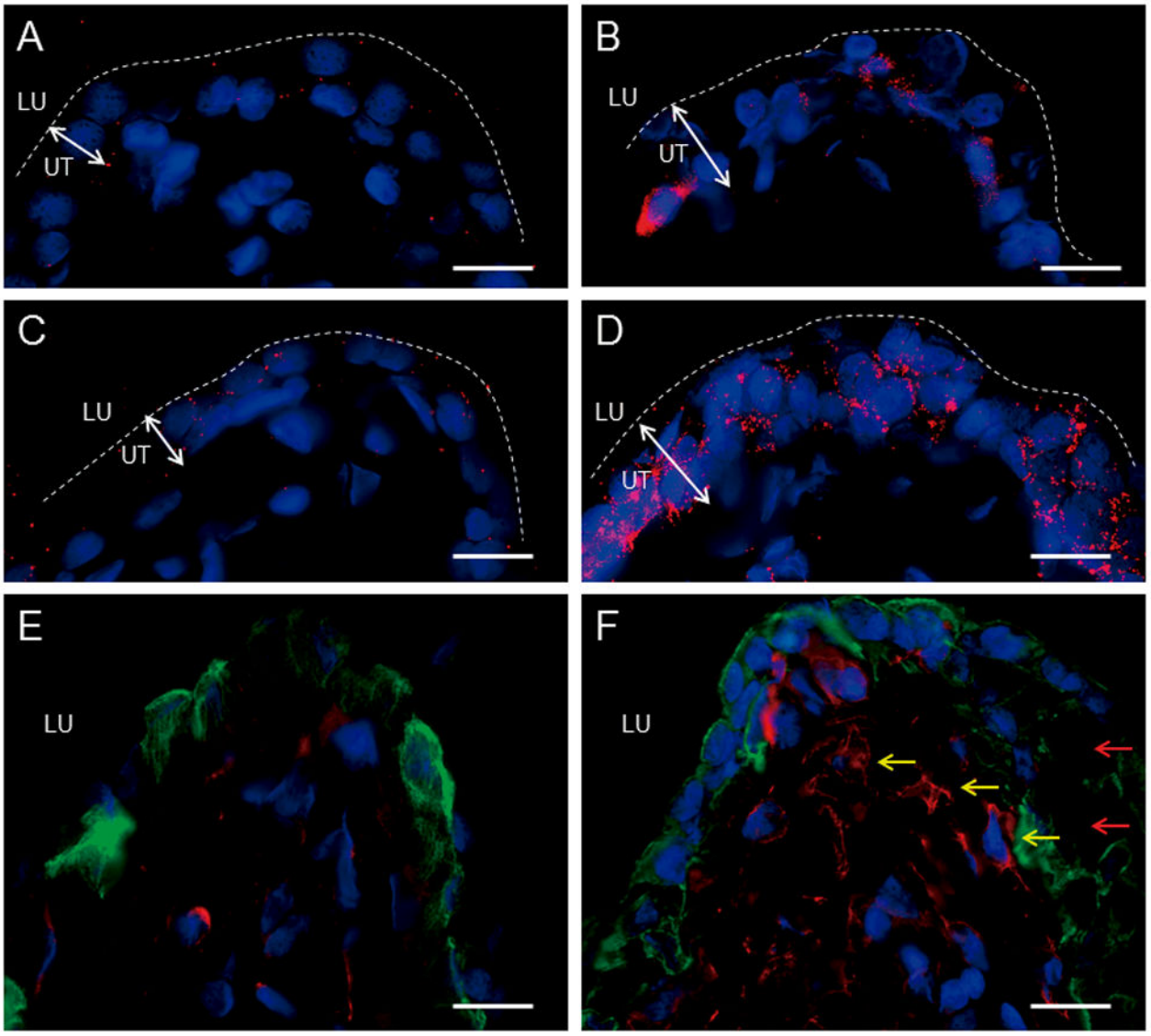

Fig. 1. Altered connexin and vimentin-immunoreactivity in the ischemic bladder

Immunohistochemical analysis of Connexin 26 (Cx26; A, B), Connexin 43(Cx 43; C, D) and vimentin (E, F) in control (A, C, E) and ischemia (B, D, F) groups (original magnification ×600: Bars indicate 20μm). Nuclei were counterstained with DAPI (blue). Cx26 (red) was expressed within the urothelial layers (UT, within the white dotted line) (A, B), and the intensity of staining was significantly increased in the ischemic bladder (B) as compared to control (A). In contrast, Cx43 (red) labeled was observed in both the urothelium as well as underlying suburothelium (C, D). Similar to Cx26, the density of Cx43 in the urothelium was significantly increased in the ischemic bladder (D) as compared to control (C). The distribution of vimentin-IR cells (red) within the lamina propria and cytokeratin 17-IR cells (a marker for basal epithelial cells; green) was shown (E,F). The basal cells layer was partially broken in ischemia (red arrows, F). Vimentin-IR cells with typical ‘stellate’ shape in ischemia were indicated (yellow arrows, F), which may be indicative of lamina propria interstitial cells. The number of vimentin-positive cells that

were also DAPI-positive was significantly increased in ischemic (F) versus that of control (E). LU: lumen.

Table 1

Ischemia induced alterations in proteins involved in mucosal barrier and sensory functions

Vimentin and Connexins (Cx) 26 and 43 were examined using immunocytochemistry, Zona occludens-1 (ZO-1) and Uroplakin III (UP3) were measured by western immunoblotting, and Nerve Growth Factor (NGF) and Norepinephrine (NE) were measured by ELISA.

Connexin 26 (Cx26)	Increased 2.81±0.75 fold (p=0.0426)
Connexin 43 (Cx43)	Increased 2.84±0.27 fold (p=0.0003)
Zona occludens-1 (ZO-1)	Increased 1.88±0.25 fold (p=0.0469)
Uroplakin III (UP3)	Unchanged 1.13±0.08 fold (p=0.4536)
Nerve Growth Factor (NGF)	Increased 1.71±0.36 fold (p=0.0296)
Norepinephrine (NE)	Reduced 0.51±0.14 fold (p=0.0743)
Vimentin	Increased 2.19±0.19 fold (p=0.0001)

Changes are represented as mean ± SEM as compared to control;

* $p < 0.05$.