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The effects of cold-restraint stress on urinary bladder wall compared with interstitial cystitis morphology

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Abstract Stress is associated with many diseases of unknown aetiology. This study demonstrates the effects of cold-restraint stress on the morphology of the urinary bladder. Additionally, it compares the results obtained with the morphology of the interstitial cystitis. The animals were subjected to three hours of cold-restraint stress and then starved for 48 h. The morphology and histochemistry of the urinary bladder was investigated with light and electron microscopy. The proliferative activity was analysed via flow cytometry. Increased and degranulated mast cells in the mucosa, leucocyte infiltration in the lamina propria, vacuole formation in the urothelial cells, loose tight junction, dilated intercellular spaces and altered proliferative activity were observed in the stress group when compared with the control. The increase in the number of mast cells and especially degranulated mast cells and vacuole formation and the loose tight junction of the urothelium correlated with the histopathological findings of interstitial cystitis.

Key words Cold-restraint stress · Urinary bladder · Interstitial cystitis · Mast cell · Urothelium · Ultrastructure · Ruthenium red · Flow cytometry

Introduction

Stress may have a role in the formation of many diseases. It shows its effect via the stimulation of the immune, endocrine and nervous systems [1]. It is well known that ulcerative gastritis, psoriasis, and migraine are diseases which are often triggered by stress conditions [4, 18, 45]. Recently, interstitial cystitis (IC) has also been accepted by many researchers as being induced by stress conditions [13, 14, 32, 42, 44].

IC is a sterile bladder condition that occurs almost exclusively in women (90%). Bourque (1951) was the first to describe the clinical entity of IC. Stress conditions have been showed to increase pain in 60% of the IC patients [32]. This disease was characterised by urinary frequency, urgency, burning and suprapubic pain [5]. The combination of urinary and pelvic symptoms leads to the mistaken diagnosis of gynaecological disease, such as endometriosis, and may result in unnecessary laparoscopy and hysterectomies [25]. There is no agreement as to the cause of IC. A number of theories have been put forward, including infection [17, 26], autoimmunity [9, 41] neurogenic and hormonal factors [22, 24], defects in bladder cytoprotection, the presence of a toxic substance in the urine [23, 40] and psychiatric causes [25].

The National Institute of Health (NIH), and the National Institute of Arthritis, Digestive, Diabetic and Kidney Diseases (NIADDKD) have established morphologic criteria for research into IC. These criteria include mononuclear inflammation, mucosal haemorrhage, and deficiencies in the mucous layer of the bladder, epithelial disruption and increased mast cells in the detrusor [2, 8 , 12, 20, 25, 36, 46, 49].

In this study we aimed to show the effect of cold-restraint stress on the bladder wall morphology. Additionally, we compared the results from this study with the diagnostic criteria of IC and set out to establish an experimental reproducible model for IC.

Material and methods

Animals

Adult female Wistar strain albino rats weighing 180–200 g were used in this study. They were housed individually in light- and temperature-controlled rooms on a 12/12 light and dark cycle. They were fed on a standard pellet laboratory chow and water ad libitum. The study was designed with the permission of the ethic council of the medical faculty.

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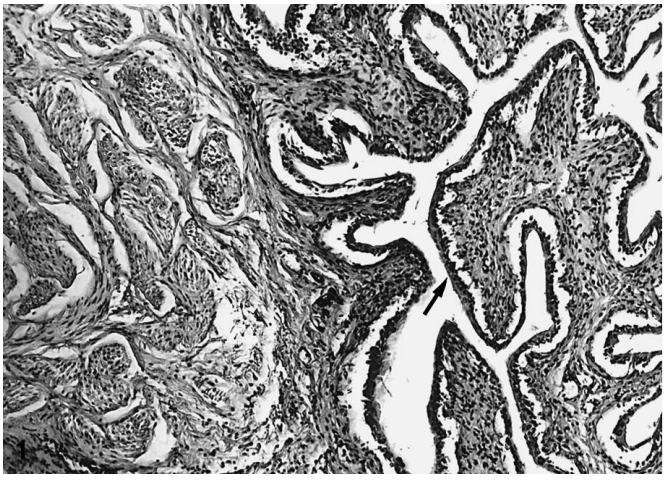


Fig. 1 Control group: the regular bladder wall (→) urothelium, H&E staining, ×33

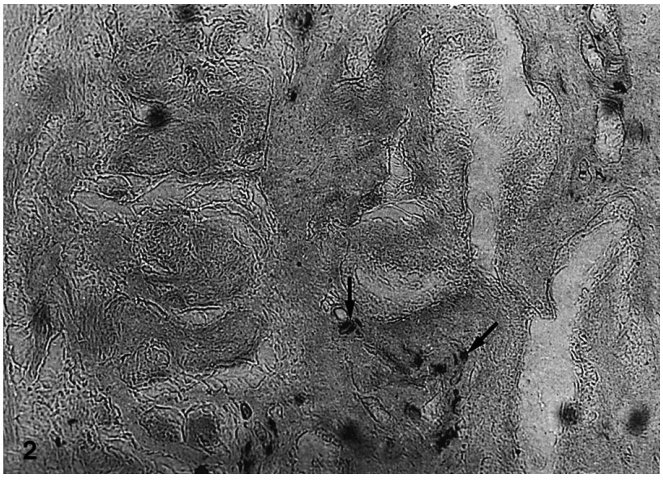


Fig. 2 Control group: a few mast cells (→) in the bladder wall. TB staining, ×33

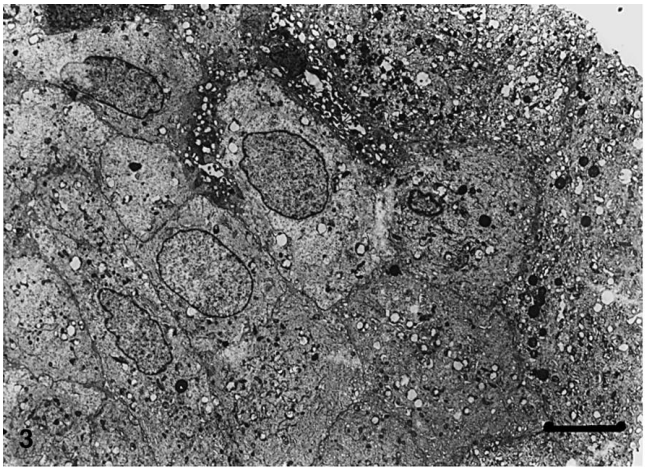


Fig. 3 Control group: regular urothelial cells with a fusiform vesicles, Transmission electron micrograph (TEM), *scale bar*: 5 μm

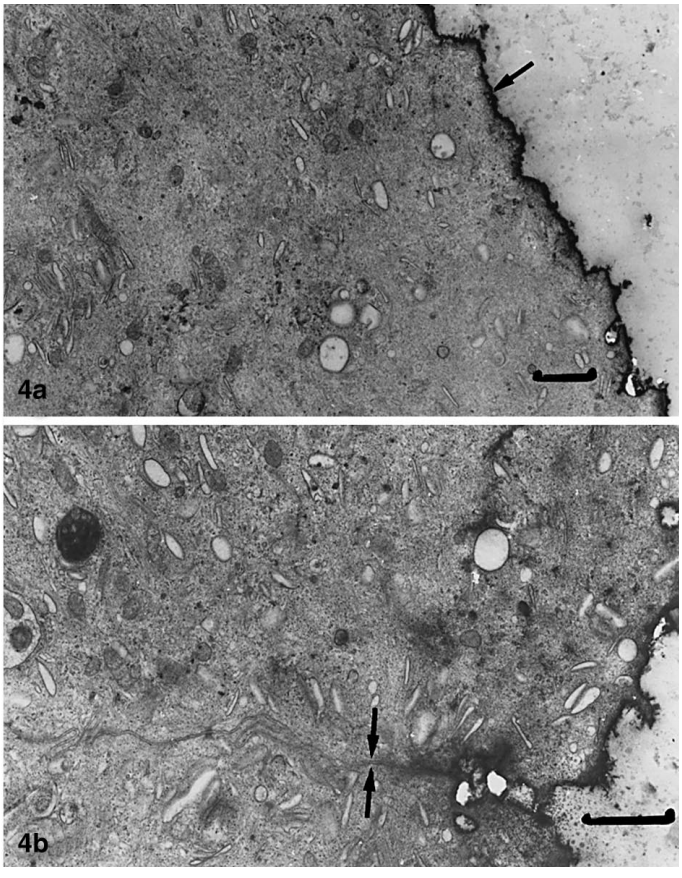


Fig. 4a, b Control group: **a** Regular GAG layer (→) and **b** impermeable tight junctions (∗). RR staining, TEM, *scale bars*: 1 μm

Experimental groups

Three groups were set up: (a) control ($n = 4$), (b) stress ($n = 8$), and (c) recovery ($n = 8$). The standard laboratory conditions were applied to the animals of the control group during the experimental procedure. The animals in the stress and recovery groups were starved for 48 h, after which they were put into restraint cages for 3 h at 4°C [43]. Following the stress conditions, the animals in the recovery group were kept under normal laboratory conditions for 48 h. All animals were sacrificed under ether anaesthesia and bladders were removed for microscopic and flow cytometric investigations.

Light microscopic preparation

The specimens were fixed in normal 10% buffered formalin for 48 h, dehydrated in an ascending alcohol series and embedded in

paraffin wax. Approximately 7-μm thick sections were stained with hematoxylin and eosin (H&E) for general morphology. Toluidine blue (TB) 0.5% in 0.5 M hydrochloric acid (pH 0.5) for 30 min was used for the identification of mast cells [12, 47].

Electron microscopic preparation

For transmission electron microscopic (TEM) investigations the specimens were fixed in 4% phosphate buffered gluteraldehyde (0.13 M and pH 7.4) for 4 h and postfixed with 1% OsO_4 for 1 h dehydrated in a graded alcohol series and embedded in epon 812. In order that the glycosaminoglycan (GAG) layer at the TEM level could be observed, the specimens were then stained en bloc with ruthenium red (RR) and postfixed with OsO_4 (ratio used 1 part of stock RR solution: 4 parts of 1% OsO_4). These samples were stained en bloc within a range of 1/5 stock RR solution and 2% uranyl acetate [10] and prepared for TEM investigations. Thin sections were stained with uranyl acetate and lead citrate and observed at the Jeol 1200 SX TEM (Tokyo, Japan).

For scanning electron microscopic (SEM) investigations samples were fixed and dehydrated as above, dried with liquid CO_2 under pressure with critical point dryer (Bio-Rad E 3000, Hertfordshire, UK) and covered with gold particles (Bio-Rad SC 502, Hertfordshire, UK).

These samples were observed under a Jeol JSM SEM (Tokyo, Japan).

Flow cytometry preparation

Paraffin embedded samples were cut 100 μm thick, deparaffinised with toluene and rehydrated using a decending alcohol series. They were then were put into proteinase K/phosphate buffer solution (1/10) at 37°C for 30 min, washed with PBS and centrifuged. Then the cells were stained with propidium iodide at 4°C for 30 min. The stained cells were investigated with the facscan instrument (FACScan Becton Dickinson, USA). The instrument was standardised with chicken erythrocytes.

Results

Morphological observations

In the control group, a regular mucosal layer of the bladder was observed with H&E staining (Fig. 1). Few mast cells were observed in the lamina propria and detrusor muscle layer with light microscopy (Fig. 2). TEM investigations showed polygonal shaped apical urothelial cells with a few microvilli. RR stained sections showed impermeable and regular tight junctions (Fig. 4). SEM observations revealed mucosal foldings and regular polygonal cells with microridges (Fig. 5).

In the stress group, H&E stainings showed polymorphonucleated leucocytes and oedema in the lamina propria (Fig. 6). TB stainings showed increased granulated and degranulated mast cells in the mucosa – especially in the lamina propria – and detrusor muscle (Fig. 7). TEM observations showed migrated mast cells between the urothelial cells, large spaces around the perinuclear area, vacuole formation in the cytoplasm, polymorph shaped nuclei (Fig. 8a) and dilatations of intercellular space (Fig. 8b). RR stained sections showed irregular GAG layer and RR penetration towards the basal cells of the urothelium (Fig. 9). SEM observations showed irregular surface and degenerate urothelial cells and some of them protruded towards the lumen (Fig. 10).

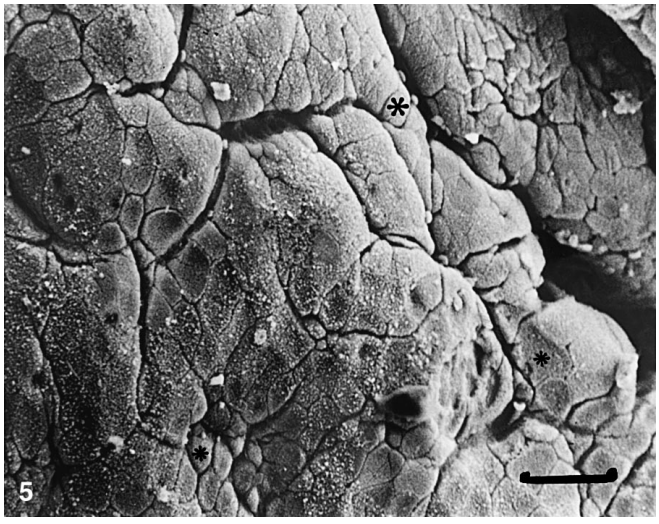


Fig. 5 Control group: polygonal shaped regular urothelial cells with microridges (∗). Scanning electron micrograph (SEM), *scale bar*: 50 μm

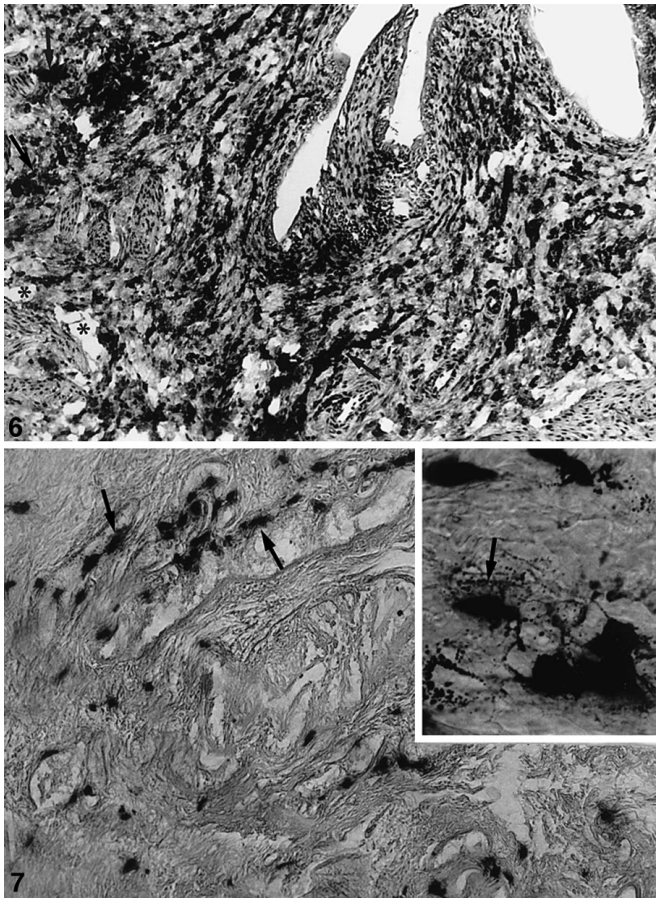
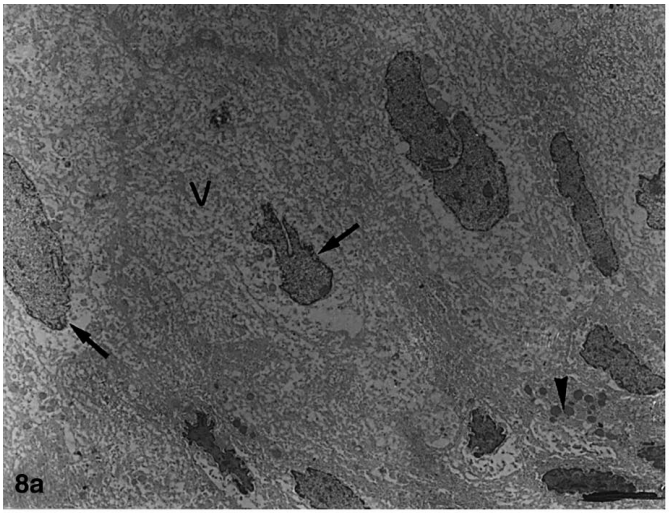


Fig. 6 Stress group: polymorphonucleated leucocytes (→) and oedema in the lamina propria (∗). H&E staining, ×33

Fig. 7 Stress group: Increased number of granulated and degranulated mast cells in the bladder wall (→). TB staining, ×33, *inset*: ×133



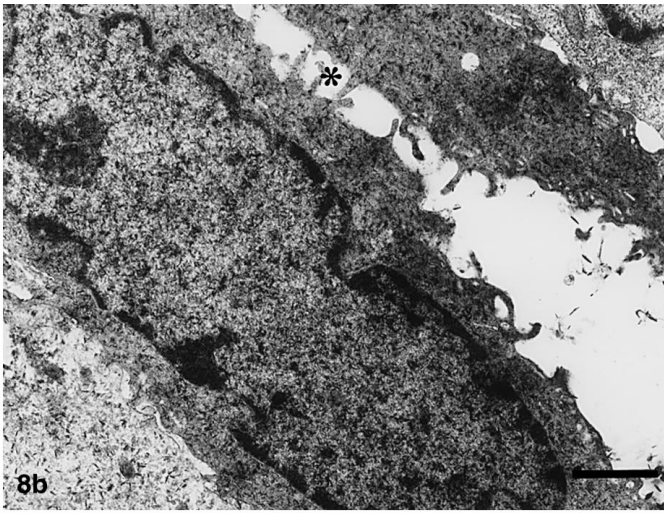


Fig. 8a, b Stress group: Dilated perinuclear space (→), vacuole (*v*) formation in the cytoplasm **(a)** and dilated intercellular junctions (∗), and migrated mast cells between the urothelial cells (ä) (**b**). TEM, *scale bars*: 1 μm (**a**), 500 nm (**b**)

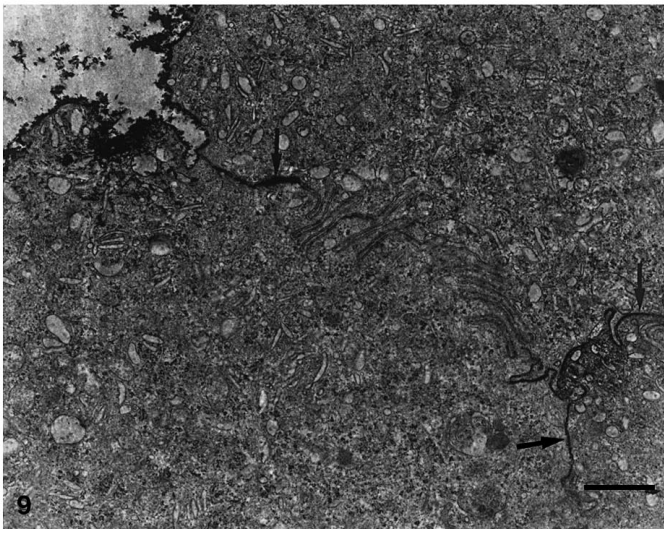


Fig. 9 Stress group: Irregular GAG layer at the luminal surface and penetration of the ruthenium red between the tight junctions (→), RR staining, TEM, *scale bar*: 1 μm

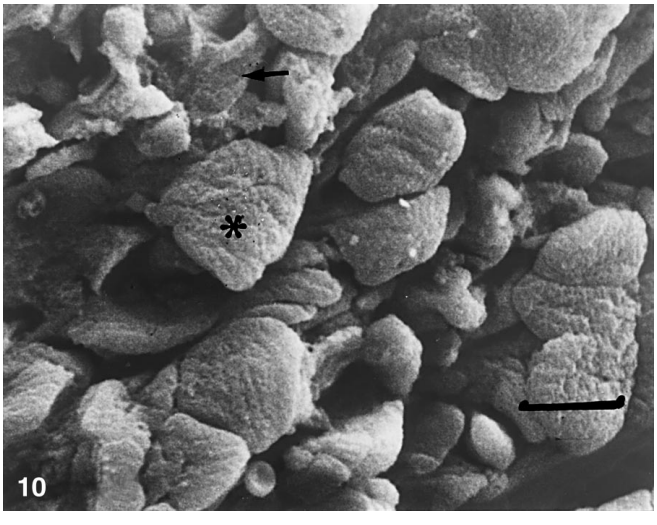


Fig. 10 Stress group: Protrusions of the luminal urothelial cells (∗) towards the lumen and degenerated urothelial cells (→). SEM, *scale bar*: 10 μm

In the recovery group, light microscopic observations showed almost regular mucosa (Fig. 11). Additionally granulated and lightly stained and a decreased number of mast cells was observed when compared with the stress group (Fig. 12). TEM observations showed irregular tight junctions with numerous spaces, which were filled with electron dense material (Fig. 13). Some urothelial cells consisted of an increased number of multivesicular bodies. The RR staining showed absence of a GAG layer in some areas and penetration of RR into the intercellular space was observed (Fig 14). SEM observations showed regular polygonal surface cells and microridges on the surface of the cells (Fig. 15).

Flow cytometric observations

Flow cytometric evaluations showed a peak at the G_1 phase in the histograms of control and recovery groups, but in the histograms of stress group evaluations showed two peaks at the G_1 and $G_2 + M$ phase (Fig. 16).

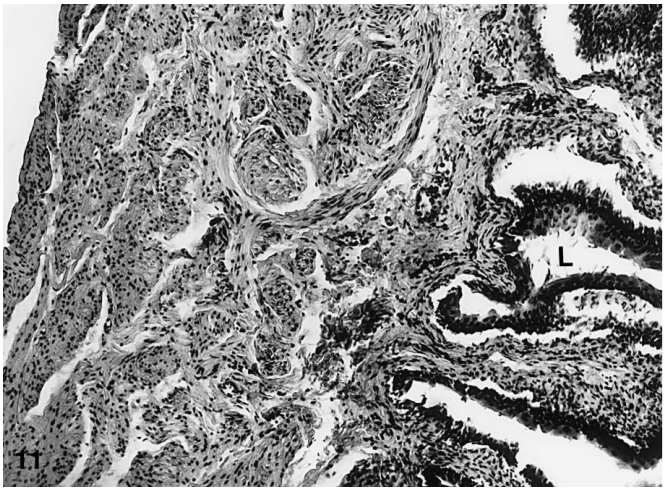


Fig. 11 Recovery group: Regular bladder wall, lumen (*L*). H&E staining, ×033

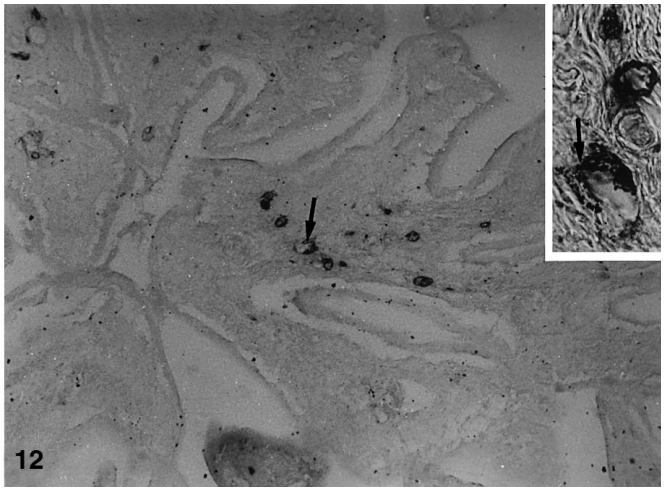
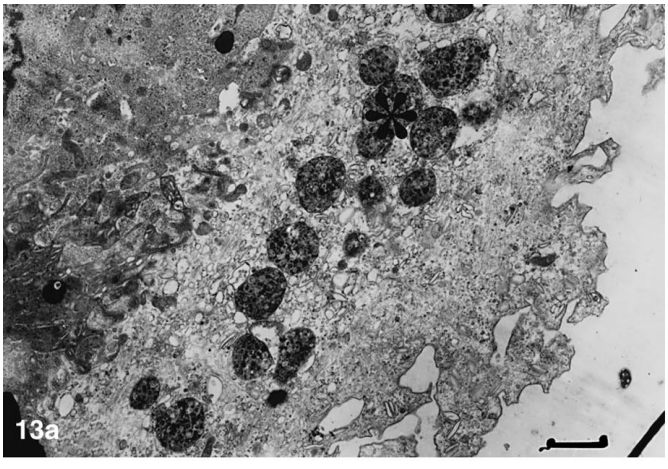


Fig. 12 Recovery group: Decreased number of mast cells in the bladder wall with fewer granules (→). TB staining, × 33, *inset*: × 132



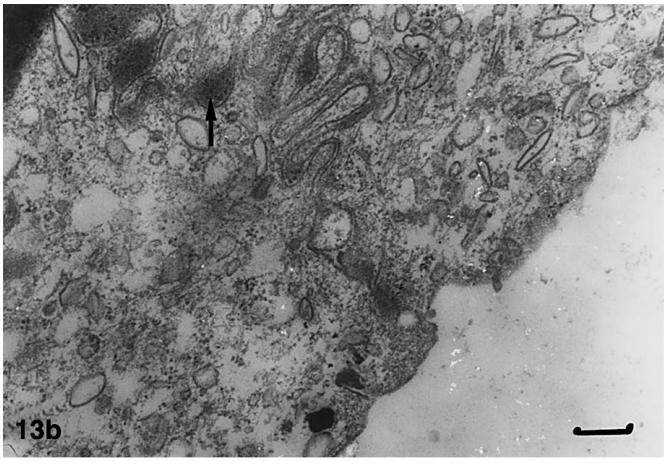


Fig. 13 Recovery group: **a** Many multivesicular bodies (∗) and **b** accumulation of the electron dense material in the intercellular spaces (→). TEM, *scale bars*: 1 μm

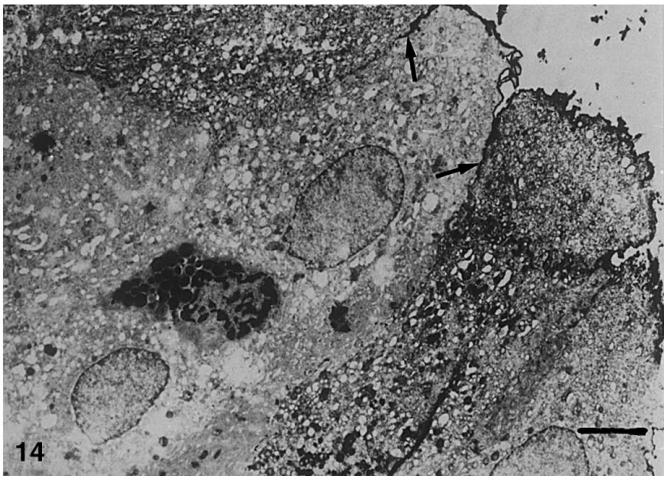


Fig. 14 Recovery group: The ruthenium red penetration in some area of the tight junctions (→). RR staining, TEM, *scale bar*: 2 μm

Discussion

Many diseases are triggered by stress; ulcerative gastritis and psoriasis are two of well-known examples [4, 18, 45]. Recently, it has been suggested that IC is also induced by stress conditions [13, 32, 42, 44].

Cold [42] and isolation stress [6] have been shown to trigger mast cell secretion. Both of our previous quantitative experimental study on mast cells [14] and the study by Spanos et al. (1997) showed an increase in the number of granulated and degranulated mast cells in the bladder under stress conditions [44]. In the IC patients, an increase in the number of mast cells was also observed in

Fig. 15 Recovery group: The regular luminal urothelial cells with microridges (→). SEM, *scale bar*: 10 μm

the detrusor and in the mucosa of the urinary bladder [2, 8, 12, 20, 36, 46, 49). Theoharides postulated that IC is related more to increased activation rather than an increased number of mast cells. Our results showed an increase in both activation and in the number of mast cells. Additionally, migrated mast cells were also observed under the stress conditions. The findings from our experimental stress group correlated well with the bladder morphology of the IC patients. Studies showed that rat and human bladder mast cell secretion is triggered by neuropeptide substance P [21, 34, 46]. Further, microscopic observations showed that mast cells are closely related to the substance P containing nerve fibres [28, 38]. An increased level of substance P under stress conditions was observed [38]. The increased level of substance P and the increased number of mast cells are the two important clinical signs of IC [38]. These findings suggest that stress conditions may cause the release of substance P, which may play a role in the induction of the morphological alterations of the bladder wall. Our previous studies on the denervation of substance P containing nerve fibers with capsaicin (unpublished data) and applying substance P antagonists cp 99994 [15] on the bladder wall prior to exposure to cold-restraint stress confirmed the close relationship between substance P and mast cells. In the stress group, two of our important findings were oedema and leucocyte infiltration. This may due to the activation of mast cells under the stress conditions.

In the recovery group the mast cells were mainly granulated. These findings suggest that if the stress factors are absent, the mast cells return to their normal morphology.

The impermeability of the bladder transitional epithelium is thought to be important for the protection of the bladder against the contents of the urine, since urine contains toxic metabolites and is frequently hyperosmotic. It is also important that this hypertonicity is maintained so that water is conserved. [33]. It has been

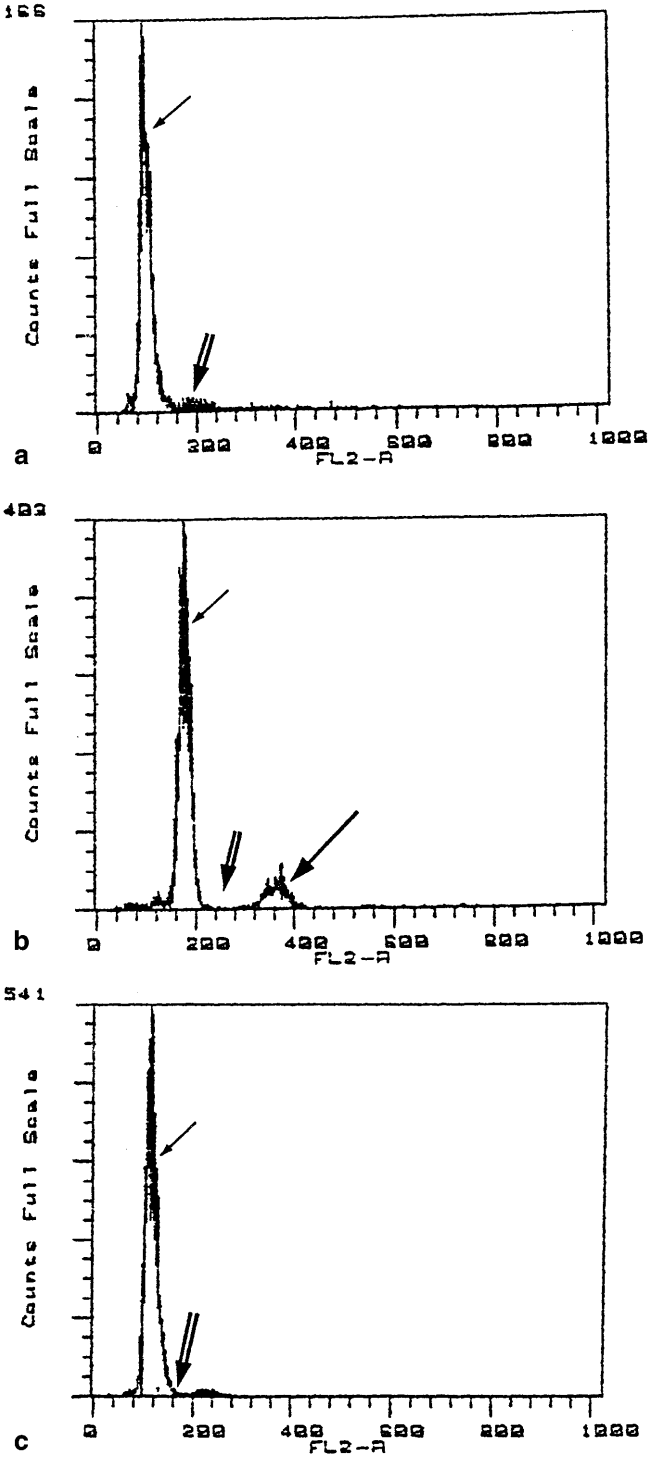


Fig. 16 a–c Histogram of flow cytometry: The peak of G1 phase in the control (**a**) and recovery groups (**b**) (→), and G_1 (→) and G_2+M phase (⇒) in the stress group (**c**)

postulated that the principal mechanisms for the relative impermeability of the urothelium may be due, at least in part, to the mucin (or glycosaminoglycan) layer covering the urothelium [33, 39] and the tight junctions of the urothelium [11]. Our observation of the penetration of the RR towards the urothelial cells under stress condi-

tions showed dilatations of the tight junction. This could be a factor that enables urine and the substances within the urine to penetrate towards the inner layers of the bladder, and cause the inflammation of the bladder wall.

Glutathione is an important constituent of cellular protective mechanisms against a number of noxious stimuli, including oxygen-derived free radicals [48]. It has been previously reported that reduction of cellular glutathione is accompanied by lipid peroxidation [19, 31, 48]. The experimental stress studies on the stomach showed erosion of the epithelial cells and ulceration due to the increased lipid peroxidation [4, 16, 37]. The effect of cold-restraint stress on the bladder morphology results in swollen urothelial cells, vacuole formation in the cytoplasm, penetration of the RR towards the basal layer of the urothelium and protrusion of urothelial cells towards the lumen. The former morphological changes were similar to the experimental stress on the stomach. Thus, morphological alterations in the bladder wall may be related to the degeneration of unit membranes, due to the formation of oxidative free radicals. Similar morphological changes were observed in the urothelium of the IC patients, such as ulceration of the mucosa [27], an increase in microvillar structures [3, 29], changes to the GAG layer [30] and the irregular tight junctions [11].

Bushman et al. (1994) found abnormal flow cytometry profiles in IC patients [7]. They revealed functional abnormalities of the urothelium and bladder mucosa in IC patients, but the pathogenesis of these changes remains unexplained. We found an increased $G_2 + M$ phase in the stress group. We were unable to define the cell type and/or types responsible for proliferation in the stress group with this technique. Although there are limited studies on flow cytometry of IC patients, we believe a mechanism exists and results in a change in the proliferative activity of cells under physiological stress conditions.

In the recovery group, it was interesting to observe morphological changes such as RR penetration of the intercellular space and an increase in the number of mast cells. The observations of numerous multivesicular bodies in the urothelium may be related to the degradation of the degenerated unit membranes. Experimental studies on the stomach have indicated that keeping an animal under normal laboratory conditions for 18 h after exposure to stress conditions was enough time for the the stomach morphology to recover [35]. For the bladder 48 h was insufficient time for recovery to occur. We suggest that the proliferation of the urothelium is slower than in the stomach.

In conclusion, our morphological, cytochemical and flow cytometric findings showed the effects of stress conditions on the rat urinary bladder. The rat urinary bladder can be affected by stress conditions, the outcome of which correlated highly with the morphology of IC. We believe that physiological stress conditions can be one of the factors causing IC. Additionally, this cold-restraint stress model can be introduced as a model for IC. This will enable extended and reproducible experimental studies on the IC.

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