INTRODUCTION

Cyclophosphamide (CYP) treatment causes mucosal inflammatory response as indicated by macroscopic and microscopic changes in the urinary bladder and the presence of inflammatory cell infiltrations. Such acute and chronic CYP treatment induces cystitis with overactivity. The aim of this study was to estimate the effect of CYP on rat urinary bladder function, histological structure and mastocytes numbers following acute and chronic CYP treatment. Forty two female rats were divided into four groups: I (control), II (acute cystitis), III (chronic cystitis), IV (sham group). Acute and chronic cystitis were induced by CYP in single dose and four doses (1st, 3rd, 5th, 7th day), respectively. In group I–III the cystometric evaluation was performed. Sections of the bladder were stained with HE and toluidine blue for the detection of mastocytes. The severity of inflammation was examined according to mucosal abrasion, haemorrhage, leukocyte infiltration and oedema. Acute and chronic CYP treatment caused inflammatory macroscopic and microscopic changes (mucosal abrasion, haemorrhage, oedema) and increased infiltration of inflammatory cells in urinary bladder. Acute treatment induced the infiltration of mastocytes within bladder wall contrary to chronic one decrement. Acute treatment caused more severe mucosal abrasion, whereas chronic one revealed more developed haemorrhage changes. Additionally, cystometric evaluation revealed urinary bladder overactivity development in both types of cystitis. Basal pressure and detrusor overactivity index after acute treatment increased considerably in comparison with the increase obtained after chronic one. Our results proved that acute model of CYP-induced cystitis in rats is more credible for further evaluation of neurogenic inflammation response in pathogenesis of overactive bladder as compared to chronic one.

Key words: overactive bladder, cyclophosphamide, mastocytes, cystitis, cystometry, afferent sensory fibers

MATERIAL AND METHODS

Animals

Experiments were performed on forty-two adult female Wistar rats (weight: 195-275 g). Rats were housed individually per cage. The animal room was maintained at a constant temperature of 23°C, humidity and a 12:12-hours alternating light-dark cycle. They were fed with animal food (Labofeed; Kcynia, Poland) without any restraint to water. The study has been approved by the Animals Ethical Committee of Jagiellonian University (Cracow, Poland).

Animal models of overactive bladder (OAB)

Acute and chronic chemical cystitis leading to OAB was induced by CYP. CYP (Endoxan, BaxterOncology, Germany) was administrated intraperitonealy in a single dose of 200 mg/kg and four doses of 75 mg/kg (in 1st, 3rd, 5th, 7th day of experiment), respectively (1, 7).

Bladder catheter implantation

Under urethane anaesthesia, the abdomen was opened through a midline incision and the bladder end of the
polyethylene catheter (o.d. 0.97 mm/ i.d. 0.58 mm; BALT, Poland) was passed through a small incision at the apex of the bladder dome and secured in place by silk ligature 4-0, as previously described (2, 8). The implantation was performed after 5 hours of CYP administration in group II and after 24 hours of the 4°CYP dose administration in group III.

Study protocol

All animals were randomly divided into four groups: group I – control (n=12), group II – acute cystitis (n=12), group III – chronic cystitis (n=12), group IV – sham group (n=6). In group I – III cystometry was performed under urethane anaesthesia (dose: 1.2 g/kg urethane - Sigma-Aldrich, St. Louis, USA) after a one-hour recovery period from the catheter implantation. Saline solution at room temperature was infused at a rate of 0.046 ml/min continuously into the bladder. The free end of the implanted catheter was connected via T-stopcock to a pressure transducer (UFI, MorroBay, CA, USA) and injection pump (Unipan340A, Poland). Cystometry was recorded using ML110-B-Brige/Amp (ADInstruments, Australia) hardware and PowerLab/SP (ADInstruments, Castle Hill, Australia) software, as previously described (1, 9). The measurements in each animal represent the average of five bladder micturition cycles, after obtaining repetitive voiding. The following parameters were recorded: BP - basal pressure (cmH20), PT – threshold pressure (cmH20), MVP – micturition voiding pressure (cmH20), ICI – intercontraction interval (min), compliance (ml/cmH20), fBC – functional bladder capacity (ml), MI – motility index (cmH2O x s/min) DI – detrusor index (cmH2O/ml) in group I and DOI - detrusor overactivity index (cmH2O/ml) in group II and III (2). After cystometric evaluation the rats were sacrificed by pentobarbital overdose (200 mg/kg) and urinary bladders were removed in group I-III. The bladders from sham group (group IV) were also removed to estimate the effect of bladder catheter implantation of hyperemia degree within bladder walls.

Histological evaluation

The bladder was removed through a lower midline abdominal incision. After the ureters were ligated the bladder was stored in 10% formalin solution. The specimen was split longitudinally and processed for histological examination.

Staining: Specimens were 10% formalin fixed and embedded in paraffin. Thin sections of the bladder were cut and stained with HE and toluidine blue for the detection of mastocytes. In each fragment 10 consecutive cross section were examined.

Severity of inflammation: Previously described techniques (Cayan 2002, Bjorling 1999) were used to determine the severity of inflammation resulted from CYP administration. The severity of inflammation was examined using optical microscope Axioshot (Zeiss, Germany) in each section according to 4 criteria, including mucosal abrasion, haemorrhage, leukocyte infiltration and oedema (10, 11).

Mucosal abrasion: Mucosal abrasion was defined as erosion of the mucosa. The presence or absence of mucosal abrasion per individual field was determined at 100x magnification on a scale of 0 - no abrasion and 1 - visible abrasion. The total score of all view fields was divided by the maximum possible score and multiplied by 100.

Haemorrhage: The presence or absence of haemorrhage per individual field was determined at 100x magnification on a scale of 0 - no haemorrhage and 1 - visible haemorrhage. The total score of all view fields was divided by the maximum possible score and multiplied by 100.

Leukocyte infiltration: Leukocyte infiltrations (neutrophils and mononuclear cells) were evaluated in each of the view fields at 400x magnifications on a scale of 0 - no extravascular leucocytes, 1 - less than 20, 2 - 20 to 45 and 3 - greater than 45 leucocytes per high power field. The total score of all view fields was divided by the maximum possible score and multiplied by 100.

Oedema: Oedema in each view fields was scored at 200x magnification on a scale of 0 - no oedema, 1 - mild oedema (no alteration in the width of the submucosa), 2 - moderate oedema (an increase of less than twice the width of the submucosa) and 3 - severe oedema (an increase of more than twice the width of the submucosa). The total score of all view fields was divided by the maximal possible score and multiplied by 100.

Mastocytes: The total number of mastocytes was counted at 200x magnification in 10 random sections of the bladder of each rat.

Statistical analysis

The results were expressed as mean and standard deviation (±S.D.). Kruskal-Wallis test was used to compare between groups and “post hoc” multiple comparison tests for statistically significant results. Statistical significance was set at p≤0.05 for all tests.

RESULTS

After acute CYP treatment, rats display piloerection and the characteristic immobile rounded back posture with the head aligned with the body axis, while none of control rats and rats during chronic CYP treatment displayed this position. These behaviours were interpreted to be pain related condition (3).

The CYP-treated rats exhibited macroscopical signs of urinary bladder inflammation, i.e. redness, oedema (in group II, III) and also wall thickening, mucosal erosions, ulcerations, petechial haemorrhages on the serosal surface (in group III). In some animals of the group III the urine contained blood. Rats in the groups I and IV had healthy bladders and normal urine. Microscopic evaluations of acute and chronic cystitis revealed increased severity of inflammatory cells infiltration of bladder wall (neutrophils and mononuclear cells) - 32.65±18.14 vs. 69.43±18.22 vs. 64.58±15.09 (p<0.017), respectively. Compared to the control group, a single dose of CYP caused increased number of mastocytes from 0.73±0.29 to 6.73±2.09 vs. 1.48±0.71, p<0.002). Furthermore, CYP-treated rats showed clear signs of inflammation; however, the alteration of bladder structure depends on the mode of CYP administration. Acute model caused more severe mucosal abrasion when compared to chronic one which revealed more developed haemorrhage changes within bladder wall. Additionally, in acute and chronic cystitis we observed similar tissue oedema changes (Fig. 1A-3AB).

Optional comparison of bladder architecture and hyperemia degree between rats after (group I) and without (group IV) bladder catheter implantation showed no significant changes.

Compared to control group, acute and chronic CYP treatment caused significant decrease of MVP (27.41±4.86, 21.51±3.12, 21.67±1.94 cmH20, p<0.001), ICI (5.278±1.549, 1.503±0.736, 2.149±0.350 min, p<0.001), IBC (0.243±0.071, 0.069±0.034, 0.099±0.016 ml, p<0.001), compliance (0.059±0.019, 0.036±0.031, 0.030±0.007 ml/cmH20, p<0.005), and increase of BP (1.40±0.60, 4.57±1.20, 3.31±1.85 cmH20, p<0.001), DOI (121.92±32.98, 824.36±327.57, 384.03±181.68 cmH20/ml, p<0.001), MI (185.64±45.95, 309.90±135.03, 478
261.00±33.92 cmH₂O x s/min, p=0.004). The PT was not significantly changed in acute treatment (5.68±1.22 and 7.50±2.11 cmH₂O, p=0.050). Significant changes of BP and DOI between acute and chronic CYP treatment were observed. The percentage changes of cystometric parameters were presented in Table 2.

**DISCUSSION**

CYP treatment is associated with various urological complications in rats including reduction of micturition voiding pressure, intercontraction intervals and functional bladder capacity, as well as an increase of non-voiding contraction.

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**Table 1.** Histological evaluations of urinary bladder wall and mastocytes in control group and with (acute & chronic) cystitis rats.

<table>
<thead>
<tr>
<th>Histological parameters</th>
<th>I Control</th>
<th>II Acute cystitis</th>
<th>III Chronic cystitis</th>
<th>Kruskal-Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastocytes</td>
<td>6.73 ± 2.09</td>
<td>11.97 ± 6.02</td>
<td>1.48 ± 0.71</td>
<td>p=0.002 *</td>
</tr>
<tr>
<td>Mucosal abrasion</td>
<td>8.33 ± 6.45</td>
<td>52.08 ± 16.61</td>
<td>31.25 ± 18.96</td>
<td>p=0.003 **</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>16.67 ± 17.08</td>
<td>33.33 ± 24.58</td>
<td>83.33 ± 20.41</td>
<td>p=0.004 ***</td>
</tr>
<tr>
<td>Edema</td>
<td>26.38 ± 11.07</td>
<td>75.70 ± 11.00</td>
<td>82.67 ± 7.18</td>
<td>p=0.002 ****</td>
</tr>
<tr>
<td>Leukocyte infiltration</td>
<td>32.65 ± 18.14</td>
<td>69.43 ± 18.22</td>
<td>64.58 ± 15.09</td>
<td>p=0.017 ****</td>
</tr>
</tbody>
</table>

* statistically significant differences between group I and group II, III, as well as between group II and III (p≤0.05); ** statistically significant differences between group I and group II (p≤0.05); *** statistically significant differences between group I and group III, as well as between group II and III (p≤0.05); **** statistically significant differences between group I and group II, III (p≤0.05).

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**Fig. 1A.** A histological appearance of urinary bladder wall in control rats (hematoxylin and eosin staining).

**Fig. 1B.** A histological appearance of urinary bladder wall with mastocytes in control rats (Toluidine Blue staining).

**Fig. 2A.** A histological appearance of urinary bladder wall in rats with acute cystitis (hematoxylin and eosin staining).

**Fig. 2B.** A histological appearance of urinary bladder wall with mastocytes in rats with acute cystitis (Toluidine Blue staining).
frequency (1). These signs are strongly correlated with bladder cystitis.

Our results showed that acute and chronic CYP treatment caused inflammatory response as indicated by macroscopic and microscopic changes in bladder (mucosal abrasion, oedema, haemorrhages) and increased infiltration of inflammatory cells (neutrophils, mononuclear cells) of bladder wall. These results are close to that reported previously by several authors despite a slight modification of experimental protocol (10, 11). Also short-term CYP administration induced the infiltration of mastocytes within bladder wall (mucosa and muscular layer) contrary to chronic one which decreased the number of mastocytes.

Additionally, cystometric evaluation in acute and chronic cystitis revealed urinary bladder overactivity development. However, more severe overactivity occurred in acute cystitis. We obtained significant increase of basal pressure and detrusor overactivity index after acute CYP treatment as compared to chronic one. We postulated that this fact might be the result of two mechanisms. Firstly, that more severe mucosal damage (abrasion) in case of acute cystitis led to extensive exposition of submucosal bladder afferent C-fibres. Its activation by acrolein, as well as wide range of mediators elicited by inflammatory cells stimulated these nerves leading to generation of non-voiding bladder contractions (NVCs), which was reflected in the larger increment of detrusor overactivity index. Secondarily, increased number of mastocytes, the cells which are pivotal in afferent C fibres stimulation, enabled their intensive stimulation and in consequence more frequent bladder NVCs generation. Conversely, in chronic cystitis the changes in basal pressure and detrusor overactivity index are softer than in acute one. Nevertheless, the mastocytes number decreased the mucosal damage (abrasion) occurred. Therefore, it could be stated that extensive bladder afferent C-fibres exposition caused by mucosal damage (abrasion) seems to be crucial in bladder NVCs generation. On the other hand rhythmic detrusor muscle bundles spontaneous action potentials are initiated in non typical ICC-like cells situated at the border of the bundles (12). Therefore the increased frequency of NVCs could be a result of overstimulation of ICC by acrolein.

Neurogenic inflammation responses have recently been linked to overactive bladder in animals and humans. Keith et al. study revealed a close relationship between mastocytes and peptidergic afferent nerve fibres of urinary bladder (5). He revealed that stimulation of vanilloid (TRPV1) and anceryn (TRPA1) transient potential receptors by vanilloids as well as other agents (bradykinin, protons) caused neuropeptidase release like substance P (SP), calcitonin gene-related peptide (CGRP) and interleukins from nerve terminals. Those factors generate responses in blood vessels diameter, mastocytes and lymphocytes activation causing neurogenic inflammation and in consequence overactive bladder (3, 4). Previous studies suggest that CGRP acts synergistically with SP in spinal cord, therefore CGRP may facilitate the SP-evoked chemonociceptive reflex (13). Additionally, TRPV1 and TRPA1 receptors are also targeted by environmental irritants (e.g. acrolein) that are associated with nociceptive and inflammatory effects of smog, cigarette smoke and the metabolic product of chemotherapeutic agents – CYP (14, 15). Ercan et al. (16) study showed that SP localized in capsaicin-sensitive sensory nerve endings (TRPV1 positive nerves) is important in stress response. Inactivation of sensory neurons with capsaicin prevents acute stress-induced epithelial damage and mastocytes proliferation in the rat bladder. It was postulated by Saban et al. (17) that it is very likely that during stress, discharge of neuropeptidase (particularly SP) from primary afferent fibres activates mastocytes in the bladder and induces inflammation. Purinerigic molecules such as ATP, ADP and adenosine play a role of a neurotransmitter in nonadrenergic, noncholinergic nerves supplying the urinary bladder (18). Additionally, mastocytes seem to be equally important in initiation and progression of inflammation in the bladder. Increased numbers of mastocytes were observed in bladder biopsies obtained from patients with interstitial cystitis (19). Many

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**Table 2.** The percentage changes of cystometric parameters following acute and chronic cystitis in comparison with control group.

<table>
<thead>
<tr>
<th>Cystometric parameters</th>
<th>Acute cystitis</th>
<th>Chronic cystitis</th>
</tr>
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<tbody>
<tr>
<td>BP</td>
<td>↑ 226.4% *</td>
<td>↑ 136.4% *</td>
</tr>
<tr>
<td>PT</td>
<td>↑ 32.0%</td>
<td>NS</td>
</tr>
<tr>
<td>MVP</td>
<td>↓ 21.5%</td>
<td>↑ 21.0%</td>
</tr>
<tr>
<td>ICI</td>
<td>↓ 71.5%</td>
<td>↓ 59.0%</td>
</tr>
<tr>
<td>Compliance</td>
<td>↓ 39.0%</td>
<td>↓ 49.1%</td>
</tr>
<tr>
<td>DOBC</td>
<td>↓ 71.6%</td>
<td>↓ 59.2%</td>
</tr>
<tr>
<td>DOI</td>
<td>↑ 576.1% *</td>
<td>↑ 215% *</td>
</tr>
<tr>
<td>MI</td>
<td>↑ 66.9%</td>
<td>↑ 40.6%</td>
</tr>
</tbody>
</table>

* significant changes between acute and chronic cystitis.

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Fig. 3A. A histological appearance of urinary bladder wall in rats with chronic cystitis (haematoxylin and eosin staining). Fig. 3B. A histological appearance of urinary bladder wall with mastocytes in rats with chronic cystitis (Toluidine Blue staining).
experiments show that SP can induce mastocytes degranulation (20) as well as it mediates the inflammatory responses induced by mastocyte tryptase (21). The responses to SP and other tachykinins are regulated by G protein-coupled neurokinin receptors. Among these receptors neurokinin-1 receptor (NK1R) is the predominant receptor subtype activated by SP which is involved in inflammation and is expressed by endothelial cells, submucosal glands, and circulating leukocytes. NK1R seems also to modulate plasma extravasation in the urinary bladder induced by SP (22). The fundamental role of NK1R in cystitis was demonstrated in NK1R knockout mice. These mice were resistant to bladder inflammation induced by antigen challenge (20). Stress- and SP-injected centrally, increased the number of both granulated and degranulated mast cells. Consequently, it leads to urethral degeneration with vacuolization in the cytoplasm and dilated intercellular spaces. Both central and peripheral injection of agonist of SP receptor (NK1R) - CP 99994 prevented stress-induced urethral degeneration as well as stress-induced mast cell degranulation (23). These findings imply a link between substance P and mastocytes in the pathogenesis of bladder inflammation. The decrease of mastocytes number in reaction to chronic CYP administration seems to be the result of three mechanisms. Firstly, the direct cytotoxic effect of CYP acting on mastocytes occurred. Secondly, permanent irritating stimulation by CYP and its metabolites caused complete degranulation of mastocytes, which became invisible in toluidine blue staining. Thirdly, chronic CYP administration led to depletion of bladder afferent C-fibres. Hence, the probable, coexisting pathomechanism of mastocytes restraint depended on dramatic decrement of substance P released peripherally (within bladder) by C-fibres endings. Although, Vizzard et al. (24) study demonstrated dramatic changes in the expression of CGRP and SP in micturition pathways following chronic CYP-induced cystitis. However, both neuropeptides were present in lumbarosacral (L6-S1) spinal cord segments and dorsal root ganglia involved in micturition reflexes in control animals, urinary bladder inflammation dramatically increased CGRP and SP immunoreactivity in these regions. In summary, the acute and chronic CYP treatment caused inflammatory macroscopic and microscopic changes and increased infiltration of inflammatory cells in urinary bladder. Acute CYP treatment induced the infiltration of mastocytes within bladder wall which was not observed in a chronic one. Additionally, cystometric evaluation revealed urinary bladder overactivity development in both types of cystitis. Our current functional and morphological results proved that acute model of CYP-induced cystitis in rats is more credible for further evaluation of neurogenic inflammation response in overactive bladder as compared to chronic one.

Conflict of interests: None declared.

REFERENCES


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