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Osteopontin and fatty acid binding protein (FABP), as biomarkers of cyclophosphamide nephrotoxicity, in an experimental model of cystitis in rats

Osteopontyna i białko wiążące kwasy tłuszczowe (FABP), jako biomarkery nefrotoksyczności cyklofosfamidu, w eksperymentalnym modelu zapalenia pęcherza moczowego u szczurów

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Aim: Cyclophosphamide (CP) is a cytostatic agent, which evokes numerous side effects, including well-known cystitis. Acrolein released during CP biotransformation exerts both urotoxic and nephrotoxic effects, therefore CP may cause renal dysfunction. The aim of the study was to assess kidney function in experimental models of acute and chronic cystitis.

Material/Methods: The studies were carried out on 40 rats (4 groups; n = 10), in which acute (single dose of 150 mg/kg CP; group 2) or chronic (four doses of 75 mg/kg CP; group 4) cystitis was induced with appropriate control groups (group 1 and 3). Renal function was assessed with standard (diuresis, urea, creatinine) and new (fatty acid binding protein – FABP and osteopontin) laboratory parameters as well as histopathologically.

Results: The histopathological assessment confirmed the presence of acute and chronic cystitis and did not reveal coexisting significant kidney disorders in groups 2 and 4. Group 2 retained urea and creatinine in the blood. Both groups 2 and 4 showed an increase in diurnal diuresis, and a decreased concentration of urea and creatinine was found in the urine, which was accompanied by significant proteinuria. The daily urinary excretion of small-molecule nitrogen compounds did not differ from the values found in the control groups. In addition, both groups 2 and 4 showed an increase in urinary concentration and excretion of FABP and osteopontin with urine.

Conclusions: The experiment revealed the renal dysfunction in the course of cyclophosphamide-induced cystitis with the tubulopathy character, expressed by the increased production and release into the urine two markers reflecting acute kidney injury – FABP and osteopontin.

Keywords: **cyclophosphamide • nephrotoxicity • fatty acid binding protein (FABP) • osteopontin**

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Abbreviations: **AKI** – Acute Kidney Injury, **BWW** – Bladder Wet Weight, **CKD** – Chronic Kidney Disease, **CL** – Clearance, **CP** – Cyclophosphamide, **HC** – Hemorrhagic Cystitis, **FABP** – Fatty Acid Binding Protein, **GFR** – Glomerural Filtration Rate, **KIM-1** – Kidney Injury Molecule 1, **KWW** – Kidney Weight Weight.

INTRODUCTION

The initial stage of various kidney and urinary tract disorders are symptomless and no significant disturbances of laboratory and imaging parameters, enabling quick identification of the ongoing disease, are observed for a long time [4]. Hence, there is a need to have reliable diagnostic methods to assess renal function also in the early, subclinical stage of numerous kidney diseases. The panel of currently used laboratory parameters assessing the kidney function is, in fact, still relatively poor and has some drawbacks. It involves: diuresis assessment, quantitative and qualitative assessment of urine sediment, presence, type and severity of proteinuria, plasma electrolyte concentration, urea nitrogen (BUN), and the most important marker reflecting the main function of kidney – glomerular filtration – creatinine [34]. However, some of those parameters are influenced by number of factors (e.g. age, sex, diet) and they are not solely markers of renal disturbances but also reveal other abnormalities, such as liver diseases [6, 18, 19, 28]. In addition, abnormalities in laboratory parameters usually reflect the severity of kidney diagnosed disorders with a time lag. Therefore, it justifies conducting research aimed at introducing new parameters to diagnostic practice, characterized by better sensitivity and specificity in relation to the ones currently used [25, 34]. Those characteristics are to meet the new protein parameters, currently colloquially called as "kidney troponins". They were introduced mainly for the purpose of more accurate diagnosis of both acute kidney injury (AKI) and acute renal failure, as well as chronic kidney disease (CKD). Many proteins are currently being investigated, but literature data most often mentions cystatin C, the kidney injury molecule -1 (KIM-1), neutrophil gelatinase-associated lipocaline-1 (NGAL-1), fatty acid binding protein (FABP) and osteopontin [2, 39, 40, 48]. They have also been briefly characterized in our review works [10, 11]. Quantitative and qualitative disturbances of the aforementioned markers, found in plasma and urine, appear in the early stages of kidney damage, analogously to the early classic troponins used in the diagnostics of acute coronary syndromes.

Cystitis is one of the significant disorders of the lower urinary tract, conditioned by an infectious or non-infectious agents. Among the important non-infectious factors causing bladder inflammation of the haemorrhagic cystitis (HC), one can list the chemotherapy and radiotherapy [15]. Classic alkylating chemotherapeutics agents, also used in autoimmune diseases treatment, such as: cyclophosphamide and isosfamide, are regarded to be an important etiologic factor of HC [20, 35]. The incidence of cyclophosphamide-induced HC varies between 4–36% in the group of patients receiving this drug, assuming a different degree of advancement and symptomatology, from macroscopic haematuria with symptoms of secondary bladder overactivity, to toxic necrosis of urothelium with severe haematuria [14, 32]. The pathophysiology of cyclophosphamide-induced cystitis is complex, initiated by unsaturated aldehyde – acrolein, released in the process of metabolism of cyclophosphamide [29]. CP biotransformation is mediated by hepatic CYP2B6, CYP2C9 and CYP3A4 [49, 51]. However, CP may also be partially metabolised in the kidneys due to the fact that this organ is one of the possible extra-hepatic locations of CYP2B6 isoenzymes [26, 55]. Therefore, highly toxic and reactive acrolein can also be synthesized locally in the kidneys, contributing to their damage and dysfunction.

The aim of the study was to assess kidney function in rats with a model of acute and chronic cyclophosphamide-induced cystitis, performed by histopathological and laboratory analysis, including the one based on two new protein biomarkers – fatty acid-binding protein (FABP) and osteopontin.

MATERIALS AND METHODS

The experiment described in this manuscript was accepted by the II Local Ethical Committee for animal experiments operating at the Institute of Pharmacology of the Polish Academy of Sciences in Cracow. The procedures were implemented in accordance with the EU Directive 2010/63 on the protection of animals used for

scientific purposes and the Polish law – the Act of January 15, 2015 on the protection of animals used for scientific or educational purposes.

Animals and test groups: The experiment was carried out on 40, 10-week-old, albino Wistar rats; half male and half female. During the entire experiment, the animals were housed in the animal house, provided with a ventilation system allowing 8–10 times air exchange per hour; in standardized conditions (temperature 20–24°C, humidity 60–70%, lighting 130–325 lux, noise <30 dB, laboratory living feed Labofeed and water *ad libitum*). Upon arrival, the animals were subjected to 10 days' quarantine, staying in a living room in collective, same-sex cages of 5 individuals each. During this period, they were not subjected to any interventions. After the end of the quarantine period, on the 11th day after the animals were obtained, a random randomization of individuals was performed to individual groups (each with 10 animals; half male and half females): group 1 – control acute, group 2 – with acute cyclophosphamide-induced cystitis, group 3 – control chronic and group 4 – with chronic cyclophosphamide-induced cystitis. The adopted experiment plan assumed one-time (in group 2) or four-time (in group 4) administration of cyclophosphamide (Endoxan, Baxter Oncology) along with the sham treatment of control animals (group 1 and 3, respectively) and individual 24-hour monitoring in metabolic cages on specific days of the experiment to assess the basic living parameters (body weight, feed and water consumed), daily diuresis of the study animals and acquisition of urine for laboratory tests. Finally, euthanasia of rats was performed, with blood collection for biochemical determinations and the performance of nephrectomy/cystectomy followed by histopathological analysis of the collected organs (to assess tissue changes in the kidneys and verify inflammatory changes in the bladders).

Procedures in group 1 (control acute group): individuals forming group 1, on the 11th days of the entire experiment, were placed in individual metabolic cages, constituting a control for animals from group 2. Before placement in metabolic cages, these animals received intraperitoneal saline injection as the "sham treatment", in volumes corresponding to those ones used in group 2. During their stay in metabolic cages, each animal had unrestricted, but monitored access to water and feed. After 24 hours, on the 12th day of the experiment and after quantitative evaluation of diuresis, individuals from this group were subjected to pentobarbital euthanasia (Morbital, Biowet, Puławy). Initially, each rat was subjected to isoflurane and then an anaesthetic dose of 60 mg/kg b.w. was administered and blood samples were collected by direct intracardiac puncture in the conditions of deep general anaesthesia. Then, an additional dose of the drug was administered to obtain a lethal dose of pentobarbital 200 mg/kg b.w. After confirming the cessation of vital signs, laparoscopy was performed along with bilateral nephrectomy and cystectomy. Urinary bladders, in order to preserve their integrity, were taken together with a 2-3 mm proximal part of the urethra, then gently dried and emptied of residual urine by gentle pressure. The kidneys were harvested from the surrounding fat sac. The collected organs were weighted on the analytical scales immediately after the collection and then placed in containers with a 4% formalin solution.

Procedures in group 2 (acute cyclophosphamide cystitis): animals forming group 2, on the 11th day of the experiment received a single dose of 150 mg/kg b.w. CP intraperitoneally to cause acute, cyclophosphamide-induced cystitis. According to literature data, this dose is a sub-lethal dose of CP, manifested by nephrotoxicity [44, 53]. Each time the drug was administered, the individual was weighed to calculate the precise dose. Immediately after administration of CP, the animals were placed in individual metabolic cages, with unrestricted, but monitored access to water and feed. Further procedures were analogous to those described for group 1 above.

Procedures in group 3 (control chronic group): subjects forming group 3, received the injection of saline four times (on the 11th, 13th, 15th and 17th day of experiment) intraperitoneally, in volumes comparable to those used in group 4 ("sham treatment"). After receiving the fourth, last dose of saline, on the 17th day of the entire experiment, they were placed in individual metabolic cages, constituting control for animals from group 4. During their stay in metabolic cages, each animal had unrestricted, but monitored, access to water and food. After 24 hours of monitoring, on the 18th day of the experiment, the quantitative assessment of diuresis was performed, and then blood was collected and animals were euthanized according to the method described above in the description of group 1.

Procedures in group 4 (chronic cyclophosphamide cystitis): animals forming group 4 received an intraperitoneal dose of 75 mg/kg b.w. CP four times (on 11th, 13th, 15th and 17th day of experiment). The admission of such a dosing schedule, according to literature reports, leads to the progressive development of cystitis in the studied rats within 7-10 days [1, 46]. As in the case of group 2 animals, each time the drug was administered, the individual was weighed to calculate the precise dose. After the admission of the next CP doses, the animals stayed in the current collective cages. After the admission of the fourth, last CP dose, rats were placed in individual metabolic cages. Further procedures were analogous to those described for group 1.

Biochemical determinations: blood was collected "on a clot" and after centrifugation, the serum was frozen at temp. -80°C until the assays were made. Analogously, the urine samples obtained were centrifuged and frozen. The biochemical determinations were performed using the SIEMENS ADVIA 1200 laboratory analyser. In

serum, the classic small-molecule nitrogen compounds were determined assessing the kidney functions, as: urea [mmol/L] (with conversion to urea nitrogen – BUN [mg/dL]) and creatinine [μmol/L].

Urea [mmol/L], creatinine [μmol/L] and total protein [g/L] were also determine in urine, and taking into consideration the value of the daily diuresis, the 24-hour excretion of these compounds was additionally calculated, expressing them, respectively, as [mmol/24 h], [μmol/24 h] [μmol/24 h] and [mg/24 h]. The clearance of urea CL_{urea} and creatinine CL_{cr}, was also calculated based on the formulas:

$$\mathbf{CL_{cr} = \frac{Cr\ urine\ [\mu mol/L] * diuresis\ [mL/min]}{Cr\ plasma\ [\mu mol/L]}}$$

$$\mathbf{CL_{urea} = \frac{urea\ urine\ [mmol/L] * diuresis\ [mL/min]}{urea\ plasma\ [mmol/L]}}$$

In addition, in the urine samples obtained, the concentrations [ng/mL] of two proteins (new AKI markers) were assessed with the immunoenzymatic method (ELSA): osteopontin and fatty acid binding protein ("liver-type" isoform of the protein, also found in kidneys), using commercially available ELISA kits (Shanghai SunRed Biological Technology Co) and acting strictly according to the manufacturer's instructions. In addition, taking into account the values of the diuresis found, the diurnal excretion of the above-mentioned proteins with urine was estimated [ng/24 h].

Measurement of wet weight of the kidneys and bladders collected during laparoscopy and their histopathological evaluation: immediately after cystectomy and nephrectomy, organs collected were weighed on the analytical scales to find the so-called bladder wet weight (BWW) [mg] and kidney wet weight (KWW) [mg]. The measurement of the so-called wet weight is an indirect, indicative parameter evaluating the functional state of the organ, reflecting, among others, oedema as the level of advancement of inflammation [33, 41, 56].

By starting microscopic preparations for histopathological analysis, the collected bladders and kidneys were rinsed in physiological saline, then fixed for 24 hours in 8% formalin in a phosphate buffer solution (PBS pH7.4). The collected sections were then rinsed in running water for 2 hours, and then dehydrated in ethanol with increasing concentrations (50–100%). Before paraffin embedding, the preparations were moved through the xylene solutions to be cleared. The sections were moved from xylene to a mixture of xylene and paraffin in a 1:1 ratio and incubated at temperature of 37°C for 2h. Then, individual tissue fragments were transferred twice to pure paraffin and incubated at temperature of 62°C. After 2 hours, the preparations were embedded into blocks, which after setting were cut on a Leica RM2135 microtome, into 4 micron thick sections, which when placed on a slide, were dried in a heating oven at temperature of 37°C. The final preparations were stained by the haematoxylin-eosin method (HE).

Histopathological evaluation was carried out by a pathomorphologist, using a classic light microscope (Delta Optical) at 40x magnification (in the case of bladder evaluation) and 100x (for the evaluation of kidney preparations).

Statistical analysis of laboratory results: the values of the determined living parameters as well as laboratory parameters were subjected to statistical analysis, comparing the results obtained in groups 2 and 4 with those obtained in the appropriate control groups 1 and 3. In the first stage, the normality of the distribution of results of the evaluated parameters was verified using the Shapiro-Wilk test. In the next stage, the intergroup differences were analysed with the t-Student's test (when the values of the tested parameter were characterized by a normal distribution) or the Mann-Whitney test (when the normality of distribution was found to be inaccurate).

RESULTS

Basic living parameters: The body weight of rats treated with CP (both after a single dose in group 2 and after administration of 4 doses in group 4) showed a downward trend, although the differences were not statistically significant compared to the respective control groups.

In addition, rats with both acute and chronic CP-induced cystitis were characterized by lower daily feed intake compared to the corresponding control subjects, and the differences were statistically significant. Additionally, the 24-hour water intake was statistically significantly lower in rats with significantly lower in rats with chronic cyclophosphamide-induced cystitis. Despite the lower water intake, 24-hour diuresis in animals treated with CP was almost three times (in group 2) and two times (in group 4) higher compared with the corresponding control groups 1 and 3.

Detailed results of the discussed parameters are presented in Table 1.

Concentrations of the evaluated compounds in blood and urine: In the model of acute cyclophosphamide-induced cystitis, the retention of nitrogen compounds has been demonstrated in blood (statistically significant increase in urea and creatinine concentration accompanied by BUN increase). For animals with chronic cystitis, this relationship was revealed only in the case of increased concentration of creatinine in serum.

Table 1. Results of parameters obtained during 24-hour monitoring in individual metabolic cages

	Group 1 11th day	Group 2 11th day	Statistic groups 1–2	Group 3 17th day	Group 4 17th day	Statistic groups 3–4
Body weight [g]	288.60 ±32.29	268.30 ± 15.06	NS	257.45 ± 63.18	209.73 ± 74.84	NS
24-hour water intake [mL/24 h]	26.33 ± 3.81	22.50 ± 11.47	NS	30.00 ± 3.21	18.57 ± 7.09	0.02
24-hour feed intake [g/24 h]	23.77 ± 5.76	1.98 ± 1.37	<0.01	20.38 ± 2.39	9.63 ± 7.69	<0.01
24-hour diuresis [mL/24 h]	6.01 ± 2.27	16.70 ± 8.31	0.04	5.73 ± 1.31	11.94 ± 4.14	<0.01

Urea and creatinine concentration in urine were significantly lower in animals with both acute and chronic cystitis, which was accompanied by a significant increase in protein concentration in the urine of these animals.

Values of the calculated urea and creatinine clearance were comparable in all groups, except for the urea clearance value, which was significantly lower in animals in group 4.

Detailed values are presented in Table 2.

Analysis of FABP (namely" liver-type fatty acid binding protein") concentration in urine showed a statistically significant, twofold increase in the value of this parameter in animals with acute cyclophosphamide-induced cystitis compared to the appropriate control (group 1) – respectively, 21.69 ± 4.87 vs. 10.69 ± 3.68 [ng/mL]. For animals administered with four doses of CP, the concentration of FABP in urine was also significantly higher in comparison with the value found in the appropriate control group (18.56 ± 2.92 vs. 12.45 ± 4.19 [ng/mL]).

When assessing the concentrations of the next biomarker of kidney damage in the urine – osteopontin, again, a statistically significant twofold increase in this parameter was found in the urine of animals with acute cyclophosphamide-induced cystitis compared to the corresponding control group 1 (48.23 ± 4.87 vs. 24.43 ± 7.91 [ng/mL]). This dependence was also disclosed for animals with chronic CP-induced cystitis, compared

Table 2. Concentrations of the parameters assessed in the blood and urine of the study animals and calculated clearance values

	Group 1	Group 2	Statistic p value groups 1–2	Group 3	Group 4	Statistic groups 3–4
Serum						
urea [mmol/L]	6.34 ± 0.70	8.98 ± 1.53	0.02	6.09 ± 0.74	6.76 ± 1.07	NS
BUN [mg/dL]	17.81 ± 1.97	25.19 ± 4.31	0.02	17.76 ± 2.10	18.95 ± 3.01	NS
creatinine [µmol/L]	28.86 ± 1.66	38.45 ± 7.96	0.05	27.62 ± 2.31	31.99 ± 2.81	0.01
Urine						
urea [mmol/L]	1003.96 ± 176.71	414.35 ± 115.32	<0.01	1058.63 ± 193.43	411.54 ± 163.01	<0.01
creatinine [µmol/L]	6100.05 ± 1028.40	3350.00 ± 1173.31	<0.01	8162.50 ± 2285.85	3575.00 ± 1356.20	<0.01
total protein [g/L]	0.81 ± 0.48	2.51 ± 0.92	0.01	1.01 ± 0.65	3.88 ± 1.44	<0.01
Calculated clearances						
urea clearance [mL/min]	0.87 ± 0.32	0.91 ± 0.25	NS	0.66 ± 0.16	0.47 ± 0.14	0.01
creatinine clearance [mL/min]	0.67 ± 0.31	0.49 ± 0.11	NS	1.10 ± 0.37	0.85 ± 0.26	NS

to the corresponding control group 3 (44.63 ± 10.39 vs. 21.37 ± 5.42 [ng/mL]).

A summary of the comparisons discussed above is presented in Figures 1–4.

24-hour urinary excretion of evaluated compounds: Animals with acute cyclophosphamide-induced cystitis and control ones excreted similar amounts of urea and creatinine in the urine within 24 hours (although there was a trend of increased excretion in rats with acute cystitis). Rats with chronic CP-induced cystitis were characterized by the statistically significant decreased urea excretion in the urine within 24 hours, compared to the corresponding control subjects. Both in the group with acute and chronic cystitis, statistically significant proteinuria was found, which reached nearly identical values in both groups of CP-treated animals and was approximately 8-fold higher compared to the corresponding control groups.

Analysis of 24-hour urinary excretion of two assessed new biomarkers of kidney damage – FABP and osteopontin revealed significant and crucial differences in the study groups. Rats with acute cystitis excreted about 6-fold higher amounts of both FABP and osteopontin compared

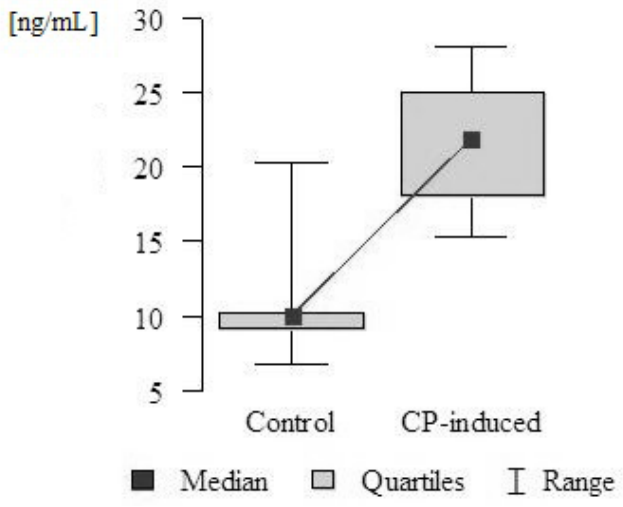

Fig. 1. Urinary concentration of L-FABP [ng/mL] in group 1 – controlanimals (left) and in group 2 – animals with cyclophosphamide-induced acute cystitis (right);

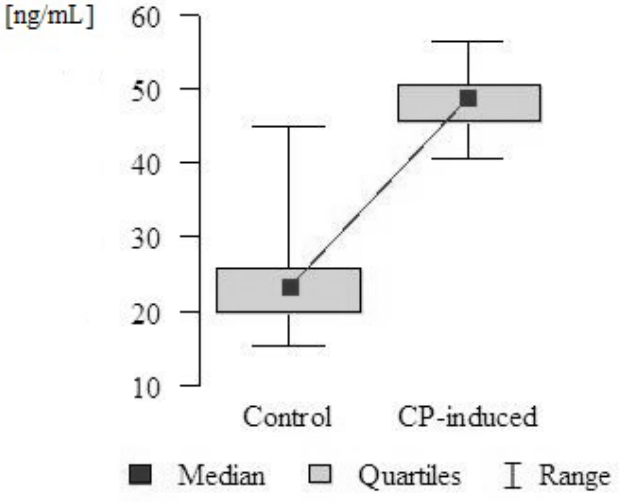

Fig. 2. Urinary concentration of osteopontin [ng/mL] in group 1 – controlanimals (left) and in group 2 – animals with cyclophosphamide-induced acute cystitis (right); $p < 0.01$

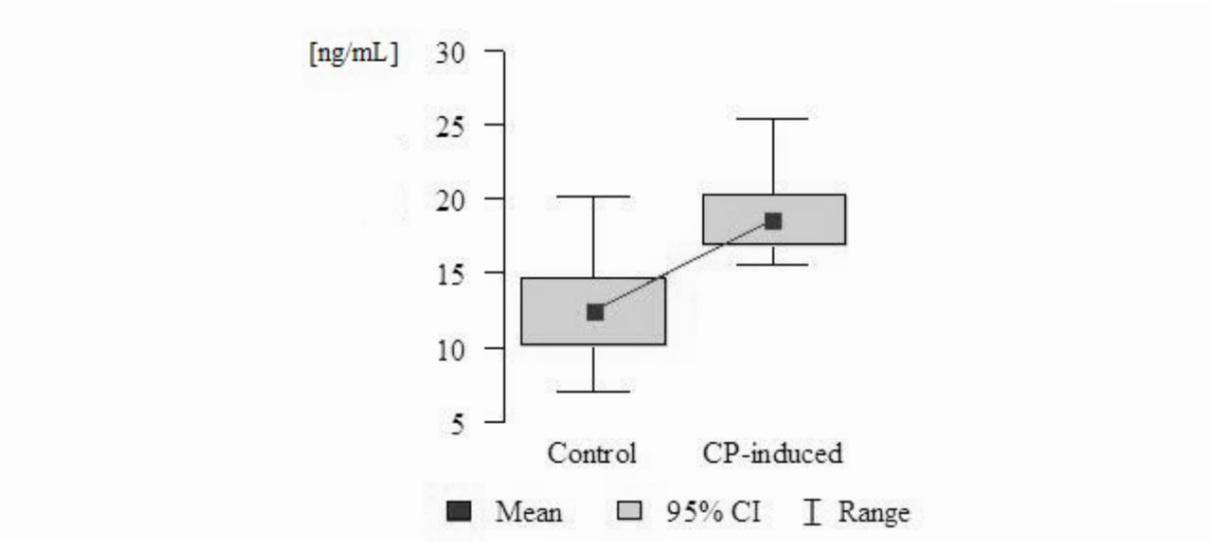

Fig. 3. Urinary concentration of L-FABP [ng/mL] in group 3 – control animals (left) and in group 4 – animals with cyclophosphamide-induced chronic cystitis; p

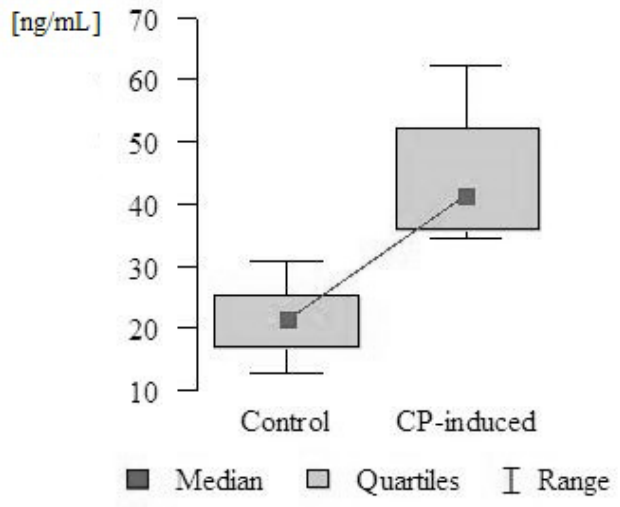

Fig. 4. Urinary concentration of osteopontin [ng/mL] in group 3 – control animals (left) and in group 4 – animals with cyclophosphamide-induced chronic cystitis (right); p <0.01

to those found in the appropriate control group (group 1). Animals with chronic cystitis also excreted significantly higher amounts of both proteins in the 24-hour collection of urine – about 3 times more FABP and about 4 times more osteopontin, compared to the results disclosed in the appropriate control group (group 3).

Detailed values of the 24-hour excretion of these compounds are presented in Table 3.

Macroscopic and histopathological evaluation of bladders and kidneys: The assessment of wet weight of the collected bladders did not reveal any significant intergroup differences. BWW [mg] values were found in individual groups: 120 ± 54; 145 ± 19; 150 ± 40 and 161 ± 45, respectively for groups 1–4.

Histopathological analysis of the prepared preparations in rats with acute cyclophosphamide-induced cystitis has shown the extension of its light with the features of congestion and oedema in the lamina propria of the mucosa, visible small inflammatory infiltrates composed of small lymphocytes and single granulocytes, compared to the image found in control group 1.

Bladder preparations of animals with chronic cyclophosphamide-induced cystitis were also characterized by dilation of the bladder light with congestion fea-

Table 3. 24-hour urinary excretion of assessed compounds in the study animals

	Group 1	Group 2	Statistic groups 1–2	Group 3	Group 4	Statistic groups 3–4
urea excretion [mmol/24h]	5.97 ± 2.28	6.36 ± 1.91	NS	5.93 ± 1.28	4.45 ± 1.14	0.01
creatinine excretion [µmol/24h]	36.23 ± 13.33	50.50 ± 18.12	NS	45.56 ± 14.14	39.38 ± 12.52	NS
total proteinuria [mg/24h]	5.34 ± 4.19	41.92 ± 17.12	0.02	5.72 ± 4.15	45.42 ± 24.15	<0.01
L-FABP [ng/24h]	64.18 ± 28.48	370.60 ± 197.33	<0.01	74.68 ± 31.44	218.61 ± 64.10	<0.01
osteopontin [ng/24h]	146.38 ± 61.42	801.51 ± 288.04	<0.01	128.95 ± 50.66	521.86 ± 143.15	<0.01

tures and remarkably increased mucosal oedema and the entire bladder wall, accompanied by minor haemorrhages and small, mainly perivascular, inflammatory infiltrates made of small lymphocytes.

The wet weight of the kidneys did not differ significantly in the individual groups. KWW [mg] values in individual groups were: group 1: left kidney 870 ± 211; right kidney 882 ± 220, group 2: left kidney 965 ± 274; right kidney 942 ± 244, group 3: left kidney 936 ± 257; right kidney 951 ± 269, group 4: left kidney: 998 ± 357; right kidney: 986 ± 265.

Histopathological analysis of kidney preparations taken from rats with both acute and chronic CP-induced cystitis did not reveal significant abnormalities compared to the images of the respective control groups, with the exception of poor to moderate congestion.

DISCUSSION

The main conclusions from our experiment can be summarized in the points below:

1. An expression of unspecific, generalized CP toxicity was the significantly lower daily intake of water by animals with chronic CP-induced cystitis, and feed in both groups with acute and chronic cystitis.

2. Histopathological analysis confirmed the presence of inflammatory changes in the bladder of animals with acute and chronic CP-induced cystitis. On the other hand, there were no coexisting significant histological disturbances found in light microscopy in the kidneys of animals with both models of cystitis.

3. Animals with acute CP-induced cystitis were characterized by a significant increase in diuresis despite normal daily water consumption, which was accompanied by a significant proteinuria and increased urinary excretion of FABP and osteopontin. In addition, the urinary concentration of both FABP and osteopontin was higher than in the control group. Urine concentrations of small-molecule parameters reflecting the kidney function (urea, creatinine) were lower than those found in the control group, which was most likely caused by the increase in diuresis, while the expression of nitrogen compound retention in blood was the increase in the concentration of these compounds in blood.

4. Animals with chronic CP-induced cystitis, as in the group with an acute model of inflammation, were also characterized by significantly higher diuresis compared to the control group, despite the smaller 24-hour water consumption, and the increase in diuresis was also a potential cause of the decrease in urinary concentration of classic parameters, like urea and creatinine. Analogously to the acute CP-induced cystitis group, in rats with chronic cystitis a significant proteinuria was revealed and the increase in both the concentration and urinary excretion of both protein biomarkers of kidney function – FABP and osteopontin.

5. The conducted study, revealing the increase in the concentration and urinary excretion of both AKI markers – FABP and osteopontin, confirmed their potential clinical use as laboratory markers for monitoring nephrotoxicity in patients treated with cyclophosphamide.

Generally, nephrotoxicity is a common phenomenon occurring during therapy with the use of many drugs. Estimates indicate that complications of pharmacotherapy even in 25% of cases lead to the development of acute kidney injury [7].

Drugs perceived as having significant potential of nephrotoxicity include compounds with various pharmacological effects, such as: non-opioid analgesics – non-steroidal anti-inflammatory drugs, some antidepressants (amitriptyline, doxepin, fluoxetine, lith-

ium), cytostatics (cisplatin, mitomycin C, gemcitabine, methotrexate, ifosfamide), antibacterial agents (aminoglycosides, colistin, ciprofloxacin, sulphonamides, vancomycin, rifampicin), antivirals (acyclovir, ganciclovir, indinavir), antifungal (amphotericin B), radiological contrast agents, immunosuppressants (cyclosporine, tacrolimus), loop and thiazide diuretics, proton pump inhibitors (omeprazole, pantoprazole), H2 antihistamines (ranitidine), drugs use in cardiovascular disease (statins, clopidogrel, ticlopidine, angiotensin convertase inhibitors, AT1 receptor antagonists for II angiotensin). The risk of nephrotoxicity also increases as a result of drug interactions, including herbal preparations and OTC drugs, the use of which in self-treatment continues to increase [8, 27, 52].

Clinically, drug-induced kidney damage takes different forms; expressing the symptoms of AKI and acute renal failure [5] as well as chronic glomerulopathy or/and tubulopathy leading over time to the development of chronic kidney disease [37].

The pathogenesis of drug-induced nephrotoxicity is manifold and includes prerenal effects that interfere with renal blood flow; intrarenal, such as microangiopathic thrombosis, acute tubular necrosis, immunologically mediated glomerulonephritis and/or kidney interstitial inflammation; as well as postrenal abnormalities, e.g. obstructive nephropathy in the urinary tract [5, 37].

CP is considered to be a pharmacological compound characterized by significant systemic toxicity, with the most important cystitis, which was mentioned in the introduction and which was confirmed once again in our experiment. Early adverse effects of CP include the majority of classic toxic effects of cytostatics, such as: dyspeptic disorders, myelosuppression with the development of leukopenia and an increased risk of opportunistic infections, thrombocytopenia and anaemia, alopecia and the above-mentioned haemorrhagic cystitis. Distant and long-term toxic effects of CP include infertility, the development of congestive heart failure and interstitial pneumonitis with fibrosis [3, 22]. As for many other cytostatics, along with the prolongation of the therapy time and the increase in cumulative CP dose, there is a risk of developing secondary tumours (myelodysplastic syndromes, acute leukaemia, skin cancer) [12].

Generally, in contrast to the increased susceptibility of the bladder to damage, the kidneys were not perceived as a significant organ damaged during CP therapy, and the drug itself was regarded as not too nephrotoxic against the other cytostatic oxazaphosphorines [45]. On the other hand, glomerular and tubular abnormalities manifesting in proteinuria, glomerular filtration disorders and urinary compulsion disorders have been reported in patients treated with CP and in experimental studies [16, 38, 42]. The results of our present experiment, however, indicate the possibility of significant kidney damage also by CP, which is consistent with the selected literature reports, describing potential pathomechanisms of cyclophosphamide nephrotoxicity [16, 42, 47].

One of the proposed CP nephrotoxicity mechanisms is associated with the increased oxidative stress in the kidneys, induced by acrolein. This unsaturated aldehyde, released during the biotransformation of CP, is also formed outside the bladder, in the kidneys, with the participation of CYP2B6, which was mentioned in the introduction [26, 55]. The acrolein overproduced locally in the kidneys breaks the defensive antioxidant systems (glutathione, antioxidant enzymes), causing the secondary synthesis of malonic aldehyde, nitric oxide and peroxynitrate. These compounds initiate the peroxidation of lipids and proteins, contributing to damage of kidney tissues [38]. According to the above-mentioned concept of free-radical damage to kidney tissues, antioxidant compounds had a nephroprotective effect in the experimental studies in animals treated with CP [43, 52].

The increase in urinary excretion of FABP in rats treated with CP, demonstrated in our experiment, may also be an expression of compensation for increased oxidative stress. FABP is an endogenous protein that regulates the intracellular distribution of fatty acids, also present in kidney tubule cells, in the isoform also found in the liver ("liver-type fatty acid binding protein"; L-FABP) [21]. It is believed that this protein, binding the excess of free fatty acids and being a scavenger of free radicals, plays an antioxidant role in many organs in the course of oxidative stress [57], including kidney damage of various aetiologies [30]. Both after single and fourfold CP dose, we observed an increase in urinary FABP concentration, and its 24-hour excretion. Similarly, our experiment showed an increase in the concentration and 24-hour urinary excretion of the second study protein – osteopontin. According to its name, this protein is mainly involved in the regulation of bone mineral density. However, it is also found outside the bones, including the kidneys (in the distal and collective tubules), where, similar to uromodulin, it plays the role of a physiological inhibitor of crystallization and nucleation, inhibiting the formation of kidney stones [31, 54]. Osteopontin is also an overproduced protein in the kidney in the course of free radical damage, inhibiting the activity of nitric oxide synthase, which proves its similar antioxidative functions, as those found for FABP [17, 50].

Considering the increase in renal excretion of both FABP, and osteopontin, produced in the kidney tubules in response to various damaging factors, including free radicals, it can be assumed that these proteins determined in the urine of rats treated with CP are laboratory evidence of developing tubulopathy in the course of acute kidney injury. Hence, it appears that FABP and osteopontin may be useful markers for monitoring co-

renal dysfunction in patients treated with CP. It should also be noted that the disturbances of these proteins were observed in the absence of significant histopathological disturbances in the kidneys, which justifies the thesis that overproduction and release of FABP and osteopontin into the urine may precede morphological disorders of the kidneys. Moreover, both FABP and osteopontin seem to be the decisive biomarkers for the diagnosis of early (functional) stage of CP-induced tubulopathy in the event of a lack of full interpretation compatibility of the results of the classical, lower molecular laboratory parameters assessing kidney functions. In the urine of the study animals, proteinuria was found – considering it in the context of the observed increase in FABP and osteopontin, it can be classified as a tubular one. During our experiment, we also found an increase in blood creatinine in animals with both acute and chronic cystitis, with increased urea concentration, but only in the group with an acute inflammation model. However, irrespective of the CP dose administered, urea and creatinine concentrations were reduced in the urine of rats, which was not accompanied by significantly different diurnal urinary excretion of these compounds. Additionally, the calculated values of urea and creatinine clearances did not differ significantly in the control groups and those treated with CP (with the exception of urea clearance in group 4). Therefore, the results of classical parameters assessing kidney function were divergent and did not allow for an unequivocal biochemical diagnosis of evident acute kidney injury. Moreover, a significant observation made in our experiment was polyuria in both groups of animals treated with CP. Literature reports are not consistent with our observations – there is even described an antidiuretic effect of CP on the distal part of the nephron [52], due to the induction of vasopressin-independent increased expression of tubular water canals – aquaporins [23, 24], as well as the development of CP-induced syndrome of inappropriate antidiuretic hormone release [36]. Therefore, the issue of diuresis disturbances after CP requires further refinement. However, considering the increased excretion of osteopontin, released in further and collective tubules, in our opinion, the polyuria detected in CP-treated rats may also be an indirect evidence of tubulopathy development, as an expression of the inability to concentrate urine in the distal part of the nephron. In addition, in one of our previously published papers [9] dedicated to assessment of another protein biomarker – kidney-1 injury molecule, we have also showed polyuria in analogous groups of animals, which was accompanied by increased 24-hour urinary excretion of KIM-1, both in animals with an acute and chronic model of cystitis. Thus, the present experiment once again demonstrated the nephrotoxic potential of CP and proved the usefulness of new protein biomarkers in assessing this phenomenon.

In summary, parallel impairment of kidney functioning develops in the course of cyclophosphamide-induced cystitis, including the increased production and the release of some of the acute kidney injury markers – FABP and osteopontin. Therefore, taking into account the results of our experiment and, according to literature suggestions, laboratory diagnostics of cyclophosphamide-induced nephrotoxicity should now be based not only on the assessment of classical parameters (creatinine, urea), but on the panel of new protein biomarkers (L-FABP and osteopontin, KIM-1, NGAL-1 and cystatin C), which are characterized by better sensitivity and specificity in comparison to routinely assessed small molecule nitrogen compounds. It should be expected that these new AKI markers will be increasingly used in clinical practice, expanding the possibilities of laboratory diagnosis of kidney diseases. Moreover, our results indicate the need to monitor kidney function based on the evaluation of these proteins in patients treated with cyclophosphamide.

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