The Effect of Acetylcysteine on Renal Function in Experimental Models of Cyclophosphamide- and Ifosfamide-Induced Cystitis

Lukasz Dobrek, Klaudia Nalik-Iwaniak, Kinga Fic, Zbigniew Arent

Abstract

Introduction: Urotoxicity is a characteristic attribute of cyclophosphamide and ifosfamide. Acetylcysteine is perceived as a uroprotective and possible nephroprotective compound. The purpose of the study was to assess the effect of acetylcysteine treatment on the morphology of the kidneys and the urinary bladder, and renal function in rats with cystitis induced by cyclophosphamide or ifosfamide. Methods: Cystitis was induced in rats belonging to groups 2 and 3, as well as 4 and 5, by five administrations of cyclophosphamide (75 mg/kg) or ifosfamide (80 mg/kg) respectively. Additionally, groups 3 and 5 received acetylcysteine (200 mg/kg). Group 1 was "sham treated" as a control. Upon conclusion of the experiment, the animals were euthanized and their kidneys and urinary bladders were collected for histopathological analysis. The assessment of renal function was based on classic nitrogen blood parameters (urea, creatinine, and uric acid), as well as proteinuria and cystatin C (CysC) and kidney injury molecule-1 (KIM-1) urinary concentrations, and their 24-hour elimination with urine. Results: Reduction of blood urea nitrogen and uric acid, and urinary pH with a significant increase of CysC and KIM-1 urinary concentrations, and their 24-hour elimination with urine were observed in groups 2 and 4. The acetylcysteine treatment did not cause a significant change of blood parameters, but significantly decreased 24-hour elimination of CysC and KIM-1 with urine, and accounted for alleviation of the histopathological abnormalities of urinary bladders, with no significant effects on the structure of the kidneys. Conclusions: Acetylcysteine used in the experimental model of cyclophosphamide- and ifosfamide-induced cystitis had a uroprotective effect and also reduced renal dysfunction, which suggests its potential use as a nephroprotective compound in cyclophosphamide/ifosfamide therapy.

Introduction

Both cyclophosphamide and its analogue ifosfamide are commonly used alkylating cytostatics in chemotherapy for various tumors and in autoimmune disorders [1–4]. Cyclophosphamide and ifosfamide are prodrugs, and their pharmacological effect is associated with their metabolites being synthesized in the course of the metabolic transitions occurring both within and outside the liver, with the participation of cytochromes CYP3A4, CYP2B6, and CYP2C9. Bio-transformation of cyclophosphamide and ifosfamide leads to the formation of intermediate, unstable 4-hydroxyl derivatives that are further transformed to phosphoric yperite and unsaturated aldehyde acrolein. The transformation also involves the production of another metabolite – chloroaacetdehyde. Finally, intermediate metabolites are oxidized with dehy-
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Experimental studies have demonstrated that chloroacetaldehyde reacting with sulphydryl groups decreases the extracellular glutathione level, thus sensitizing cells to free radical damage. Chloroacetaldehyde also causes inhibition of the caspase system, thus accounting for the disturbance of the equilibrium between cell proliferation and apoptosis, and inhibits the process of oxidative phosphorylation in mitochondria, which leads to the arrest of intracellular ATP production and an energetic deficit. Those disturbances lead to the necrosis of cells of renal tubules [15, 16]. For this reason, despite the fact that the nephrotoxic effect of cyclophosphamide/ifosfamide may be observed in any segment of a nephron, intrarenal disorders of a structural and/or functional tubulopathic character are most commonly reported [17, 18]. Clinically, this is manifested by isolated loss of compounds that are normally resorbed in the renal tubes (glucose, protein, phosphates, and hydrocarbons) with urine, a full-symptomatic proteinuria or the Fanconi syndrome. Urine condensation disorders and polyuria were also reported to be caused by the development of renal diabetes insipidus following the use of ifosfamide. Contrary reports are associated with cyclophosphamide treatment, suggesting the development of oliguria and the syndrome of inappropriate antidiuretic hormone hypersecretion [19].

One possibility for reducing urotoxicity during cyclophosphamide/ifosfamide therapy is to introduce concomitant Mesna (sodium-2-mercaptoethanesulfonate). This compound is bio-transformed into a metabolite eliminated with urine, containing numerous thiol groups that have an antagonistic effect on acrolein [20]. Therefore, the use of Mesna is currently a basic uroprotective procedure applied during cyclophosphamide/ifosfamide treatment, although studies are under way on the use of other compounds (antioxidants, free radical scavengers, such as melatonin, NSAIDs, and steroids) in the chemoprevention of cystitis [20, 21]. Acetylcysteine is one of these studied compounds, considering its character as a donor of -SH groups, similar to Mesna. Moreover, acetylcysteine is described as an antioxidant optimizing cells against oxidative injury and inflammation due to its ability to directly bind hydroxyl radicals and increase glutathione production [22]. Acetylcysteine has long been used as a mucolytic agent and also as an antidote in paracetamol intoxication [23]. Due to its strong antioxidant effect, acetylcysteine has also been studied for its efficacy in the supportive treatment of hepatic cirrhosis [24] and colitis [25]. There are also grounds to suggest that due to the anti-inflammatory and antioxidant properties of acetylcysteine, this compound also has nephro-

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protective properties. Data on the effect of acetylcysteine used for nephroprotection in cyclophosphamide- or ifosfamide-induced cystitis, however, are scarce. A review of the literature in the PubMed database for the last 20 years (completed in September 2018 with the following search criteria: publication date 1998–2018, any type of English-language paper, search terms “acetylcysteine” or “kidney” with the “and” quantifier) returned 773 items, broadly associated with the search terms. A detailed analysis of titles and abstracts of the search results indicated only 204 original, review or clinical trial reporting on the effect of acetylcysteine on renal function in the course of various nephrological disorders (mostly 140 items on contrast media-associated nephropathies, 18 reports on acute kidney injury of other etiologies, 21 papers on dialyzed patients with chronic kidney failure, and a single report on nephrotoxic kidney injury caused by cisplatin or amphotericin B and the hepatorenal syndrome).

Therefore, the aim of this study was to perform a laboratory and histopathological assessment of renal function in the experimental model of cystitis induced by cyclophosphamide or ifosfamide, and to assess any potential changes in renal function with the administered uroprotective treatment with acetylcysteine.

Materials and Methods

Animals, Study Groups, and General Outline of the Experiment

Fifty 9-week-old albino Wistar rats (male and female in equal ratios) obtained from the Central Animal House of the Pharmacy Faculty in Krakow, were used for the experiment. After being transported to the Experimental and Innovative Medicine Centre, the animals were quarantined for 10 days, in order to become accustomed to their new housing. Environmental parameters were automatically monitored and maintained in accordance with recommended zoo-technical requirements for rats (temperature 20–24°C, air humidity 60–70%, constant ventilation ensuring 8–10 total exchanges of air per hour, lighting 130–325 lux with a 12/12 light/darkness cycle, and noise < 30 dB). During the quarantine period and the experiment, the rats were kept in single-sex cages, 2 animals in each, except for days when the animals were kept in individual metabolic cages. Throughout the duration of the experiment, the animals had access to standard feed (Labofeed B from “Morawski” Feed Production, Keynia, Polska) and water ad libitum. Following the quarantine, the animals were randomly divided into 5 study groups (10 animals in each group, male to female ratio 1:1): control receiving normal saline (Group 1), group with cystitis induced by cyclophosphamide (Group 2), group with cystitis induced by cyclophosphamide and receiving a simultaneous supportive treatment with acetylcysteine (Group 3), group with cystitis induced by ifosfamide (Group 4), group with cystitis induced by ifosfamide and receiving a simultaneous supportive treatment with acetylcysteine (Group 5). Upon conclusion of the treatment, rats were placed for 24 hours in individual metabolic cages for the assessment of their basic life parameters and collection of urine. After monitoring in metabolic cages, blood was collected from live animals under deep anesthesia. Finally, following blood drawing, the animals were euthanized and their organs were collected for histopathological examination.

Procedures Used in Study Groups

In groups 2 and 3, cystitis was induced by five intraperitoneal administrations of cyclophosphamide (Endoxan, Baxter, powder for solution for injection, European Article Number – EAN code: 5909990240920) at a dose of 75 mg/kg, every 2 days, up to a total dose of 375 mg/kg. The cyclophosphamide solution was prepared ex tempore prior to administration, in order to avoid hydrolysis of the drug. The accepted cyclophosphamide dosage was consistent with the methodology developed by other authors inducing a model of chronic, cyclophosphamide-induced cystitis [26, 27]. Additionally, animals in Group 3 intraperitoneally received a dose of acetylcysteine at 200 mg/kg an hour before administration of cyclophosphamide (Acetylcysteine Sandoz 300 mg/3 ml, ampoules containing solution for injection, EAN code: 5909990789313). The acetylcysteine dose used was consistent with the dosing range (200–500 mg/kg) administered in other experimental studies [28, 29].

Rats in groups 4 and 5 received five intraperitoneal doses of ifosfamide (Holoxan, powder for solution for injection, EAN code: 5909990241118) at a dose of 80 mg/kg, using the same scheme as Group 2. The amount of ifosfamide administered was consistent with the dosing range described in other experimental studies on ifosfamide-induced cystitis [30, 31]. Similarly to cyclophosphamide, the ifosfamide solution was prepared ex tempore. As with Group 3, animals in Group 5 received intraperitoneally acetylcysteine, using the same dosing scheme.

Control animals in Group 1 received a “sham treatment” consisting in an injection of normal saline, in volumes corresponding to the amount of drugs applied in groups 2–5. We did not study an additional control group with acetylcysteine alone due to literature reports that acetylcysteine administered in other experimental studies at doses up to 500 mg/kg did not have any adverse effects [32]. Each animal was weighed prior to administration of each cyclophosphamide/ ifosfamide/acetylcysteine/saline dose, for dose calculation.

The Assessment of Living Parameters

Twenty-four hours after the last dose of cyclophosphamide/ ifosfamide/acetylcysteine/saline, the animals were placed in individual metabolic cages in order to collect urine for further clinical chemical tests and assessment of basic living parameters. While placed in metabolic cages, the animals had unlimited access to water and standard laboratory feed as in normal cages. The following parameters were assessed: body weight (g), 24-hour water consumption (ml/24h), 24-hour feed consumption (g/24h), and 24-hour diuresis (ml/24h).

Determined Serum Laboratory Parameters

Basic parameters reflecting renal function were determined in serum samples obtained after the end of the experiment: creatinine (Cr) (mg/dl), blood urea nitrogen (BUN) (mg/dl), BUN/Cr ratio, uric acid level (mg/dl), and total protein (g/dl). Determinations were made using the automated Siemens Advia 1200 analyzer.
Table 1. Results of parameters obtained in study groups during 24-hour monitoring in metabolic cages

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<tr>
<td></td>
<td>1</td>
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<tr>
<td>Body weight, g</td>
<td>298.64 ± 88.28</td>
<td>269.20 ± 92.11</td>
</tr>
<tr>
<td>24-hour water intake, ml</td>
<td>12.65 ± 6.46</td>
<td>18.75 ± 7.81</td>
</tr>
<tr>
<td>24-hour feed intake, g</td>
<td>19.1 ± 8.33</td>
<td>16.75 ± 10.84</td>
</tr>
<tr>
<td>24-hour diuresis, ml</td>
<td>5.80 ± 3.05</td>
<td>9.00 ± 5.18</td>
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*p ≤ 0.05.

Table 2. Biochemical parameters assessed in the serum of study rats

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<tr>
<td></td>
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<tr>
<td>Cr, mg/dl</td>
<td>0.22 ± 0.08</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>24.00 ± 1.55</td>
<td>17.00 ± 2.68</td>
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<tr>
<td>BUN/Cr</td>
<td>126.33 ± 56.99</td>
<td>66.67 ± 19.52</td>
</tr>
<tr>
<td>Uric acid, mg/dl</td>
<td>1.93 ± 0.71</td>
<td>0.68 ± 0.32</td>
</tr>
<tr>
<td>Total protein, g/dl</td>
<td>5.57 ± 0.49</td>
<td>5.30 ± 0.47</td>
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*p ≤ 0.05; **p ≤ 0.01.

Table 3. Biochemical parameters assessed in the urine of study rats

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<tr>
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<tr>
<td>Protein, g/l</td>
<td>0.58 ± 0.36</td>
<td>0.62 ± 0.42</td>
</tr>
<tr>
<td>pH</td>
<td>7.05 ± 0.55</td>
<td>6.50 ± 0.65</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.01 ± 0.01</td>
<td>1.01 ± 0.01</td>
</tr>
<tr>
<td>CysC, pg/ml</td>
<td>354.89 ± 227.38</td>
<td>638.61 ± 359.97</td>
</tr>
<tr>
<td>KIM-1, pg/ml</td>
<td>163.09 ± 55.39</td>
<td>307.34 ± 78.35</td>
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*p ≤ 0.05; **p ≤ 0.01.

Determined Urine Laboratory Parameters

Urine samples obtained after the end of monitoring in metabolic cages were subjected to preliminary and semi-quantitative analysis using urine strips (Insight, ACON Laboratories, REF U031-105). According to the producer’s instructions, specific gravity was determined 45 seconds after placing the urine sample on a strip, and pH and proteinuria values (mg/dl) 15 seconds later (at 60 seconds). Further, cystatin C (CysC) (pg/ml) and kidney injury molecule-1 (KIM-1) (pg/ml) were determined in urine samples using commercial ELISA tests (Rat CysC ELISA kit FineTest, Wuhan Fine Biological Technology Co. Ltd., catalogue No ER0891; range 0.0156–1 ng/ml; sensitivity < 0.0094 ng/ml, and Rat KIM-1 ELISA kit FineTest, Wuhan Fine Biological Technology Co. Ltd., catalogue No ER0043; range 62.5–4,000 pg/ml; sensitivity < 37.5 pg/ml), strictly following the manufacturer’s instructions. Also, based on the measured diuresis (ml/24h), the 24-hour elimination of the abovementioned proteins was calculated (pg/24h).
Animal Euthanasia
Following the completion of monitoring in metabolic cages, the rats were subjected to deep general anesthesia and thoracotomy for the collection of blood from the heart. The animals were first placed in the anesthetic chamber and subjected to inhalational anesthesia (isoflurane). Under general anesthesia, the animals additionally received intraperitoneally pentobarbital (Mobital, Biowet, Puławy) at a dose of 50 mg/kg. After deep general anesthesia was confirmed, a thoracotomy was performed and blood was collected from the heart to a clot tube. The obtained samples were centrifuged (2,000 rpm, 5 minutes) and the resulting serum was stored at -80°C awaiting tests. Following the collection of blood, the animals received an additional pentobarbital dose (50 mg/kg, intraperitoneal) up to a total lethal dose of 100 mg/kg. Upon confirmation of the absence of vital signs, laparotomy was performed. The kidneys were extirpated from the clots. The obtained result was expressed as a ratio (%) of the total body weight of the particular individual. The assessment was made at 100× magnification.

Histopathological Assessment of Collected Organs
Microscopic slides were made of the collected organs. Four-micron thick slices were cut with a Leica RM2135 microtome and stained with hematoxylin and eosin (H&E). The detailed methodology is described in one of our earlier published papers [34]. The histopathological analysis was performed by an expert pathomorphologist working for the Animal Pathomorphology Laboratory “ALAB” in Warsaw. The assessment was made at 100× magnification.

Statistical Analysis of Obtained Results
The results were compared for statistical significance, initially for 5 groups (Group 1: control; Group 2: cyclophosphamide; Group 3: cyclophosphamide + acetylcysteine; Group 4: ifosfamide; Group 5: ifosfamide + acetylcysteine), and analyzed with Bartlett’s parametric test. Then, intergroup differences were analyzed, comparing results for groups: 1 vs. 2; 1 vs. 3; 1 vs. 4; 1 vs. 5; 2 vs. 3; and 4 vs. 5) with the Fischer-Snedecor parametric test. The value of p 0.05 was accepted as the level of statistical significance.

Results
Animals Lost During the Experiment
Forty-seven animals completed the experiment. One individual was lost in the cyclophosphamide group and two in the ifosfamide group. Therefore, the final number of animals in individual groups was: Group 1 (n = 10), Group 2 (n = 9), Group 3 (n = 10), Group 4 (n = 8), Group 5 (n = 10).

Living Parameters of Study Animals
No significant differences in body weight of rats in individual groups were observed in the intergroup analysis. The animals were also characterized by a similar 24-hour water consumption. Rats treated with acetylcysteine were characterized by a significant lower 24-hour consumption of feed, compared to the control. Values of 24-hour diuresis were similar in all groups, except for rats with ifosfamide-induced cystitis treated with acetylcysteine. These animals excreted lower amounts of urine. A trend (not statistically significant) was also observed for increased micturition in rats with cyclophosphamide-induced cystitis. The detailed results are presented in table 1.

Parameters of Renal Function Determined in the Serum
No statistically significant differences in Cr levels were found among all study groups. Comparing control rats and rats with cyclophosphamide-induced cystitis, a significant reduction of the uric acid level, BUN, and the BUN/Cr ratio was observed. The level of total protein was similar in both groups. A similar correlation regarding BUN and uric acid was also found comparing results for groups 1–3. Animals with ifosfamide-induced cystitis demonstrated a significant reduction of the BUN level and the BUN/Cr ratio, but uric acid and total protein levels were comparable to those in the control group. Animals with ifosfamide-induced cystitis treated with acetylcysteine only demonstrated a significant reduction of the BUN/Cr ratio value, compared to the control.

Comparing results for cyclophosphamide- and ifosfamide-induced cystitis groups and the additional acetylcysteine treatment (groups 2–3 and 4–5), no significant intergroup differences were noted. The detailed results are presented in table 2.

Parameters of Renal Function Determined in Urine
Semi-quantitative urinalysis made with strip tests demonstrated no significant proteinuria or specific gravity abnormalities in study groups. A trend for reduced urine pH was observed in groups 2–5, which was statistically significantly different in the case of values achieved in control animals compared to groups 2, 3, and 4.

Analyzing the concentration of CysC in urine, a statistically significant increase was found in rats in Group 2,
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compared to the control (Group 1). Also, rats in Group 4 demonstrated a marked trend for increased CysC levels, although it was not statistically significant. Administration of acetylcysteine to rats with cyclophosphamide-induced cystitis (Group 3) did not significantly change the urine content of the protein, as compared to corresponding values in groups 1 and 2. Animals with ifosfamide-induced cystitis treated with acetylcysteine (Group 5), however, demonstrated the lowest CysC levels in all groups, and the difference in comparison to non-treated animals (Group 4) was statistically significant.

Analyzing urinary concentrations of the second protein – KIM-1 – a significant increase of the value compared to the control (Group 1) was found in all groups. Comparing KIM-1 levels, however, in animals with cyclophosphamide- or ifosfamide-induced cystitis to values determined in animals receiving acetylcysteine (groups 2–3 and 4–5), no significant differences were found (table 3).

Assessing 24-hour elimination of CysC, a nearly 3- and 1.5-fold increased elimination of the protein with urine was found in groups 2 and 3, respectively, com-

Fig. 1. A, B 24-hour urinary CysC and C, D KIM-1 excretion (pg/24h) in study groups (*p ≤ 0.05; **p ≤ 0.01; NS = non-significant).
compared to Group 1. Comparing results obtained for animals with cyclophosphamide-induced cystitis treated (Group 3) or non-treated with acetylcysteine (Group 2), a significantly lower 24-hour elimination of CysC was observed in animals receiving the acetylcysteine treatment.

A significant 1.5-fold increase of CysC elimination was also found in rats with ifosfamide-induced cystitis (Group 4) compared to the control (Group 1). Administration of acetylcysteine (Group 5) significantly decreased elimination of the protein in comparison to non-treated animals (Group 4), down to a value lower than that observed in the control group (Group 1).

For KIM-1, over a 3-fold and double, statistically significant increase of 24-hour elimination of the protein by animals with cyclophosphamide-induced cystitis, without and with the acetylcysteine treatment, respectively, was observed compared to the control group (Group 1). The difference was still significant comparing groups 2 and 3.

Animals with ifosfamide-induced cystitis (Group 4) also demonstrated a significant, double 24-hour elimination of KIM-1, compared to the control (Group 1). Treatment with acetylcysteine (Group 5) caused a reduction of 24-hour elimination of the protein to a level comparable to that observed in the control group. Also, comparing 24-hour KIM-1 elimination in animals with ifosfamide-induced cystitis treated with acetylcysteine (Group 5) and receiving no such treatment (Group 4), a significantly lower result was found in Group 5. Detailed results are presented in figure 1.

Weight Assessment of Urinary Bladders and Kidneys

The absolute wet weight of urinary bladders (g) was not different in compared groups. Expression of the value as a ratio (%) of body weight of the study rats demonstrated a significant increase in individuals with cyclophosphamide-induced cystitis not treated with acetylcysteine (comparing groups 1 and 2). No significant differences were noted for those parameters comparing the results obtained in groups 1, 4, and 5.

The assessment of the absolute “wet weight” of kidneys, and expression of those values as the ratio of body weight, demonstrated no significant intergroup differences, except for a reduction of the wet weight of the right kidney in groups 2, 3, and 4, compared to the control (table 4).

Table 4. The bladder wet weight and kidney wet weight and their percentage share in the total body weight of the study animals

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<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Bladder wet weight, g</td>
<td>0.182±0.094</td>
<td>0.190±0.063</td>
</tr>
<tr>
<td>Bladder weight to body weight, %</td>
<td>0.059±0.017</td>
<td>0.073±0.014</td>
</tr>
<tr>
<td>Left kidney wet weight, g</td>
<td>0.925±0.239</td>
<td>0.924±0.318</td>
</tr>
<tr>
<td>Left kidney wet weight to body weight, %</td>
<td>0.317±0.022</td>
<td>0.344±0.023</td>
</tr>
<tr>
<td>Right kidney wet weight, g</td>
<td>0.962±0.270</td>
<td>0.906±0.275</td>
</tr>
<tr>
<td>Right kidney wet weight to body weight, %</td>
<td>0.327±0.023</td>
<td>0.342±0.029</td>
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*p ≤ 0.05.
a particular emphasis on the disappearance of urothelial dysplasia. A marked blood congestion was still observed in the cystic wall. Improvement of the morphological condition of the urinary bladder was also observed in the group with ifosfamide-induced cystitis and simultaneous acetylcysteine treatment (Group 5). Epithelial cells had a normal structure, with minor anisokaryosis and signs of renewal. The discussed histopathological lesions of the urinary bladders observed in all study groups are presented in figure 2.

Kidneys from rats in Group 2 demonstrated exfoliation of apical parts of the tubular epithelium and a moderate edema of tubular epithelial cells in the part of the cortex close to the medulla. These lesions were accompanied by a mild to moderate congestion of renal glomeruli. The histopathological presentation of kidneys from rats in Group 4 was similar to that mentioned above.

Acetylcysteine administration did not significantly alter the histopathological presentation of kidneys from animals treated with cyclophosphamide (Group 3). In rats receiving acetylcysteine treatment, changes were still present but they were minor and probably had no effect on renal function. Similarly, no significant changes indicating pathological lesions within glomeruli, tubules, stroma, or renal vessels were observed in Group 5. The discussed histopathological changes of the kidneys observed in all study groups are presented in figure 3.

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**Fig. 2.** Urinary bladders in study groups (A–E: H&E staining, magnification 100×). Some histopathological abnormalities are marked with arrows.
Discussion

The analysis of laboratory results and histopathological assessment demonstrated the development of the impairment of renal function secondary to experimental cyclophosphamide- and ifosfamide-induced cystitis. The laboratory assessment of renal function in our experiment was based on the determination of classic nitrogen compounds in the serum, and on the assessment of new renal function markers – CysC and KIM-1 in the urine. Modern nephrological diagnostics increasingly apply new biomarkers, that demonstrate better sensitivity and specificity in comparison with classic nitrogen parameters. These proteins appear in the urine or blood at early, clinically mute periods of renal dysfunction, not yet associated with hypercreatininemia. Moreover, demonstration of disturbance of these proteins allows the drawing of inferences about the etiology of developing kidney injury, which is particularly important in the case of acute kidney injury [35]. Therefore, these proteins are referred to as “renal troponins”, in analogy to parameters determined in the course of acute coronary syndromes [36]. Compounds most commonly used in modern nephrological laboratory diagnostics are: CysC, KIM-1, neutrophil gelatinase-associated lipocalin-1, fatty acid-binding protein, osteopontin, interleukin-18, and other inflammatory mediators and enzymes released from injured tubules, such as N-acetyl-β-D-glucosami-
dase. The detailed description and diagnostic role of the abovementioned biomarkers were discussed in numerous reviews [37, 38], including one written by us [39]. It has to be noted that the assessment of drug-induced nephrotoxicity is currently based on the analysis of this type of parameters [40].

The analysis of classic low-molecular nitrogen parameters of renal function assessed in our study yielded inconclusive results. No differences in serum Cr levels were found among the groups, accompanied by reduction of BUN and consequential reduction of the BUN/Cr ratio. Reduction of serum BUN is an observation of minor pathophysiological significance, particularly in the absence of any co-existing Cr disorders [41]. According to the physiological description, urea is filtered along with Cr, and then undergoes resorption and tubular secretion. The mechanisms of the tubular transport of the compound account for the condensation of urine and determine diuresis [42]. We did not determine the urinary urea level, which would allow the drawing of conclusions regarding the tubular transport (secretion and absorption) of urea. The magnitude of 24-hour diuresis in animals with both cyclophosphamide- and ifosfamide-induced cystitis, however, was similar to that observed in the control rats (although there was a trend of increasing value in Group 2), which indirectly implies that the apparent decrease in serum urea was not associated with its excessive loss with urine and did not contribute to quantitative disorders of diuresis. Hence, reduced serum urea may be considered a manifestation of simultaneously occurring liver failure in animals treated with cyclophosphamide/ifosfamide, and of impaired synthesis of urea, rather than of loss of the resorptive properties of tubules. In clinical nephrological diagnostics, the BUN/Cr ratio is used to assess the etiological azotemia, and reduction in this ratio may indicate acute kidney injury in the intrarenal mechanism [41, 43]. Lower BUN/Cr values observed in cyclophosphamide/ifosfamide-induced cystitis groups, as compared to the control group, however, cannot be unequivocally perceived as expression of intrarenal damage, but may also have resulted from impaired liver function in the test animals. In summary, in our opinion, serum levels of creatinine and urea do not allow for unequivocal interpretations. An important observation made in our experiment, however, was the reduction of serum uric acid in rats with cyclophosphamide-induced cystitis both not treated with acetylcysteine (Group 2) and treated with acetylcysteine (Group 3). Uric acid is a product of catabolism of purines, demonstrating strong antioxidative properties [44, 45]. As mentioned above, acrolein formed in the course of transformations of cyclophosphamide is a compound that initiates significant oxidative stress, associated with a compensatory antioxidative response, expressed, among others, with the “consumption” of endogenous antioxidants. This type of association was not demonstrated for animals with ifosfamide-induced cystitis, which may be a result of quantitative differences in the biotransformation of cyclophosphamide and ifosfamide. Despite the fact that both compounds share some common metabolic pathways and are sources of uro- and nephro-toxic intermediary metabolites, biotransformation of cyclophosphamide yields higher amounts of acrolein, compared to the metabolic pathway of ifosfamide [46]. Hence, the reduction of serum uric acid in animals receiving cyclophosphamide may be perceived as a manifestation of the “consumption” of uric acid as an antioxidant. A similar correlation was not noted in rats with ifosfamide-induced cystitis, which is consistent with the general concept that ifosfamide has lower urotoxic potential, compared to cyclophosphamide.

In our opinion, the most significant results obtained in our study were those related to the levels and 24-hour elimination of CysC and KIM-1 with urine. CysC is an inhibitor of cysteine proteases and a modulator of the activity of immune cells. It is produced by all cells of the organism, with constant, physiological dynamics. The protein is filtrated in the kidneys. Then, it is resorbed, undergoing lizosomal degradation in proximal tubules, with Megalin participating in the process [47]. In nephrological diagnostics, CysC is increasingly perceived as a parameter that reflects the excretory function of the kidneys better than Cr, and the calculated Cr-based clearance rate [48, 49]. Clinical trials have demonstrated that CysC is a much more sensitive marker of decreased glomerular filtration rate in chronic renal disease, than Cr, and that increased CysC is considered as a minor disorder of glomerular filtration (glomerular filtration rate at a level of 70–90 ml/min) at which blood Cr is still normal [50]. It is, therefore, believed that plasma CysC is a sensitive marker of renal filtration disorders allowing early diagnosis of asymptomatic stages of kidney injury [51]. CysC may be determined both in the blood and urine. As mentioned above, blood CysC levels depend on the filtration rate. Therefore, this protein is an alternative to Cr in the assessment of the filtration fraction, and urine CysC levels are a result of glomerular filtration and the resorptive capacity of proximal tubules. Therefore, considering the absence of any coexisting disorders of serum Cr (indicating maintenance of a normal filtration fraction), the increased presence of CysC in the urine obtained from
animals with cyclophosphamide- and ifosfamide-induced cystitis observed in our experiment suggests that after the filtration stage, CysC does not undergo tubular reabsorption. For this reason, the assessment of CysC levels justifies a hypothesis of early cyclophosphamide/ifosfamide-induced tubulopathy in animals with cystitis. The diagnosis of early renal dysfunction is also a result of the absence of significant proteinuria in the study groups, which principally excludes their advanced tubulopathy. Moreover, reduction of urine pH was demonstrated in animals with cyclophosphamide- and ifosfamide-induced cystitis, compared to the control animals. The final pH mostly depends on both reabsorption of alkaline hydrocarbons in the proximal segment of nephron, and active secretion of hydrogen ions with K+\text/H+\text-ATP-ase antport present in the luminal membranes of distal tubules [52, 53]. Excessive acidity of the urine observed in rats with cyclophosphamide/ifosfamide-induced cystitis may also be a manifestation of dysfunction of the renal tubules in terms of the mechanisms engaged in urine pH control, which fits into the general hypothesis of the development of tubulopathy in those animals.

Our hypothesis is also strongly supported by the analysis of KIM-1 results obtained in our study. KIM-1 is a glicoprotein anchored in the apical membrane of proximal tubules, and absent in glomeruli and renal parenchyma. Renal tubular metaloproteinases release KIM-1 in response to ischemia, hypoxia, septic, or toxic damage of the tubules, and the development of local inflammation. KIM-1 rapidly appears in urine and its level is maintained until the regeneration of the kidneys, which makes the protein a sensitive early marker of renal injury [54, 55]. The significance of KIM-1 in nephrological diagnostics was confirmed by two international drug agencies – Food and Drug Administration and European Medicines Agency. They both recommend assaying the protein as a laboratory marker of nephrotoxicity in studies of novel drugs [56]. Considering the fact that KIM-1 is not found in urine from healthy individuals, any significant increase of the protein urinary level has to be perceived as a manifestation of kidney injury. In our study, we also demonstrated increased 24-hour elimination of KIM-1 with urine. Along with results of CysC, this protein also confirms the hypothesis of the development of tubulopathy in animals receiving cyclophosphamide or ifosfamide. Quantitative CysC and KIM-1 level disorders were more pronounced in rats with cyclophosphamide-induced cystitis. These animals had higher urine CysC and KIM-1 levels, and demonstrated higher values of 24-hour elimination of these proteins with urine. In our opinion, this suggests that compared to ifosfamide, cyclophosphamide is characterized by equal or even higher nephrotoxic potential than previously believed. It is accepted, however, that ifosfamide is characterized by relatively preferentially accentuated nephrotoxicity resulting from overproduction of nephrotoxic chloroacetaldehyde in the course of ifosfamide transformations, as well as from active uptake of the compound by renal tubules with participation of the organic cation transporter 2 [57].

The analysis of the results of the experiment also demonstrated that treatment with acetylcysteine, in addition to the expected uroprotective effect manifested by the improved histopathological presentation of the bladders, resulted in some improvement of renal function. The analysis of the results obtained for groups of animals treated and non-treated with acetylcysteine (groups 2–3 and 4–5) demonstrated no significant changes of serum low-molecular-weight nitrogen parameters (except for the increase of the BUN/Cr ratio in Group 3). In assessing the results of 24-hour elimination of new biomarkers with urine, however, it was demonstrated that both rats with cyclophosphamide- and ifosfamide-induced cystitis, and simultaneously treated with acetylcysteine, were characterized by reduced 24-hour elimination of both CysC and KIM-1, compared to animals with the same model of cystitis but receiving no additional treatment. This observation suggests that treatment with acetylcysteine may improve the resorptive function of tubules (resulting in lower elimination of CysC with urine) and reduce their structural damage (expressed by lower exocytosis of KIM-1 into urine). The nephroprotective effect of acetylcysteine was not associated with improved histopathological presentation of the kidneys. In our opinion, this may result from the limited observation time and acceptance of the assumption that improved renal function precedes structural improvement. Moreover, the morphological changes of the kidneys that determine their functional disturbances may be discrete. Therefore, the classic histopathological examination of the kidneys may be extended by immunohistochemistry and evaluation of the microstructure of the kidney in an electron or confocal microscope, which we did not perform in our study. This assumption opens the field for further, advanced histopathological studies that can unequivocally confirm our conclusion.

Analogous to its uroprotective effect, the mechanism of the nephroprotective effect of acetylcysteine is associated with the marked antioxidative property of the compound as a donor of thiol groups, and with its anti-inflammatory action, associated with the inhibition of
selected paracrine proinflammatory mediators and oxidative enzymes [22, 23, 58, 59]. Detailed research has also demonstrated that acetylcysteine reduced apoptosis of renal tubule cells as a result of downregulation of the pro-apoptotic Bax protein, and upregulation of the inhibitor of apoptosis – the Bcl-2 protein [60].

To our knowledge, this study is the first experimental trial on the effect of acetylcysteine on renal function in in vivo models of cyclophosphamide- and ifosfamide-induced cystitis, based on the analysis of novel laboratory markers (CysC, KIM-1). The only other similar study was reported by Chen et al. [61]. They demonstrated that acetylcysteine, administered simultaneously with ifosfamide, caused reduction of plasma Cr levels, as well as a reduction of beta-2-microglobulin levels, which is associated with increased glutathion content and a reduced amount of malonic aldehyde – a marker of lipid peroxidation in homogenates of rat kidneys. The presence of inflammatory changes in the urinary bladder was not verified in that study [61]. No reports regarding a similar assessment for cyclophosphamide are present in the literature. Therefore, we cannot directly compare our results to results obtained by other authors.

Study Limitations

In our laboratory assessment of kidney function, our analysis was based on selected classical parameters (Cr, urea, 24-hour diuresis, and urine pH). We did not determine the electrolyte, bicarbonate, or glucose levels. The lack of a full kidney laboratory panel (which despite all reservations, is still routinely performed in the biochemical assessment of the kidneys) could be considered as a limitation of our study. Our main conclusions have been based on the results of the evaluated proteins (CysC and KIM-1). In addition, taking into account the new markers of renal function mentioned in the discussion, a more comprehensive assessment of kidney function could have been made by determining other proteins, such as neutrophil gelatinase-associated lipocalin-1, fatty acid-binding protein, and osteopontin. These limitations of our study may be the starting point for continuing research in subsequent studies.

Conclusions

In summary, it has to be stated that acetylcysteine is a drug currently experiencing a “renaissance”, as new mechanisms of its action and indications are still being discovered. The present study demonstrated a potentially beneficial uro- and nephro-protective effect of acetylcysteine administered during treatment with cyclophosphamide and ifosfamide. In future, this observation may extend the official clinical indication for the use of the drug and protect patients undergoing chemotherapy with cyclophosphamide/ifosfamide against damage not only to the bladder but also to the kidneys. Nevertheless, it should be emphasized that the results of our study have to be verified, especially in clinical trials, to confirm explicitly the suitability of acetylcysteine as a uro- and nephro-protective compound in patients undergoing cyclophosphamide or ifosfamide treatment.


