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## Original Research Article

# The influence of oxazaphosphorine agents on kidney function in rats

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## ABSTRACT

**Background and objective:** The application of cytostatic oxazaphosphorines such as cyclophosphamide (CP) and ifosfamide (IF) is associated with the risk of kidney damage that, depending on the type of drug, dose and route of administration, adopts a different clinical entity and severity. The aim of our study was to assess the influence of CP and IF on the kidney histology and function in rats intraperitoneally treated with four doses of either CP or IF.

**Materials and methods:** A total of 30 rats were divided into three groups (10 in each group): group 1 (control), sham treated with saline solution, group 2 (treated with 75 mg/kg b.w. of CP), and group 3 (treated with 60 mg/kg b.w. of IF). After the treatment rats were sacrificed, blood was collected and nephrectomy and cystectomy were performed. Qualitative and quantitative parameters (including neutrophil gelatinase-associated lipocalin-1, NGAL-1) of kidney function were assayed in urine and plasma.

**Results:** CP-treated rats were characterized by a significant polyuria, decreased urine pH and by decreased daily urinary excretion of sodium, potassium, urea and uric acid accompanied by increased NGAL-1 excretion. A significant decrease of the plasma uric acid concentration was also observed. IF-treated animals were also characterized by decreased urine pH but with normal daily urinary excretion of assessed substances (except for reduced uric acid excretion).

Both CP and IF treated rats did not show any histopathological abnormalities in their kidneys.

**Conclusions:** CP caused more advanced kidney dysfunction and some indices suggested the development of prerenal acute kidney injury. In the CP-treated group some particularly marked urinary and plasma uric acid disturbances suggested compensation of increased oxidative stress as uric acid is considered to exert also antioxidant properties.

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## 1. Introduction

Nephrotoxicity is defined as a structural and/or functional kidney damage resulting from exposure to any noxious factor of toxic or ischemic character. Drugs are one of kidney-affecting agents, therefore the incidence of drug-induced nephrotoxicity has been continuously increasing [1]. Some general issues determining drug-induced kidney disease include glomerular or tubular dysfunction, impairment of renal blood flow or disturbances of kidney metabolic and endocrine function, accompanied by microscopic structural lesions or gross morphological changes [2]. The background for nephrotoxicity are detailed pathomechanisms, affecting renal vasculature (e.g. hemodynamic acute kidney injury or thrombotic microangiopathy), glomeruli (e.g. minimal change disease, focal segmental glomerulosclerosis) or tubulointerstitium (acute tubular necrosis, [ATN], crystal nephropathy or tubulopathies such as Fanconi syndrome, salt wasting, nephrogenic diabetes insipidus, syndrome of inappropriate antidiuresis) [3,4].

Nephrotoxicity remains an important adverse drug reaction (ADR) in case of administration of many classes of pharmacological agents, including particularly: contrast media, immunosuppressants (mostly cyclosporine A), aminoglycosides, sulphonamides, amphotericin B, non-steroidal anti-inflammatory drugs, gold and D-penicillamine, antihypertensives, with special attention to angiotensin converting enzyme inhibitors or diuretics [1]. Also, many cytostatic drugs are characterized by a significant nephrotoxicity. Therefore, despite the improvement in effectiveness of chemotherapy, drug-induced kidney damage remains a complication entangling the entire treatment [3].

Among the chemotherapeutics endowed with nephrotoxicity, oxazaphosphorine alkylating agents should be mentioned. These compounds include cyclophosphamide (CP), ifosfamide and less frequently used trofosfamide. CP is used in chemotherapy of both solid tumors and acute leukemia and is used as an immunosuppressant in nonneoplastic disorders (systemic lupus erythematosus and rheumatoid arthritis). Ifosfamide (IF) is also an effective agent used in treatment of solid tumors, including testicular cancer, rhabdomyosarcoma, Wilms' tumor, Ewing's sarcoma, bone sarcomas, osteosarcoma and neuroblastoma as well as some forms of lymphoma [3,5]. The main ADR of CP is hemorrhagic cystitis, induced by acrolein – a toxic product released during CP biotransformation. Ifosfamide shares with cyclophosphamide a toxic profile characterized by urotoxicity. Moreover, IF is considered to be more nephrotoxic compared to CP [2,3,5]. That is due to the fact that biotransformation of IF leads to a principal release of chloroacetaldehyde that is attributed to exert a more powerful nephrotoxic effect compared to acrolein [6]. To sum up, nephrotoxicity of oxazaphosphorine agents depends on the type of drug (CP vs. IF), the applied dose, the route of administration, the total time of treatment, the presence of other co-existing factors predisposing to nephrotoxicity [2,4]. That multitude of nephrotoxicity-determining factors also results in a varied clinical description of kidney damage evoked by use of those agents. Moreover, published reports of kidney damage evoked by oxazaphosphorine administration

are only partial, based on the different nature of the assessment (imaging studies, selected biochemical parameters), so any more complex laboratory analysis of the issue is still missing.

Therefore, the aim of our study was to assess the influence of two oxazaphosphorines (CP and IF) on the renal histology and function, estimated by panel of laboratory parameters assessed in urine and plasma, in rats treated with four successive doses of either CP or IF.

## 2. Materials and methods

The medical experiment described in this paper was approved by the I Local Ethical Committee in Krakow. The experiment was carried out in accordance with both the Directive 2010/63/EU on the protection of animals used for scientific purposes and with the Polish Act of 15 January 2015 on the protection of animals used for scientific or educational purposes (JL, February 26, 2015, Pos.266).

### 2.1. Examined groups of animals and a general plan of the experiment

The experiment included 30 10-week-old albino Wistar rats, in equal quantities of males and females. Animals were obtained from the Central Animal House of the Faculty of Pharmacy of the UJCM in Krakow.

Upon arrival to the local Animal House of the Department of Pathophysiology of the UJCM, they were kept during the first seven days in an isolated room in order to acclimatize to the new living conditions. After that period, the rats were randomly assigned to the study groups of the same size (10 in each group; 5 males and 5 females): group 1 (control rats), group 2 (rats with CP-induced chronic cystitis), and group 3 (rats with IF-induced chronic cystitis).

All animals survived till the end of the experiment, although the overall condition of the animals from groups 2 and 3 was deteriorating with subsequent CP/IF doses, respectively.

During the experiment (except when rats were housed in individual metabolic cages), animals were kept in single-sex cages separate for the study groups, with unlimited access to water and standard feed (Labofeed, Kcynia, Poland), in the air-conditioned room with a constant temperature and humidity, maintaining a 12/12-h day/night cycle.

The plan of the experiment assumed initial assessment of vital signs, daily diuresis, feed and water consumption of all study animals performed in individual cages. Urine samples were collected over 24 h for subsequent laboratory analysis and for qualitative and semi-quantitative analysis of urine with urine dipsticks. Then, animals in groups 2 and 3 received CP or IF treatment, respectively, to induce chronic cystitis in 7 days. Control individuals were given normal saline. After the last CP/IF/saline dose all animals were once more monitored during 24 h in metabolic cages, with assessment of the same parameters as those before the treatment and urine samples were collected again. After the second stay in metabolic cages, blood samples for further biochemical assay were also collected. Finally, the animals were sacrificed and cystectomy

and nephrectomy were performed for subsequent histopathological evaluation of urinary bladders and kidneys.

## 2.2. Chronic cystitis induced by cyclophosphamide (group 2) or ifosfamide (group 3)

Rats in the group 2 were given CP (CP monohydrate; Sigma Aldrich) intraperitoneally in the amount of 75 mg/kg b.w., dissolved in 0.5 mL of normal saline. The dose was administered four times, every two days, after the initial monitoring in metabolic cages on the first day of the experiment. After the last CP dose, animals were once again placed in separate metabolic cages. According to literature data, the approved dosing schedule can induce chronic cystitis [7,8]. Besides administration of CP animals were not subjected to any other treatment.

In the group 3, the procedure was identical, but rats received 60 mg/kg b.w. of ifosfamide. The choice of IF dose was also based on literature reports – there are studies reporting ifosfamide-induced nephrotoxicity in rats using 80 mg/kg b.w. for three days [9] or 50 mg/kg b.w. for five days [10,11]. According to Springate and Van Liew [12], the recommended urotoxic dose of IF is 40–80 mg/kg b.w. Therefore, we decided to apply the dose of 60 mg/kg b.w. of ifosfamide as a suitable dosage (the dosage regimen provided the same global IF dose as in the above cited papers). Control animals (group 1) were subjected to the same procedure as individuals of groups 1 and 2, but instead of using CP/IF, rats were sham-treated with normal saline, in accordance with the regimen, and the volumes used in groups 1 and 2.

## 2.3. Monitoring in metabolic cages

In the beginning (before receiving the first dose) and in the end of the experiment (after the last, fourth dose of CP/IF in groups 2/3 or saline in group 1, respectively) animals were placed in isolated metabolic cages over 24 h to measure daily urine output and water and feed consumption. During the stay they had a free, yet monitored access to water and food. Vital parameters were assessed: body weight (g), body temperature (°C), daily water intake (mL/24 h), daily food consumption (g/24 h) and daily diuresis (mL/24 h). Body temperature was measured using a digital rectal thermometer for rodents (Vivari, UK).

## 2.4. Urine dipstick control

After recording the volume of urine, samples were subjected to general tests. Dipsticks were used for that purpose (Insight, ACON Laboratories, REF U031-105), allowing assessment of pH and specific gravity (SG) of urine, presence of blood, leukocytes (white blood cells; WBC), protein, glucose, bilirubin, urobilinogen, ketones, nitrates. The analysis was performed strictly according to manufacturer's guidelines.

## 2.5. Laboratory parameters estimated in urine and plasma

After daily urine output was measured, urine samples was subsequently centrifuged (2000 rpm/5 min), and divided into portions. Resulting samples were stored at –20 °C until laboratory determinations completed with the ADVIA 1200

SIEMENS analyzer. Initially, sodium, potassium, urea, creatinine (Cr), uric acid and protein concentrations were determined. Moreover, blood urea nitrogen (BUN) and BUN/creatinine ratio were calculated:

$$\text{BUN} = \frac{\text{urea (mg/dL)}}{2.1428}$$

Urinary concentration (ng/mL) of novel biomarker of kidney damage – neutrophil gelatinase-associated lipocalin-1 (NGAL-1), using a commercially available ELISA kit (BioPorto Diagnostics, Denmark), strictly according to the manufacturer's instructions, was also assessed.

Then, having results of daily diuresis, 24-h elimination of sodium, potassium, urea (mmol/24 h), uric acid, creatinine (μmol/24 h), protein (mg/24 h) and NGAL-1 (ng/24 h) with urine was calculated. Creatinine clearance was also estimated, according to the formula

$$\text{Cl}_{\text{cr}} = \frac{\text{Cr urine (}\mu\text{mol/L)} * \text{diuresis (mL/min)}}{\text{Cr plasma (}\mu\text{mol/L)}}$$

Fractional sodium elimination was calculated using the formula:

$$\text{FENa} = \frac{[(\text{Na urine (mmol/L)} * \text{Cr plasma (}\mu\text{mol/L)}) * 100]}{[(\text{Na plasma (mmol/L)} * \text{Cr urine (}\mu\text{mol/L)})]}$$

and the renal failure index (RFI) was calculated with the formula:

$$\text{RFI} = \frac{[(\text{Na urine (mmol/L)} * \text{Cr plasma (}\mu\text{mol/L)})]}{\text{Cr urine (}\mu\text{mol/L)}}$$

Similarly to urine, collected blood was centrifuged and resulting plasma samples were kept frozen until the time of laboratory determinations. Plasma sodium, potassium (mmol/L) and creatinine and uric acid (μmol/L) levels were determined using the same analyzer as the one used for urine samples. Plasma protein was not assayed.

## 2.6. Sacrificing animals and collecting kidneys and bladders for histopathological analysis

After the second stay in metabolic cages, animals were killed by intraperitoneal injection of a lethal dose of sodium pentobarbital (Morbital; Biowet, Pulawy; 200 mg/kg b.w.). After confirmed cessation of vital signs, cystectomy and nephrectomy were performed in order to obtain tissue for microscopic specimens necessary for histopathological evaluation.

## 2.7. Histological evaluation of collected bladders and kidneys

Bladders and kidneys obtained during autopsy were stored in a formalin solution until preparation of microscopic slides. The histopathological assessment was performed at the Department of Anatomopathology of the non-public healthcare institution “Prosmed” in Krakow. The sections were cut at preselected thickness of 4 microns using LEICA RM2135 microtome. In order to assess the severity of histological

inflammation, the preparations were stained with hematoxylin-eosin (HE). A detailed description of the histopathological technique is described in one of our previously published paper [13].

## 2.8. Statistical analysis

Results of the quantitative analysis of urine performed with urine dipsticks were analyzed using the nonparametric test of signs, examining changes in the direction of the assessed parameter, separately for each study group in the paired comparison (pre- and posttreatment).

Results of the vital parameters and daily water and feed intake were analyzed both for intragroup (at the beginning and the end of the experiment) and intergroup differences, comparing groups 1 and 2 as well as groups 1 and 3.

Differences concerning results of plasma and urine laboratory parameters were estimated for intergroup differences, comparing groups 1 and 2 as well as groups 1 and 3.

In the statistical analysis of vital parameters and laboratory parameters estimated in plasma or urine, in the first step, a distribution of obtained results was analyzed using the Shapiro-Wilk test. If results were not normally distributed, the further analysis was performed using the Mann-Whitney test, and if tested results demonstrated a normal distribution, the Student t test was used. The level of statistical significance was set at  $P \leq 0.05$ .

## 3. Results

### 3.1. Results of vital parameters obtained from monitoring in metabolic cages

Considering changes of the study parameters in the beginning and in the end of the study, control animals were characterized by a significant increase of their body weight. The body temperature and daily water and feed intake were comparable throughout the experiment. Contrary to control ones, CP-treated animals demonstrated a statistically significant decrease of their body weight with a parallel reduction of the amount of daily feed intake in the end of the study. Other parameters demonstrated no statistically significant differences between the beginning and the end of the experiment.

IF-treated animals were characterized by a significant decrease of their body temperature after the last IF dose and, similarly to CP-treated ones, demonstrated a decreased final 24-h feed intake. Their body weight and daily water intake did not change over the time.

Considering intergroup differences, no significant, initial differences were revealed between control and animals randomized to both groups 2 and 3, thus showing lack of differences between individuals in the beginning of the experiment. In the end of the study, CP-treated rats differed with respect to all parameters compared to the control ones, whereas IF-treated animals demonstrated some significant differences concerning their final body temperature and daily feed intake compared to the control.

Detailed results of parameters mentioned above are presented in Tables 1 and 2.

**Table 1 – Parameters of the animals treated with four doses of cyclophosphamide (CP): intra- and intergroup analysis.**

	Metabolic cage measurement										P	
	Beginning of the experiment					End of the experiment						
	Body weight, g	Body temperature, °C	24-h water intake, mL/24 h	24-h feed intake, g/24 h	Body weight, g	Body temperature, °C	24-h water intake, mL/24 h	24-h feed intake, g/24 h				
Control (group 1)	A	B	C	D	E	F	G	H				E vs. A, 0.0001 F vs. B, NS G vs. C, NS H vs. D, NS
CP-treated animals (group 2)	226.66 ± 44.89	37.43 ± 0.93	26.38 ± 4.07	23.75 ± 6.16	257.45 ± 63.19	37.93 ± 0.26	30.00 ± 3.27	20.38 ± 2.39				E vs. A, 0.0002 F vs. B, NS G vs. C, NS H vs. D, 0.002
	240.03 ± 65.27	37.22 ± 0.27	29.25 ± 6.59	21.13 ± 2.36	209.73 ± 74.84	37.45 ± 0.60	22.00 ± 11.71	9.63 ± 7.67				
P	NS	NS	NS	NS	0.05	0.03	0.05	0.002				

Values are mean ± SD. NS, not significant.



**Table 2 – Parameters of the animals treated with four doses of ifosfamide (IF): intra- and intergroup analysis.**

	Metabolic cage measurement								P
	Beginning of the experiment				End of the experiment				
	Body weight, g	Body temperature, °C	24-h water intake, mL/24 h	24-h feed intake, g/24 h	Body weight, g	Body temperature, °C	24-h water intake, mL/24 h	24-h feed intake, g/24 h	
Control (group 1)	A	B	C	D	E	F	G	H	E vs. A, 0.001 F vs. B, NS G vs. C, NS H vs. D, NS
	226.66 ± 44.89	37.43 ± 0.93	26.38 ± 4.07	23.75 ± 6.16	257.45 ± 63.19	37.93 ± 0.26	30.00 ± 3.27	20.38 ± 2.39	
IF-treated animals (group 3)									F vs. A, NS G vs. B, 0.03 H vs. C, NS H vs. D, 0.001
	254.35 ± 51.49	37.44 ± 0.37	26.00 ± 8.54	22.00 ± 3.13	251.53 ± 62.62	36.98 ± 0.38	21.70 ± 15.29	11.70 ± 3.59	
P	NS	NS	NS	NS	NS	0.001	NS	0.001	

Values are mean ± SD. NS, not significant.

### 3.2. Results of the qualitative and semi-quantitative analysis of urine using dipsticks

Results of urine pH and SG indicated by the dipsticks test are presented in Table 4.

In the control group, presence of blood and glucose was demonstrated in none of urine samples in both tests, in the beginning and in the end of the study. In the first test in that group, leukocytes were assessed at ± (15 (WBC/μL)) in 88% of cases and at + (70 (WBC/μL)) in 12%, nitrates assessed at + in 50% and they were not detected in the remaining 50% of samples, urobilinogen was measured at 0.2 (mg/dl) in all samples, protein was determined at + (30 (mg/dL)) in 50% of cases and ++ (100 (mg/dL)) in other 50%, ketones were not found in 88% samples and were assayed at ± (5 (mg/dL)) and bilirubin was assessed at + (1 (mg/dL)) in 88% and at ++ (2 (mg/dL)) in 12%.

In the second test performed after the sham treatment with normal saline, the findings regarding urobilinogen, protein, blood, bilirubin and glucose were identical to the initial assessment. The presence of leukocytes in urine decreased, and the analysis demonstrated no leukocytes in 63% of samples and the parameter was assessed at ± (15 (WBC/μL)) in 25% and at + (70 (WBC/μL)) in 13%. In all samples nitrates presence estimated at + was confirmed. Ketones were not detected in 50% and in the remaining half of the samples they were measured at ± (5 (mg/dL)). Only changes in urine leukocyte, nitrate and ketones were found to be statistically significant.

In group 2, no blood and glucose were demonstrated in the beginning of the study. In all samples urobilinogen was assessed at 0.2 (mg/dL). Leukocytes were not shown in 50% of samples and were estimated at ± (15 (WBC/μL)) in 25% and at + (70 (WBC/μL)) in 25%. The presence of nitrates was not confirmed in 75%, and in the remaining 25% it was estimated at +. The urine protein was measured at ± (15 (mg/dL)) in 38%, at + (30 (mg/dL)) in 12% and at ++ (100 (mg/dL)) in 50%. In assessment of ketones, in 12% of cases they were estimated at ± (5 (mg/dL)) and other urine samples contained no ketones. Bilirubin was assessed at + (1 (mg/dL)) in 88% and at ++ (2 (mg/dL)) in 12%. No samples contained glucose.

In the second analysis performed in CP-treated animals, a presence of urobilinogen assessed at + (1 (mg/dL)) and bilirubin assessed on + (1 (mg/dL)) was found in all cases, and glucose and ketones were absent. In 25% of samples, leukocytes were estimated at ± (15 (WBC/μL)) and in 50% at + (70 (WBC/μL)) and they were not demonstrated in 25% of samples. Nitrates marked as + were present in 88%, whereas 12% of samples were free from nitrates. Presence of the urinary protein was similar to the first assessment – it was estimated at ± (15 (mg/dL)) in 38%, at + (30 (mg/dL)) in 25% and at ++ (100 (mg/dL)) in 38% of cases. There was no blood found in half of urine samples and in 12% of them it was estimated at + and at ++ in 38%. Considering differences in the urine dipsticks analysis observed before and after the treatment with CP, only changes related to the nitrates and blood were considered to be statistically significant.

Before the IF treatment, all urine samples from rats in the group 3 were characterized by absence of the glucose, ketones, blood and leukocytes in 70% of cases. In 30%, leukocytes were estimated at + (70 (WBC/μL)). Nitrates were found in 12% and

all the other urine samples were deprived of the substance. Urobilinogen was assessed at 0.2 (mg/dL) and bilirubin at + (1 (mg/dL)) in all cases. Urine protein in rats in the group 3 was not detected in 80% of cases and estimated at  $\pm$  (15 (mg/dL)) in 10% and at ++ (100 (mg/dL)) in 10%, before the treatment with IF.

The IF treatment resulted in increased urine protein level of  $\pm$  (15 (mg/dL)) in 90% of cases and of ++ (100 (mg/dL)) in the remaining 10% of the cases. Moreover, in 25% of samples, blood was also detected. All of the other parameters (leukocytes, urobilinogen, bilirubin, ketones, nitrates, glucose) were assessed in the same manner as in the initial assessment. However, the performed statistical analysis indicated that the above discussed differences were not statistically significant.

### 3.3. Results of the assayed plasma parameters

Administration of four doses of CP resulted both in significant decrease of the plasma uric acid and increase in plasma creatinine levels. However, BUN and BUN/creatinine ration did not differ significantly in control and CP-treated animals. Plasma sodium, urea, levels were comparable in CP-treated and control rats.

Similarly to administration of CP, the treatment with IF caused a significant increase in plasma creatinine level, which was accompanied by a blood urea nitrogen value increase. Other parameters assessed in plasma of IF-treated animals did not differ significantly from those in control subjects.

Detailed results are presented in [Table 3](#).

### 3.4. Results of assessed urine parameters

CP-treated rats differed in terms of the majority of estimated urine parameters from control ones. First of all, they were characterized by a higher value of daily diuresis (both total and minute) with a substantial acidification of urine. CP administration resulted also in a significant decrease in urinary excretion of sodium, potassium, urea and uric acid. In contrast, the daily urinary excretion of protein and creatinine was similar in CP-treated animals and control ones.

The IF treatment resulted only in a significant decrease of urine pH, with simultaneous increase of its specific gravity. The daily urinary excretion of assayed substances did not differ significantly from values of those parameters assessed in the control group, except for the uric acid, that was – similarly to CP-treated rats – found in the urine in diminished amount compared to control group.

Urinary NGAL-1 concentrations were not significantly different in all studied groups.

Detailed results are presented in [Table 4](#).

The value of calculated creatinine clearance was similar in both CP- and IF-treated rats ( $0.85 \pm 0.26$  and  $0.92 \pm 0.44$ , respectively) and did not differ from the value found in the control group ( $1.1 \pm 0.37$ ). Moreover, animals receiving CP demonstrated a significant decrease of calculated values of the fractional sodium excretion and RFI compared to control rats.

**Table 3 – Plasma parameters in rats treated with cyclophosphamide (CP) and ifosfamide (IF).**

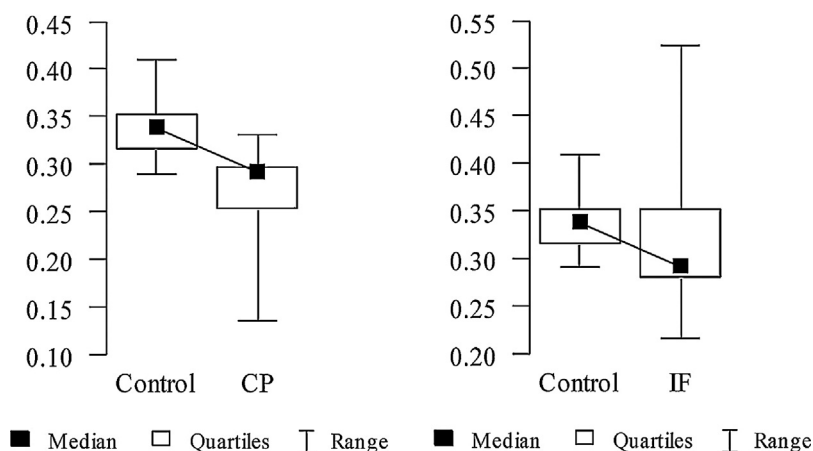
	Control (group 1)	CP-treated rats (group 2)	IF-treated rats (group 3)	P	
				Group 1 vs. 2	Group 1 vs. 3
Na, mmol/L	142.06 $\pm$ 1.79	143.80 $\pm$ 1.9	142.57 $\pm$ 1.21	NS	NS
Urea, mmol/L	6.34 $\pm$ 0.75	6.76 $\pm$ 1.07	7.28 $\pm$ 1.06	NS	NS
BUN, mg/dL	17.76 $\pm$ 2.10	18.95 $\pm$ 3.00	19.97 $\pm$ 2.87	NS	0.05
Uric acid, $\mu$ mol/L	157.96 $\pm$ 35.14	106.1 $\pm$ 57.56	189.81 $\pm$ 94.21	0.02	NS
Creatinine, $\mu$ mol/L	27.95 $\pm$ 1.77	31.99 $\pm$ 2.81	34.19 $\pm$ 3.5	0.05	0.001
BUN/creatinine, mg/dL	54.34 $\pm$ 4.40	52.67 $\pm$ 8.94	52.52 $\pm$ 10.37	NS	NS

Values are mean  $\pm$  SD. BUN, blood urea nitrogen; NS, not significant.

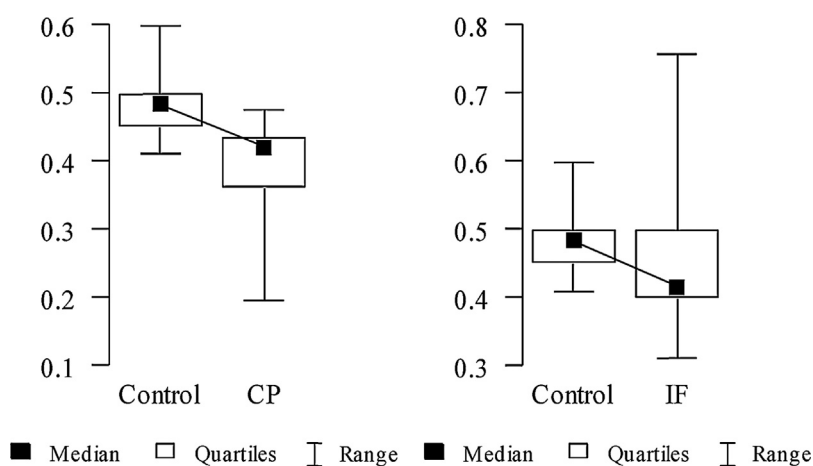
**Table 4 – Urine parameters in rats treated with cyclophosphamide (CP) and ifosfamide (IF).**

	Control (group 1)	CP-treated rats (group 2)	IF-treated rats (group 3)	P	
				Group 1 vs. 2	Group 1 vs. 3
Diuresis, mL/24 h	5.72 $\pm$ 1.31	11.94 $\pm$ 4.14	8.27 $\pm$ 5.31	0.005	NS
Diuresis, mL/min	0.004 $\pm$ 0.001	0.008 $\pm$ 0.003	0.006 $\pm$ 0.004	0.001	NS
SG	1.009 $\pm$ 0.001	1.008 $\pm$ 0.006	1.013 $\pm$ 0.006	NS	0.05
pH	9.0 $\pm$ 0.00	8.13 $\pm$ 1.03	8.25 $\pm$ 0.86	0.02	0.01
Protein, mg/24 h	5.71 $\pm$ 4.15	3.94 $\pm$ 2.47	5.29 $\pm$ 4.61	NS	NS
Na, mmol/24 h	0.76 $\pm$ 0.25	0.48 $\pm$ 0.19	0.63 $\pm$ 0.37	0.04	NS
K, mmol/24 h	2.08 $\pm$ 0.55	1.02 $\pm$ 0.66	1.68 $\pm$ 0.85	0.005	NS
Urea, mmol/24 h	5.93 $\pm$ 1.28	4.45 $\pm$ 1.14	5.05 $\pm$ 2.36	0.02	NS
Uric acid, $\mu$ mol/24 h	9.03 $\pm$ 2.85	6.07 $\pm$ 1.25	5.26 $\pm$ 2.99	0.02	0.01
Creatinine, $\mu$ mol/24 h	45.56 $\pm$ 14.14	39.38 $\pm$ 12.52	44.70 $\pm$ 19.92	NS	NS
NGAL-1, ng/mL	1.53 $\pm$ 0.80	3.34 $\pm$ 2.42	1.02 $\pm$ 0.90	NS	NS

Values are mean  $\pm$  SD. SG, specific gravity; NGAL-1, neutrophil gelatinase-associated lipocalin-1; NS, not significant.



**Fig. 1 – Fractional excretion of sodium (FENa) in CP-treated (left) and IF-treated (right) rats.  $P < 0.05$  for CP-treated rats; NS, not significant for IF-treated rats.**

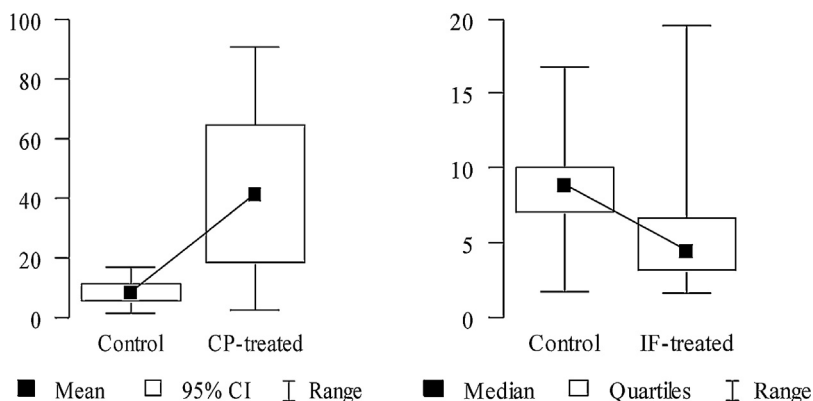


**Fig. 2 – Renal failure index (RFI) in CP-treated (left) and IF-treated (right) rats.  $P < 0.05$  for CP-treated rats; NS, not significant for IF-treated rats.**

Dependencies concerning some significant intergroup differences for abovementioned parameters of FENa and RFI are presented in Figs. 1 and 2.

Moreover, CP-treated rats were characterized by significantly greater 24-h urinary NGAL-1 excretion (ng/24 h)

compared to control ( $41.44 \pm 35.32$  vs.  $8.64 \pm 4.36$ ). In the IF-treated group, daily NGAL-1 urinary excretion was similar to the value found in the control animals ( $6.24 \pm 5.45$  vs.  $8.64 \pm 4.36$ ). The results described above are presented in Fig. 3.



**Fig. 3 – 24-h urinary excretion (ng/24 h) of neutrophil gelatinase-associated lipocalin-1 (NGAL-1) in CP-treated (left) and IF-treated (right) rats.  $P < 0.05$  for CP-treated rats; NS, not significant for IF-treated rats.**

### 3.5. Description of the histopathological assessment of bladders and kidneys

Specimens of urinary bladders collected from CP-treated rats had a largely homogeneous presentation. Reactive proliferation of urothelial epithelium with multiplication of layers up to 6 and signs of dyskaryosis (nuclear polymorphism), mostly in the basal layer of the urothelium were found in the entire group. Moreover, an inflammatory infiltration was found in stroma in all specimens. Kidneys in all CP-treated rats were at the limit of a normal presentation.

Specimens of urinary bladders collected from IF-treated animals presented an inflammatory infiltration and fibrosis of the stroma. Kidneys of IF-treated animals appeared normal.

## 4. Discussion

The main findings of the study may be summarized as follows:

1. Rats treated with CP presented a significant polyuria and acidification of urine. The administration of CP resulted also in decreased 24-h urinary excretion of sodium, potassium, urea and uric acid which was accompanied by a significantly higher excretion of NGAL-1. Disturbances of parameters estimated in urine were accompanied by a significant and profound reduction of the plasma uric acid level. Moreover, the calculated FENa and RFI had lower values in CP-treated rats compared to control ones. An overall qualitative assessment of urine with dipsticks also demonstrated some more emphasized disturbances in the group of rats treated with CP. The administration of CP was also associated with a significant wasting of animals, reflected by a significant reduction of their final body weight and food intake.
2. Rats treated with ifosfamide also presented a significant urine acidification. However, in contrast to CP-treated animals, the 24-h urinary excretion of majority of analyzed substances (including NGAL-1) did not differ from that observed in the control group – rats treated with IF were characterized only by a reduced uric acid excretion. IF administration was also associated with increased creatinine and BUN plasma level, but the calculated creatinine clearance and BUN/creatinine ratio were similar to those in the control group. Moreover, animals treated with IF were characterized by lesser abnormalities in the qualitative analysis of urine compared to CP-treated rats. Also, IF administration resulted in a much smaller cachexia compared to rats receiving CP (no final body weight decrease, less loss of the daily feed intake).

As already mentioned in the Introduction, ifosfamide being the main source of chloroacetaldehyde, is regarded to be a compound associated with a significant nephrotoxicity. Kidneys metabolize nephrotoxic chloroacetaldehyde into a less toxic chloroacetate and the rate of that detoxication determines the degree of kidney damage [14]. Both oxazaphosphorine agents (CP and IF) are hydrophilic. For that reason they do not easily diffuse through cell membranes and require a presence of specific cell carriers – organic anion (OAT) and

cation (OCT) transporters [15]. In the kidney, as well as in other tissues, OAT isoforms 1 and 3 and OCT isoform 2 are located in the basolateral membrane [16]. Both CP and IF have affinity for OATs, but IF is preferably transported further by renal OCT-2, which explains a greater accumulation of the compound in kidneys and a higher nephrotoxic effect [15].

The kidney dysfunction caused by the use of IF, described in the literature, adopts a wide spectrum, ranging from the generalized proximal tubulopathy (Fanconi syndrome) to partial sodium, potassium, glucose, amino acids and other small molecule compounds' re-absorption defects [3,4,17]. The CP-induced nephrotoxicity also involves a variable tubular dysfunction [2-5]. Moreover, the CP-induced kidney dysfunction results in reduction of tubular filtration rate and development of an inappropriate antidiuresis syndrome [18].

Results of our study indicated an abnormal daily urinary excretion of assessed substances by CP-treated rats (including increased NGAL-1 excretion) and a significant urine acidification. IF-treated animals demonstrated only an abnormally low urine pH at urinary NGAL-1 excretion remaining at a similar level as compared to control rats. Therefore, our study demonstrated a significant nephrotoxicity potential for CP. Which proves that acrolein, which is a unique compound released during metabolic processing of CP, exerts not only mainly urotoxic effects but also significant nephrotoxic ones. The most likely mechanism of the acrolein-related nephrotoxicity is complex and based on the same issues contributing to urotoxic properties of that compound, and includes a reduction of the kidney cellular glutathione dependent antioxidant system and an increase in free radical generation, mostly peroxynitrite. The compound is responsible for lipid peroxidation – a deleterious damage of the renal tubular cells [19]. Moreover, a significant drastic decrease (instead of an increase) in the activities of lysosomal enzymes was observed in kidneys of CP-treated rats. A decrease in the activities of lysosomal protein digestive enzymes may contribute to CYP-induced renal damage – the accumulation of abnormal amounts of the protein in kidneys may be at least in part due to a defect in lysosomal enzyme activity and may contribute to renal damage [20]. To sum up, the dosage and route of administration applied in our study showed a more emphasized kidney dysfunction in CP-treated rats, with the absence of any renal histopathological abnormalities. Moreover, results obtained for CP-treated animals suggest some possible disturbances of proximal tubules (impairment of re-absorption), as well as of distal ones (abnormal urine acidification). It seems that IF affects only the distal nephron function, because the agent caused only a drop of urine pH and the analysis of NGAL-1 did not reveal any significant abnormalities. The statement of dysfunction of distal tubules is supported by the finding that H<sup>+</sup> secretion into urine and, therefore, determination of the final urine pH, takes place just in the distal tubules [21,22]. Taking into account data presented in Tables 1 and 2, it should be concluded that the 24-h water and food intake in both CP- and IF-treated animals during monitoring in individual metabolic cages was almost identical (both in the beginning and in the end of the experiment). Therefore, in our opinion, it is unlikely that the final pH drop observed in the treated animals resulted from intergroup differences in water or feed consumption.



Moreover, rats treated with CP, in addition to urine acidification and abnormal NGAL-1 excretion, developed also a significant polyuria, which may be considered as an additional premise for the assumption of the distal tubule dysfunction. Polyuria demonstrated in our study is contrary to literature reports indicating development of the “antidiuresis syndrome” after the application of CP. That disorder was due to the increased, CP-induced expression of aquaporins via the vasopressin-independent mechanism [23,24]. On the other hand, however, development of IF-evoked nephrogenic diabetes insipidus was also reported [3]. Thus, the influence of the oxazaphosphorine agents on diuresis is ambiguous and requires further research, but abovementioned studies (also including the currently discussed one) suggest disturbances within the distal nephron due to CP/IF administration.

Taking into account all the results obtained in the group of animals treated with CP, it is worth noting that some of them (FENa, RFI) may suggest development of acute kidney injury (AKI). AKI, previously referred to as acute renal failure, can be defined as an abrupt deterioration of the all renal function (excretory, endocrine, metabolic). In other words, it means sudden loss of the ability of the kidneys to excrete waste, concentrate urine, conserve electrolytes, and maintain fluid balance [25]. AKI is conditioned by various factors of pre-, post- or toxic/inflammatory intra-renal origin that lead to acute kidney function decompensation [25,26]. Clinically, fractional excretion of sodium and renal failure index are regarded laboratory indices suggesting prerenal background of AKI [25,27]. Low FENa is an effective marker of hypoperfusion of the renal artery [28]. Those parameters obtained in CP-treated rats achieved lower values compared to the control group. Therefore, an additional assumption may be set that CP administration also contributes to prerenal AKI features resulting from kidney hypoperfusion, as that is the underlying mechanism of that form of AKI [26]. The presence of polyuria demonstrated in CP-treated rats, although is not a typical finding, does not exclude the possibility of AKI development, as the disorder paradoxically may be associated with polyuria (although in the classic form is characterized by oliguria or even anuria) [25–27].

Another evidence supporting kidney damage development in CP-treated animals is the analysis of urinary excretion of novel biomarker of kidney dysfunction – NGAL-1. While the urinary concentrations of the biomarker were comparable in all groups, the 24-h excretion of the compound was significantly higher in CP-treated animals. NGAL-1 is a protein synthesized and released into the plasma by many tissues: kidneys, prostate, trachea, lung, stomach, large intestine, uterus and bone marrow. In addition, it is released in greater amounts from neutrophils and macrophages during inflammatory response and endothelial injury [29–31]. NGAL-1 exhibits pleiotropic biological properties: extracellularly, it binds iron-complexed siderophores and regulates intracellular metabolism of the iron, enhances the proteolytic activity of matrix metalloproteinases (MMP-9), thereby preventing the degradation of these enzymes by tissue MMP-9 inhibitor (TIMP-1). This phenomenon is of particular importance in the processes of progression of carcinogenesis [29,32]. The augmentation of MMP-9 activity by NGAL-1 is also important in the development of acute coronary syndromes resulting

from atherosclerotic plaque destabilization [29,33]. Plasma NGAL-1 level is also elevated in patients with type 2 diabetes, thus suggesting the potential role of NGAL-1 in the pathogenesis of insulin resistance [34]. NGAL-1 is freely filtered in the kidneys and reabsorbed in proximal tubules. In addition, this protein is synthesized by Henle's lobe and distal tubules in response to various kidney damaging factors [31,35]. Therefore, the increased 24-h urinary excretion of NGAL-1 in CP-treated rats could have resulted from proximal tubules failure and reduced reabsorption of this protein and/or increased tubular synthesis. Moreover, the hypothesis of a more potent nephrotoxicity of CP compared to ifosfamide is also consistent with the results of one of our other experiments. In that study, we assessed the urinary concentration and 24-h excretion of another novel biomarker – kidney injury molecule-1 (KIM-1). Similar results were revealed – again, CP-treated animals were characterized by significantly higher urinary KIM-1 concentration and increased 24-h excretion of the protein with the urine [13]. KIM-1 is a glycoprotein secreted by the proximal tubule cells in response to any noxious factors – therefore, plays a special role of one of the “renal troponins” used for early and reliable detection of kidney injury. Under physiological conditions, urine does not contain KIM-1 – so its presence in the urine is regarded to be a quantitative biomarker of kidney damage, which appears rapidly in the urine in response to the proximal tubular damage [13].

To sum up, the increased 24-h urinary excretion of both NGAL-1 and KIM-1 biomarkers is another proof of CP-induced nephrotoxicity.

The next characteristic disturbance demonstrated in our experiment was a reduced urinary excretion of uric acid that in the case of CP-treated animals was associated with decreased plasma level of the compound. The decrease of serum uric acid may contribute to the development of polyuria, resulting from abnormalities in cortico-medullary osmotic gradient and disturbances of the countercurrent amplifier mechanism [36,37]. The reduction of serum uric acid level (and, consequently, reduced urinary excretion of the compound) may also reflect a compensation of an excessive oxidative stress, which is, as mentioned above, an essential, potential element of pathomechanism of the CP-induced nephrotoxicity. Uric acid is one of the most important, low molecular mass compounds that is now believed to exert strong antioxidant effect [38]. In vitro experiments demonstrated that uric acid is a powerful scavenger of the peroxy and hydroxyl radicals and of singlet oxygen [39]. Moreover, uric acid is thought to be an inhibitor of radicals generated by decomposition of peroxy nitrite – a compound also generated by the action of acrolein [40,41]. An important premise for the antioxidant role of uric acid is the observation that the loss of urate oxidase, resulting in increased plasma uric acid level, improves antioxidant defense [38]. Therefore, it can be concluded that the advanced oxidative stress in kidneys induced by acrolein released during biotransformation of peroxy nitrite, causes secondary “exhaustion” of the compensatory antioxidant mechanisms, including reduction of plasma uric acid level. The indirect confirmation of the role of uric acid as an antioxidant in the course of kidney and bladder damage is provided also by clinical observations: a negative correlation between plasma level of uric acid and the severity of hemorrhagic cystitis in

children treated with CP [42]. Thus, results of our study related to marked plasma uric acid decrease in CP-treated rats constitute one more evidence for a role of uric acid in the pathogenesis of kidney and bladder damage caused by CP.

## 5. Conclusions

To sum up, both CP and ifosfamide administrated four times intraperitoneally caused kidney dysfunction that was more advanced in animals treated with CP and demonstrated by reduced 24-h excretion of sodium, potassium, urea and uric acid, with a marked acidification of urine and increased NGAL-1 urinary excretion. Moreover, some indices (FENa, RFI) also suggest prerenal development of acute kidney injury.

IF-treated animals were characterized only by decreased urine pH and reduced daily uric acid excretion.

Decreased serum uric acid levels in rats treated with CP and decreased daily urinary excretion of the compound found in both studied groups may reflect participation of that compound in the antioxidant compensation phenomena that accompany renal failure induced by oxazaphosphorine metabolites.

## Conflict of interest

All the authors hereby disclose that there is no conflict of interest among the authors.

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