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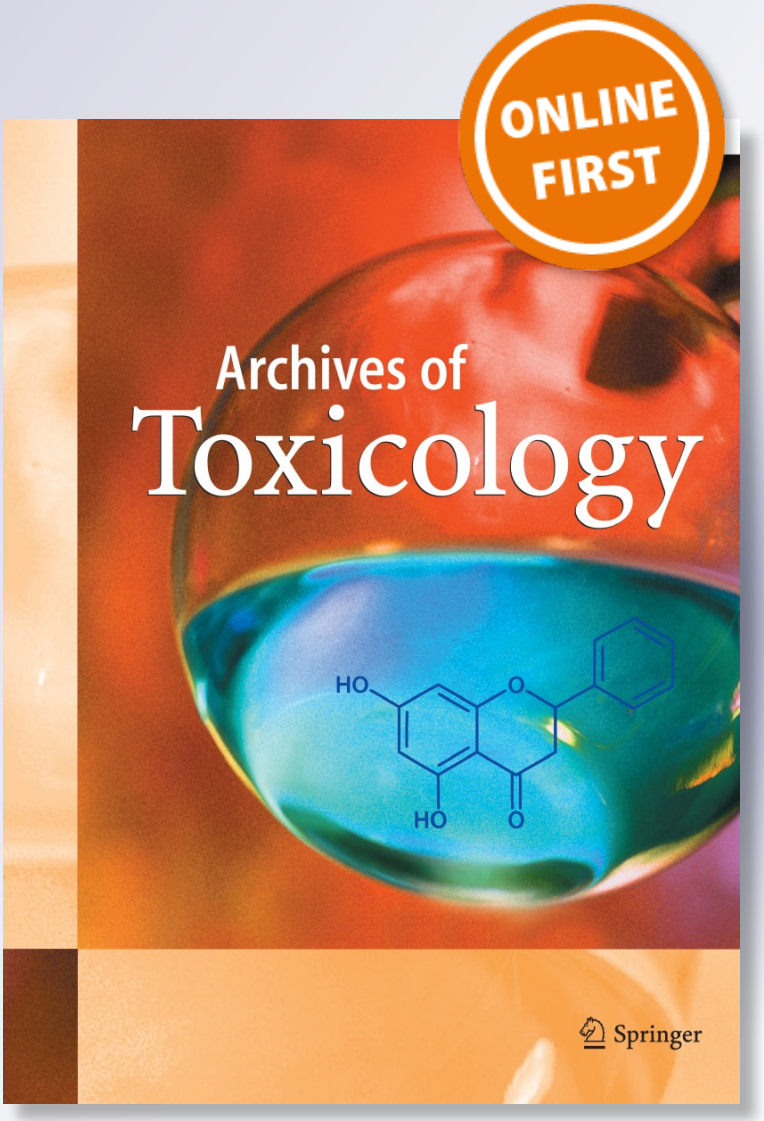

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ORGAN TOXICITY AND MECHANISMS

Time course of organic anion transporter 5 (Oat5) urinary excretion in rats treated with cisplatin: a novel urinary biomarker for early detection of drug-induced nephrotoxicity

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Abstract Cisplatin is a widely used citostatic drug employed in the treatment of many solid tumors. Its principal side-effect is nephrotoxicity. The organic anion transporter 5 (Oat5) is exclusively expressed in the kidneys. The aim of this study was to evaluate the time course of Oat5 urinary excretion and changes in conventional biomarkers, such as creatinine and urea plasma levels (Ur_p and Cr_p), and protein and glucose urinary levels (P_u and Glu_u), between others, and compared them to the onset and progression of histological changes after cisplatin treatment. Male Wistar rats were treated with cisplatin with 5 mg/kg b.w., i.p., and experiments were carried out after 2, 4, 7 and 14 days of treatment. Two days after cisplatin administration, only Oat5 urinary excretion was found markedly modified. On day 4, Ur_p, Cr_p, P_U and Glu_U were increased. By the seventh day, a severe impairment in tubular architecture was observed, and from this point and thereon, Oat5 urinary excretion and P_U showed a tendency to return to their basal values. Meanwhile, Ur_p, Cr_p and Glu_U tended to return to their basal values by the day 14 of treatment, when kidney morphology showed an important recovery. So Oat5 urinary abundance was elevated 2 days after cisplatin treatment, when no modifications of traditional markers of renal injury were still observed. Therefore, the results showed in this work, in addition to previous data obtained by our group, propose that Oat5 urinary excretion might potentially serve as a noninvasive early biomarker of cisplatin-induced nephrotoxicity.

R. P. Bulacio · A. M. Torres (✉)
Area Farmacología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET, Suipacha 531, 2000 Rosario, Argentina
e-mail: admotorres@yahoo.com.ar

Keywords Organic anions transporter · Cisplatin · Urinary biomarker · Oat5 · Nephrotoxicity

Introduction

Cisplatin (*cis*-diamminedichloroplatinum II) is a widely used citostatic drug employed in the treatment of many solid tumors, such as those of the head, neck, lung, ovary, and breast, between others. The principal side effects of cisplatin treatment in man and animals include nephrotoxicity, ototoxicity, gastrotoxicity and bone marrow suppression. Among these factors, the incidence of nephrotoxicity is extremely high, occurring in about one-third of patients undergoing cisplatin treatment (Miller et al. 2010; Uehara et al. 2011; Wensing and Ciarimboli 2013). Over the last years, intense efforts have been made in order to find less toxic but similarly effective alternatives, but cisplatin remains as a gold standard of treatment regimens for head and neck cancers, and for many others neoplasm (Miller et al. 2010).

Cisplatin nephrotoxicity increases with the dose and frequency of its administration. Volume expansion with sodium chloride has been one of the main ways to attenuate cisplatin nephrotoxicity, and many regimens also include the use of diuretics, such as mannitol or furosemide. Nevertheless, there is no good evidence that diuretics provide any added benefit and even with aggressive hydration, over a quarter of patients still develops renal problems (Miller et al. 2010; Pabla et al. 2011).

Animal studies showed that the kidney accumulates more cisplatin than other organs and that the main injury occurs in the proximal tubule cells. This compound enters renal epithelial cells principally via the organic cation transporter 2 (OCT2) (Wensing and Ciarimboli 2013).

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The mechanisms underlying cisplatin nephrotoxicity are complex and are not totally elucidated, but include oxidative stress, activation of intrinsic and extrinsic apoptotic cascades and endonucleases and inflammation. Unluckily, several of these same pathways participate in the cytotoxic actions of cisplatin on tumor cells, and some strategies intended to reduce cisplatin renal injury may reduce the anti-tumor actions of this compound (Miller et al. 2010).

Clinically, renal dysfunction often begins several days after cisplatin administration, and it can be evidenced by modifications in traditional biomarkers, such as an increase in serum creatinine and urea levels, detection of glucose and small amounts of protein in urine and reduced magnesium and potassium serum levels (Pabla and Dong 2008). None of these markers are, however, very sensitive nor very specific. Both, serum urea and creatinine lack specificity and sensitive for monitoring alterations in glomerular filtration rate, which is the best indicator of kidney functions, and there is generally an important loss of renal function before they present any significant changes (McDuffie et al. 2013).

Organic anion transporters (OATs) play an essential role, both in human and in animals, in renal excretion of organic anions compounds such as endogenous waste products and some drugs and their metabolites, mainly in the liver and the kidney (Burckhardt 2012). The organic anion transporter type 5 (Oat5, Slc22a19) has been cloned and characterized as an organic anion/dicarboxylate antiporter. This transporter is exclusively expressed in the kidneys, where it is located at the apical membrane of the proximal tubule cells, principally in the straight segment S3. Oat5 is capable of transport ochratoxin A, sulfate conjugate of steroids such as dehydroepiandrosterone sulfate (DHEA-S) and estrone-3-sulfate (ES), α-ketoglutarate, succinate and interacts with many chemically heterogeneous anionic compounds, such as diuretics (bumetanide, furosemide), non-steroidal anti-inflammatory drugs, penicillin G, and bromosulfophthalein (Youngblood and Sweet 2004; Anzai et al. 2005, 2006; Kwak et al. 2005). Our group was pioneering in detecting Oat5 in urine (Di Giusto et al. 2009). On that subject, we have reported an important increase in Oat5 in urine in ischemia and mercury induced acute kidney injury (AKI) (Di Giusto et al. 2009; Di Giusto and Torres 2010), and recently, we have demonstrated a dose-related increase in Oat5 urinary abundance in cisplatin-induced AKI (Bulacio and Torres 2013). These results suggest that Oat5 in urine might be a potential early noninvasive biomarker of AKI. The aim of this study was to evaluate the time course of Oat5 urinary excretion and changes in conventional biomarkers, such as creatinine and urea plasma levels, and protein and glucose urinary levels, between others, and compared them to the onset and progression of histological changes after cisplatin treatment.

Materials and methods

Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) and were analytical grade pure. The rabbit polyclonal antibody against Oat5 was kindly provided by Prof. H. Endou (Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan) (Anzai et al. 2005; Sekine et al. 1998). Mouse monoclonal antibody against human β actin was purchased from Alpha Diagnostic International (San Antonio, TX, USA).

Experimental animals

Male Wistar rats [120 days, 360–410 g body weight (b.w.)] were used. Animals had free access to food and tap water, and were housed in an environment of constant temperature (21–23 °C) and humidity with regular light cycles (12 h) during the experiments, as outlined in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. For surgical procedures, the animals were anesthetized with sodium thiopental [70 mg/kg b.w., intraperitoneally (i.p.)]. Rats were injected i.p. with a single dose of cisplatin with 5 mg/kg of b.w., and the studies were performed 2, 4, 7 and 14 days after the injections. This dose of cisplatin was selected considering previous studies in order to show its nephrotoxicity, and also its relation to clinical doses used in chemotherapy (Yokoo et al. 2007; Abdelrahman et al. 2010; Hosohata et al. 2012; Pinches et al. 2012; Bulacio and Torres 2013). Corresponding control groups, injected with vehicle (1 mL saline/kg b.w.), were processed at each time point evaluated. The rats were weighed at the beginning of the experiment, and just before killing, in order to monitor the possible loose of weight at different time point after the treatment. The animals were maintained in metabolic cages for 24 h before the experiments in order to collect the urine and urinary volume (V_U) was estimated by gravimetry. Animals had access to water ad libitum throughout urine collection periods. On the day of the experiments, plasma samples were obtained by cardiac puncture and kidney tissue was collected.

Two different sets of experimental animals were used: one for biochemical determination, preparation of homogenates and apical membranes from whole kidney and for urinary exosomes isolation and another for histopathological and immunohistochemical studies.

As all the tested parameters in the control groups (2, 4, 7 and 14 days after vehicle administration, $n = 4$ for each one) showed no significant differences, we decided to consider them as one group ("control group", $n = 16$) to facilitate analysis of the results, as previously described in an experimental model of obstructive nephropathy (Villar et al. 2008).

Biochemical determination

The urine samples were used for analyses of Oat5 abundance, alkaline phosphatase (AP) activity and creatinine (Cr_U), total proteins (Pr_U) and glucose (Glu_U) levels. Serum samples were used to measure urea (Urp) and creatinine levels (Crp).

Urine AP activity, creatinine, total proteins and glucose urinary levels, as well as plasma creatinine and urea levels were determined spectrophotometrically with commercial reagent kits (Wiener Laboratory, Rosario, Argentina). Oat5 abundance was determined by Western blotting.

Preparation of homogenates and apical membranes from kidney

Apical membranes were isolated from kidneys by Mg/EGTA precipitation as previously described (Di Giusto et al. 2009; Di Giusto and Torres 2010; Bulacio and Torres 2013). The kidneys were removed and homogenized in 30 g/100 mL (v/w) of ice-cold 50 mM mannitol, 2 mM Tris HCl buffer (pH 7.10), 5 mM EGTA and 1 mM phenylmethylsulfonylfluoride (PMSF) for 5 min at top speed in a *Glas-Col* homogenizer. From this preparation, we obtained total renal homogenates and aliquots were taken and stored at −80 °C until use. Then, $MgCl_2$ was added to the rest of the homogenate to a final concentration of 12 mM and the mixture was stirred in an ice bath for 15 min. The homogenate was then centrifuged (3,000×*g*, 15 min, 4 °C). The supernatant was carefully decanted and centrifuged again at 28,000×*g* for 40 min at 4 °C. The pelleted material representing apical membranes was resuspended in "experimental buffer" (50 mM mannitol, 10 mM hepes-Tris (pH 7.50) and 1 mM PMSF) and centrifuged for 15 min at 800×*g* at 4 °C. The supernatant was finally centrifuged for 45 min at 28,000×*g*. The apical membrane pellets thus obtained were resuspended in experimental buffer. Aliquots of the membranes were stored immediately at −80 °C until used. Protein quantification of samples was performed using the method of Lowry with some modifications (Lowry et al. 1951).

Isolation of urinary exosomes

Urinary exosomes were isolated from urine as previously described by Pisitkun et al. (2004), with some modifications. Briefly, collected 24-h urine samples from different experimental groups were centrifuged at 17,000×*g* for 15 min at 4 °C to remove large membrane fragments, whole cells and other debris. After that, the supernatants were ultracentrifuged at 200,000×*g* for 1 h at 4 °C (Optima™ XL-100 K Ultracentrifuge, Beckam Coulter, Inc.; Ti 80 rotor). The resulting pellets were resuspended in isolation solution (10 mM triethanolamine, 250 mM sucrose, 0.3 mM PMSF, pH 7.6).

Tamm–Horsfall protein is an abundant urinary protein that forms very high molecular weight complexes that sediment in the ultracentrifugation step unless denatured. To denature their zona pellucida domains, we added to the resuspended pellets dithiothreitol (DTT, 200 mg/mL), and then, they were incubated at 95 °C for 2 min. Subsequently, they were put into an ultracentrifuge tube and isolation solution was added in order to increase the volume up to 8 mL. The samples were then ultracentrifuged at 200,000×*g* for 1 h at 4 °C. The final pellets were resuspended in an adequate volume of isolation solution and frozen at −80 °C until used.

Electrophoresis and immunoblotting

Homogenates (20 µg of protein), apical membranes (16 µg of protein), urine samples (10 µL) and urinary exosomes samples (10 µL) were boiled for 3 min in the presence of 1 % 2-mercaptoethanol, 2 % sodium dodecyl sulfate (SDS) and separated by 8.5 % polyacrylamide gel (SDS-PAGE). After that, they were electroblotted to nitrocellulose membranes. To verify equal protein loading and transfer between lanes Ponceau Red and/or antibody against human β actin was used as previously described (Brandoni et al. 2006; Di Giusto et al. 2009; Bulacio et al. 2012). The nitrocellulose membranes were incubated with 5 % non-fat dry milk in phosphate-buffered saline containing 0.1 % Tween 20 (PBST) for 1 h. After being rinsed with PBST, the membranes were then incubated overnight at 4 °C with a non-commercial rabbit polyclonal antibody against rat Oat5 (at a dilution of 1:800) and with commercial mouse monoclonal antibody against human β actin (1.25 µg/mL). The specificity of Oat5 antibody has been described elsewhere (Anzai et al. 2005). The membranes were then incubated for 1 h with a peroxidase coupled goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA, USA) or with a peroxidase coupled sheet anti-mouse IgG (Amersham, Buckinghmshire, UK) after further washing with PBST. Blots were processed for detection using a commercial kit (ECL enhanced chemiluminescence system, Amersham, Buckinghmshire, UK). Kaleidoscope-prestained standards of molecular mass were employed (Bio-Rad laboratories). A densitometric quantification of the Western blot signal intensity of membranes was performed. For densitometry of immunoblots, samples from treated rats at different time points were run on each gel with corresponding control samples. The abundance of Oat5 in the kidney samples from the experimental animals normalized to β actin was calculated as percentage of the mean control value for that gel. For Oat5 abundance in urine and urinary exosomes, each value after normalization to urinary creatinine content was expressed as % of the mean value from control group.

Histopathological and immunohistochemistry studies

The immunohistochemistry technique was performed as previously described (Brandoni et al. 2006; Di Giusto et al. 2009; Di Giusto and Torres 2010; Bulacio et al. 2012; Bulacio and Torres 2013). Kidneys from the different experimental groups were briefly perfused with saline, followed by perfusion with periodate-lysine-paraformaldehyde solution (0.01 M $NaIO_4$, 0.075 M lysine, 0.0375 M phosphate buffer, with 2 % paraformaldheyde, pH 6.2), through a cannula inserted in the abdominal aorta. The kidney slices were immersed in periodate-lysine-paraformaldehyde solution at 4 °C overnight. After that, the tissue was embedded in paraffin. Paraffin sections were cut.

After deparaffining, some sections were used for routine hematoxylin–eosin staining, while others were incubated with 3 % H_2O_2 for 15 min (to eliminate endogenous peroxidase activity) to perform Oat5 renal immunohistochemistry. After that, the sections were incubated with blocking serum for 30 min and then with non-commercial rabbit polyclonal antibody against rat Oat5 (diluted 1:100) overnight at 4 °C. The sections were rinsed with Tris-buffered saline containing 1 % Tween (TBST). Next, the sections were incubated with horseradish peroxidase (HPR) conjugated secondary antibody against rabbit immunoglobulin for 1 h. In order to detect HPR labeling, a peroxidase substrate solution with diaminobenzidine (0.05 % diaminobenzidine in TBST with 0.05 % H_2O_2) was used. The sections were counterstained with hematoxylin before being examined under a light microscope.

Controls using preimmune serum, antiserum absorbed with excess synthetic peptide, or omission of primary or secondary antibody revealed no labeling.

Statistical analysis

The statistical analysis was performed using one way ANOVA followed by the Newman–Keuls test for comparing among more than two groups. An unpaired Student *t* test was used for comparing two experimental groups, and when variances were not homogeneous, Welch's correction was employed. *P* values of less than 0.05 were considered significant. The results are expressed as the mean ± standard error (SEM). For these analyses, GraphPad software was used.

Results

After administration of cisplatin at 5 mg/kg b.w., i.p., renal function was monitored by the measurement of urea and serum creatinine concentration at 2, 4, 7 and 14 days after treatment. There was no mortality during this study. Rats exposed to cisplatin showed no significant increases in plasma creatinine and urea levels during the first 2 days of treatment, while they were significantly increased on day 4, as compared to control animals. Peak levels for both parameters were seen 7 days after cisplatin treatment, and then, they gradually tended to return to their basal value by the day 14 (Fig. 1a, b). In recent works, where related doses of cisplatin were employed, similar time profiles have been found in plasma creatinine and urea levels (Sinha et al. 2013; Zhou et al. 2006).

As it is shown in Table 1, kidney weight remains unchanged until the fourteenth day after cisplatin administration, while a significant and progressive decrease of body weight was observed in rats throughout the study period. The loss of weight caused by cisplatin treatment has been already described (Ali et al. 2008; Abdelrahman et al. 2010). The decrease in body weight found in control animals was due to food deprivation during 24 h of lodging in metabolic cages as it has been previously described (Bulacio and Torres 2013).

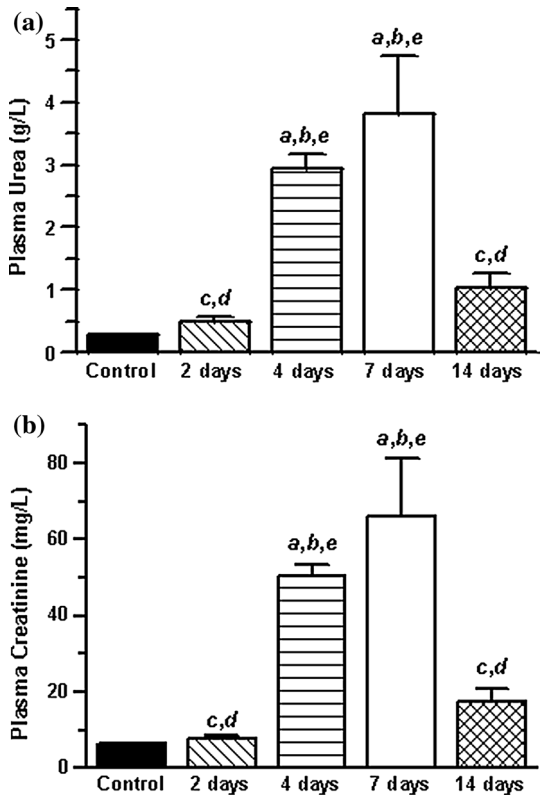

Fig. 1 Urea (**a**) and creatinine plasma levels (**b**) in control ($n = 16$) and treated animals after 2 ($n = 4$), 4 ($n = 6$), 7 ($n = 6$) and 14 ($n = 5$) days of treatment with 5 mg/kg b.w. of cisplatin. Results are expressed as mean values ± SEM. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus 2 days, (*c*) $p < 0.05$ versus 4 days, (*d*) $p < 0.05$ versus 7 days, (*e*) $p < 0.05$ versus 14 days

Table 1 Kidney weight, body weight decreased during the treatment (at different time points) and urine volume in control and rats after 2, 4, 7 and 14 days of treatment with 5 mg/kg b.w. of cisplatin

	Control ($n = 16$)	2 days ($n = 4$)	4 days ($n = 6$)	7 days ($n = 6$)	14 days ($n = 5$)
Kidney weight (g)	2.44 ± 0.04	2.47 ± 0.06	2.62 ± 0.04	2.55 ± 0.13	2.49 ± 0.04
Body weight decreased during the treatment (%)	4.66 ± 0.49	8.58 ± 0.40[d]	11.48 ± 0.56[a]	15.52 ± 3.59[a]	20.09 ± 3.48[a,b,c]
Urine volume (µL/min/100 g)	2.23 ± 0.21	2.92 ± 0.35	2.32 ± 0.79[d]	4.18 ± 0.83[a]	4.94 ± 0.55[a,c]

Body weight decreased during the treatment (%) $= -[(BW - BW_0)/BW_0] \times 100$

BW_0 = Initial body weight at time 0, just before the injection of vehicle (control rats) or cisplatin (treated animals)

BW = Body weight after 2, 4, 7 or 14 days of the injection of vehicle (control rats) or cisplatin (treated animals)

Results are expressed as means values ± SEM

[a] $p < 0.05$ versus Control, [b] $p < 0.05$ versus 2 days, [c] $p < 0.05$ versus 4 days, [d] $p < 0.05$ versus 14 days

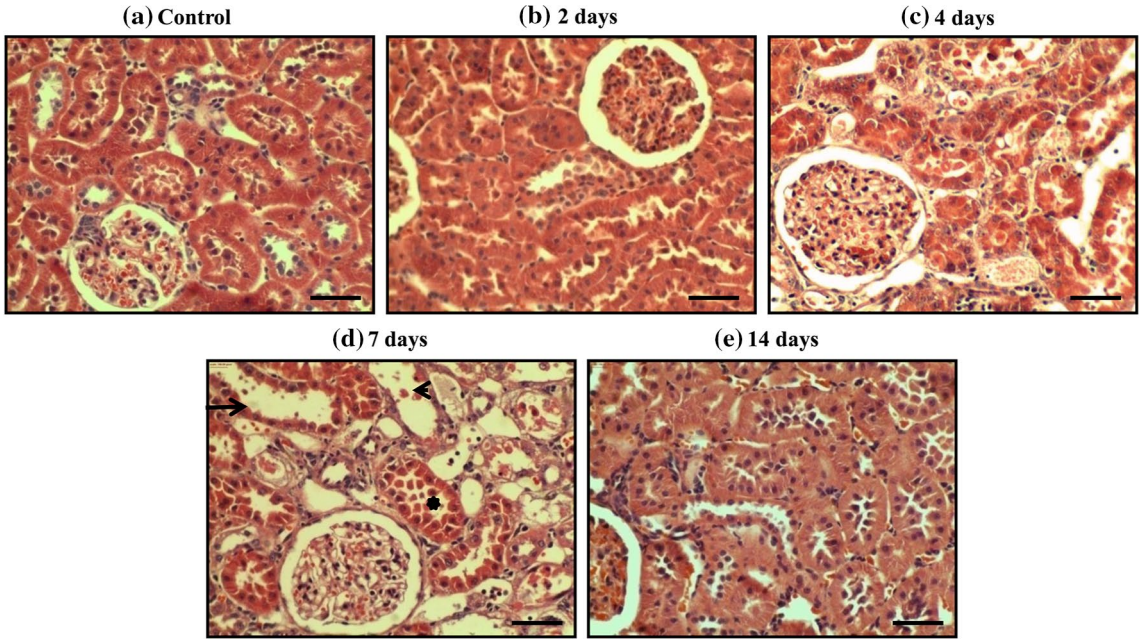

Fig. 2 Optical microscopy photos of kidney histology in control (**a**), and treated animals after 2 days (**b**), 4 days (**c**), 7 days (**d**) and 14 days (**e**) of treatment with 5 mg/kg b.w. of cisplatin (Hematoxylin–eosin staining). Two days after the treatment with cisplatin, no histological damages were detected compared to control kidneys; meanwhile, after 4 days of treatment, tubular damage becomes to be evident and was more severe on day 7, showing an important impairment in normal tubular architecture: tubular dilatation (*arrow*), tubular desquamation cells (*arrow head*) and epithelial cell detachment from basement membranes (*asterisk*) were observed (**d**). Then, after 14 days of treatment, an evident improvement of renal morphology was observed (**e**). These pictures are representatives of samples obtained from 4 animals from each experimental group. *Bars* 40 µm

Histological sections of kidneys from control and treated rats with 2, 4, 7 and 14 days of treatment with 5 mg/kg cisplatin were stained with hematoxylin–eosin. Kidney morphology from treated rats was normal on day 2, but tubular damage was evident from day 4 of treatment and become more severe on day 7, showing an important impairment in their normal tubular architecture, revealing tubular dilatation, tubular desquamation cells and epithelial cell detachment from basement membranes, as it is seen in Fig. 2. From that point on, tubule morphology gradually recover, indicating tubular regeneration and repair, as previously described. Histological findings observed in these experiments were in agreement with those previously described for cisplatin-induced renal tubular injury in rats (Zhou et al. 2006; Yao et al. 2007; Pinches et al. 2012).

Urine output was significantly increased by day 7 of treatment and remained significantly elevated thereafter, as compared to control group (Table 1). On that subject, the most prominent and persistent feature associated with the cisplatin-induced renal failure is polyuria, of which urinary concentration defect has been attributed, at least in part, to a reduced expression of AQP water channels in the

collecting duct (Kim et al. 2001; Ecelbarger et al. 2001). The increased urine output, together with the injured renal tubules, and the following loss of the tubular cells to reabsorb water leading to dehydration, might be some of the reasons that could explain the loss of body weight seen in this treatment (Ali et al. 2008).

Oat5 abundance in urine was related to urinary creatinine concentrations in order to correct for variations in urine production as previously described for urinary transporters and enzymes (Di Giusto and Torres 2010; Bulacio and Torres 2013; Hazelhoff et al. 2013). Measurements of biomarkers alone are insufficient because normal physiological variations in water excretion can dilute or concentrate urinary proteins. Normalization on the basis of total protein amount is generally unsatisfactory because total protein excretion can vary broadly among various pathological states. Creatinine is excreted in the urine at relative constant rates allowing it to be used to normalized urinary excretion of a particular protein.

Figure 3 shows that Oat5 urinary levels were markedly and significantly increased 2 days after cisplatin injection (sevenfold approximately), as compared to control values, and then continued elevated until the fourth day of treatment (threefold approximately). From day 7, Oat5 levels in urine returned to basal values. So, we found that Oat5 urinary excretion was increased before than alterations in traditional renal injury markers as serum creatinine and urea levels were detected and tubular damage was observed. To evaluate possible tubular injury, urinary AP activity and total proteins and glucose urine concentration were measured and related to urinary creatinine concentration. As it is shown in Fig. 4, these three parameters showed no significantly increases in urine samples by the day 2 of treatment, but they were significantly increased on day 4, and then they gradually tended to return to their basal values by

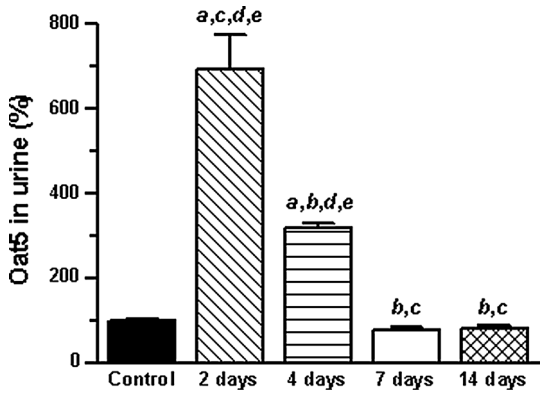

Fig. 3 Oat5 abundance in urine from control and treated rats after 2, 4, 7 and 14 days of treatment with 5 mg/kg b.w. of cisplatin. Urine samples are separated by SDS-PAGE and blotted onto nitrocellulose membranes. Densitometric quantification of Oat5 Western blotting from urine are expressed as arbitrary units related to urinary creatinine concentration in order to correct for variations in urine production. The mean of the control value was set as 100 %. Results are expressed as mean values ± SEM from experiments carried out in four different samples for each experimental group. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus 2 days, (*c*) $p < 0.05$ versus 4 days, (*d*) $p < 0.05$ versus 7 days, (*e*) $p < 0.05$ versus 14 days

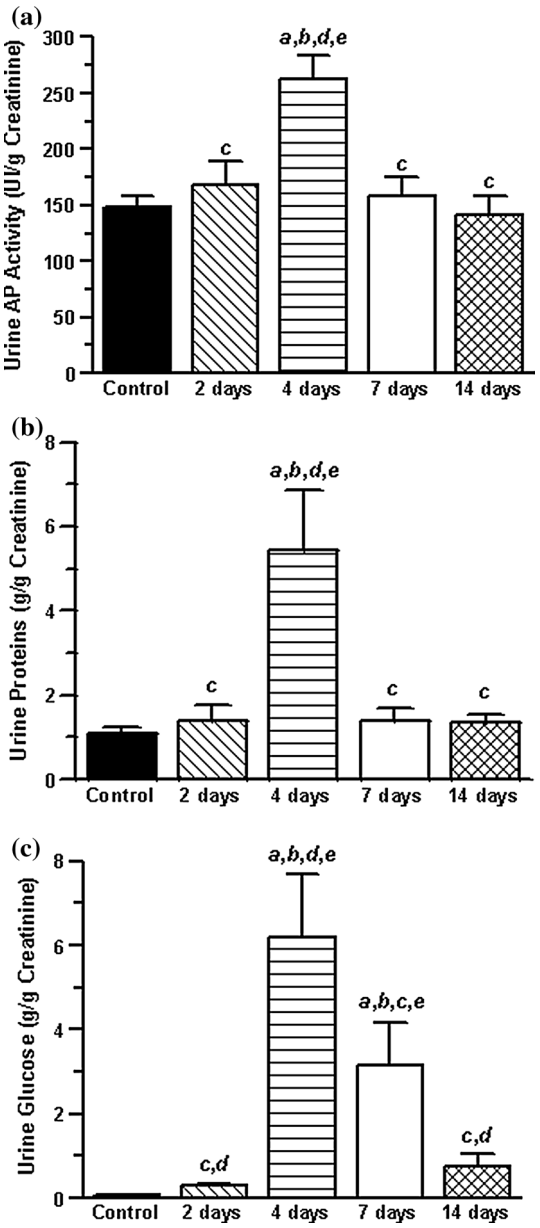

Fig. 4 **a** AP urinary activity (UI/gCr), total proteins (g/gCr) (**b**) and glucose (g/gCr) (**c**) urinary levels in control ($n = 16$) and treated animals after 2 ($n = 4$), 4 ($n = 6$), 7 ($n = 6$), 14 ($n = 5$) days of treatment with 5 mg/kg b.w. of cisplatin. These parameters are determined using commercial kits and are related to urinary creatinine concentration in order to correct for variations in urine production. Results are expressed as mean values ± SEM. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus 2 days, (*c*) $p < 0.05$ versus 4 days, (*d*) $p < 0.05$ versus 7 days, (*e*) $p < 0.05$ versus 14 days

day 14, as compared to control group. These results indicate that significant renal tubule damage was produced at this dose of cisplatin by the day 4 of treatment and then gradually tend to recover thereafter, as it was observed by histological studies.

In order to elucidate the mechanisms involved in urinary Oat5 excretion, we assessed Oat5 abundance in urinary exosomes in control and treated group with 4 days of treatment with 5 mg/kg of cisplatin by Western blotting, as preliminary results. In treated rats, Oat5 abundance in urinary exosomes, expressed as percentage, was significantly increased, compared with control group (control $= 100 \pm 4$, $n = 4$; treated $= 299 \pm 13$, $n = 4$, $p < 0.0001$).

Kidney homogenates and the apical membranes from control and treated rats were subjected to Western blotting for Oat5 protein. Figure 5a shows a significantly time-dependent decrease in Oat5 expression in treated animals in renal homogenates, observing a decrease of 26, 55, 80 and 91 % by the days 2, 4, 7 and 14 of treatment, respectively, as compared to control group. In apical membranes, Oat5 expression was also diminished in treated rats, showing approximately a 50 % of decrease by the days 2 and 4, and a 75 % on days 7 and 14 of treatment, as compared to control group (Fig. 5b).

Oat5 renal expression was also assessed by immunohistochemistry technique. In control group, strong Oat5 labeling was associated with the apical membrane and the intracellular domains in proximal tubule cells. Oat5 labeling in treated rats with 2, 4, 7 and 14 days of treatment with 5 mg/kg cisplatin was consistent with the density observed by Western blotting studies in each time point evaluated (Fig. 6). Negative controls with omission of primary antibody revealed no labeling for control and treated animals with 2, 4, 7 and 14 days after the injections.

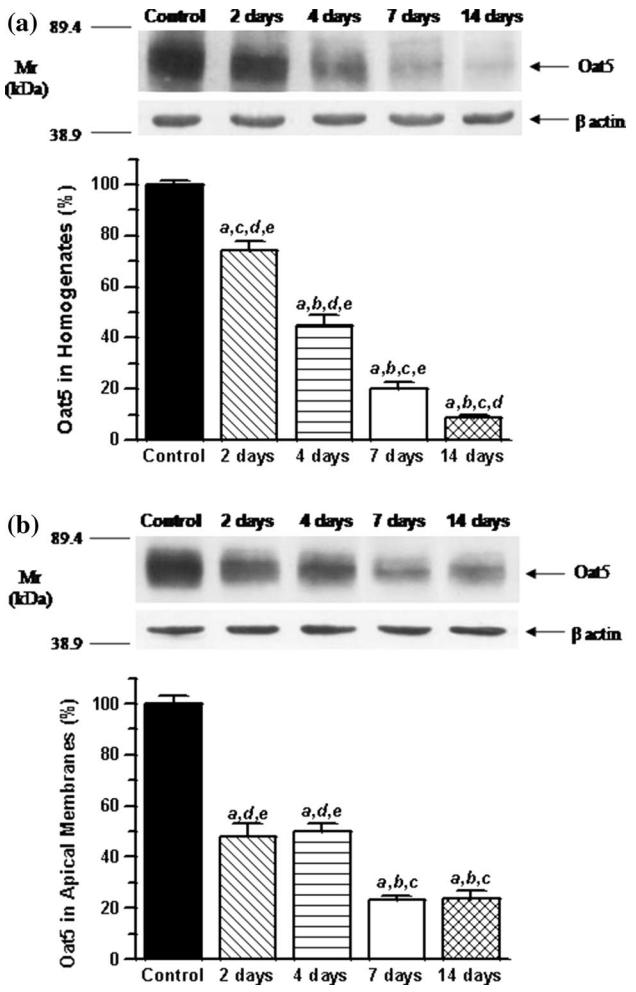

Fig. 5 Western blotting for Oat5 in homogenates (**a**, 20 µg proteins) and apical membranes (**b**, 16 µg proteins) from kidneys of control and treated rats with cisplatin, after 2, 4, 7 and 14 days of treatment. Proteins are separated by SDS-PAGE and blotted onto nitrocellulose membranes. The results are expressed as percentage, normalized for the β actin density. The mean of the control value was set as 100 %. Results are expressed as mean values ± SEM from experiments carried out in four different preparations for each experimental group. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus 2 days, (*c*) $p < 0.05$ versus 4 days, (*d*) $p < 0.05$ versus 7 days, (*e*) $p < 0.05$ versus 14 days. Kaleidoscope-prestained standards of molecular mass (Mr) corresponding to bovine serum albumin (89.4 kDa) and to carbonic anhydrase (38.9 kDa) are indicated in the *left of the figure*

Discussion

Acute kidney injury (AKI) is a prevalent and devastating condition defined by a rapid decrease (hours to days) in renal excretory function and in glomerular filtration rate (GFR). It is also accompanied by the accumulation of end products of nitrogen metabolism excreted by the kidney such as urea and creatinine. In the AKI process, several changes occur at cellular and molecular levels that finally lead to renal dysfunction and structural injury. When AKI is severe and patients requires renal replacement therapy hospital mortality can reach rates of 50–60 % (Bellomo et al. 2012; Slocum et al. 2012; Peres et al. 2013).

There are different etiologies causing AKI, including ischemia, sepsis and drugs and other kind of toxins. Drugs seem to greatly contribute particularly in critically ill patients, and some of them are cisplatin, gentamicin and cyclosporine, between many others (Bellomo et al. 2012; Peres et al. 2013).

Cisplatin is a widely used chemotherapeutic agent. Many studies showed that the kidney accumulates more cisplatin than other organs, principally in proximal tubules cells (PTC). The mechanisms of its nephrotoxicity are complex and are not completely understood and could involve oxidative stress, apoptosis or necrosis, inflammation and fibrogenesis. It has been described that 48–72 h

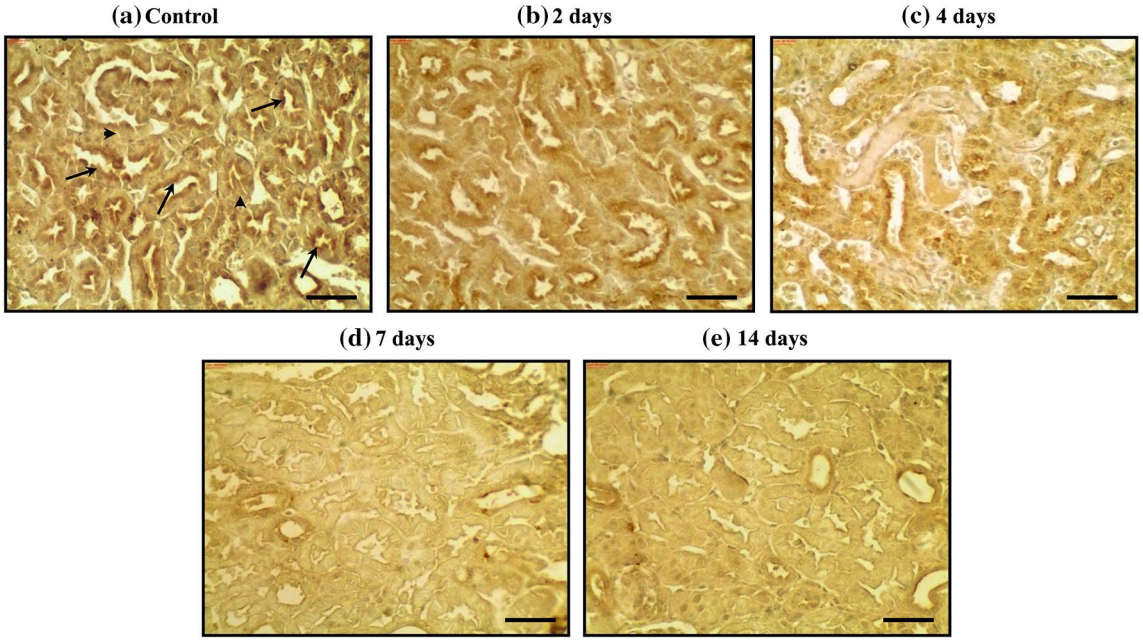

Fig. 6 Immunohistochemistry for Oat5 in renal tissue from control (**a**), and cisplatin-treated rats with 5 mg/kg b.w. after 2 (**b**), 4 (**c**) 7 (**d**) and 14 days (**e**) of treatment. Serial sections from each rat kidney are stained using a non-commercial anti-Oat5 antibody. Oat5 labeling was associated with the apical plasma membranes (*arrow*) and with intracellular domains (*arrowhead*) in proximal tubule cells (**a**). These pictures are representatives of typical samples obtained from 4 animals from each experimental group. *Bars* 40 µm

after cisplatin administration, there is impaired proximal and distal tubular reabsorption and increased vascular resistance. Then, a second phase starts (between 72 and 96 h after cisplatin administration) and is characterized by a decreased in GFR (Yao et al. 2007; Miller et al. 2010). Although cisplatin nephrotoxicity can be attenuated by diuretics and pre-hydration protocols, its prevalence is still high, occurring in about one-quarter to one-third of patients undergoing cisplatin treatment (Pabla and Dong 2008; Wensing and Ciarimboli 2013).

Two serum biomarkers remain as the markers of choice in preclinical and clinical studies in kidney injury: serum creatinine and urea levels (SCr and SUr, respectively). However, both biomarkers have important limitations relating to sensitivity and specificity, resulting in a delayed diagnosis and missing early therapeutic interventions. Alterations in SCr are often not evident until 48–72 h post-injury, and age, gender, muscle mass and nutritional status all also affect SCr levels (Ferguson et al. 2008; Slocum et al. 2012; Bonventre et al. 2010). SUr is not a reliable measure of kidney injury because it may be affected by several factors. For example, an increase in plasma urea levels can be seen with volume depletion in the absence of any tubular injury, and increased levels of uremia can be detected if urea production is increased (Bonventre et al. 2010).

Urine is also an ideal source of biomarkers because it provides a noninvasive and quick way for assessment of AKI. Nevertheless, conventional urine markers (fractional excretion of sodium, casts, urine osmolality, etc.) are nonspecific and insensitive (Zhou et al. 2006). Over the last years, tubular proteins released during tubular insult have garnered much attention as better biomarkers as they can potentially be true, early, real time and proportionate to the injury (Sinha et al. 2013). Some of the more promising AKI urine biomarkers includes neutrophil gelatinase-associated lipocalin (NGAL) (Mishra et al. 2003), kidney injury molecule-1 (Kim-1) (Han et al. 2002) and *N*-acetyl-D-glucosaminidase (NAG) (Westhuyzen et al. 2003), whose have demonstrated to be early predictors for diagnosis or outcome of AKI in human as well as in animal models (Ferguson et al. 2008).

Oat5 has been defined as a probenecid-sensitive organic anion/dicarboxylate exchanger that localize exclusively in apical membranes of renal proximal tubules. Its renal expression may have a physiological role in homeostasis of ES and DHEA-S (Kwak et al. 2005; Anzai et al. 2005).

The aim of this study was to evaluate Oat5 urinary excretion for its ability to identify cisplatin-induced AKI earlier than traditionally renal biomarkers of kidney injury. To accomplish that objective, the time course of Oat5 urinary excretion and conventional biomarkers changes were compared to the onset and progression of histological changes after a single dose of 5 mg/kg b.w., i.p. of cisplatin. Two days after cisplatin administration, Oat5 urinary

excretion was markedly increased (sevenfold), meanwhile no modifications at any traditional renal injury markers and no relevant histological findings have still been observed. On day 4 of treatment, Oat5 in urine still remained higher than control values (threefold), hemodynamic (SUr and SCr) and tubular parameters were significantly increased, and tubular damage was observed. By the seventh day of treatment, a severe impairment in normal tubular architecture was found, as assessed by histological studies. From this time point and thereon, tubular parameters (with the exception of Glu_U) showed a tendency to return to their basal value and Oat5 urinary excretion showed no differences as compared to control group. On the day 14 of treatment, kidney morphology showed an important recovery, and hemodynamic parameters and Glu_U tended to return to their basal values. So, in this experimental model, Oat5 urinary excretion was modified earlier than any traditional marker of renal injury.

Oat5 renal expression was also assessed. In cisplatin treated rats, Oat5 renal abundance was decreased, both in renal homogenates and apical membranes. The decreased Oat5 renal expression suggests an increase in its degradation or a decrease in its synthesis, probably due to tubular damage caused by cisplatin oxidative stress.

In previously works, we have also assessed Oat5 urinary excretion in ischemic and mercuric induced AKI (Di Giusto et al. 2009; Di Giusto and Torres 2010). In bilateral mild subclinical ischemia, there was a dramatic increase in urinary Oat5 abundance, while no alterations in traditional kidney injury markers were detected (Di Giusto et al. 2009). Also, in a $HgCl_2$ dose–response study, Oat5 in urine was increased at a low dose of this compound (0.2 mg/kg, s.c.), where no other significant alterations were observed (Di Giusto and Torres 2010). It is important to emphasize that inorganic Hg and platinum, usually in the form of cisplatin, and many other metals, such as Cd, Cr, Pb and V, shares oxidative stress with associated lipid peroxidation, apoptosis and necrosis as a common phenomenon in the course of renal injury of these metals. Oxidative stress, taking place principally in PTC, is manifested by depletion of GSH, inhibition of free radicals-detoxifying enzymes and increased production of ROS. Most of these events also occur in the hypoxia-induced proximal tubule injury. Other phenomena, for example, impaired intracellular vesicle recycling, increased intracellular concentration of Ca^{2+}, selective loss of transporters from basolateral and apical membranes from PTC and damage in mitochondria have been demonstrated in the course of action of Hg, cisplatin and Cd (Zalups 2000; Sabolić 2006; Miller et al. 2010). Hence, after the preclinical animal results showed in this work, together with the data shown in our previous reports (Di Giusto et al. 2009; Di giusto and Torres 2010; Bulacio and Torres 2013), we can propose that Oat5 urinary excretion might potentially serve as a noninvasive early biomarker of ischemic, mercuric and cisplatin-induced AKI. Probably, common features involved in their intracellular mechanisms of toxicity in PTC are responsible for the increase in the release of Oat5 from apical membranes to urinary space after the toxic insults.

It is possible to postulate that Oat5 excretion into urine involves exosomal pathway. Exosomes are small (20–100 nm) membrane vesicles that originate as the internal vesicles of multivesicular bodies in many cell types. They are released into the extracellular space, including plasma and urine, by fusion of its outer membrane with the cell surface (Higashijima et al. 2013; Fang et al. 2013). Urinary exosomes contain proteins that are characteristic of every renal tubule epithelial cell type. They are rich in potential biomarkers, particularly membrane proteins such as transporters and receptors that may be down- or upregulated during different disease states (Gonzales et al. 2010). So far, Pisitkun and colleagues have identified 1,160 exosomal proteins, of which at least 34 are related to kidney diseases such as Bartter syndrome (sodium–potassium–chloride cotransporter 2), autosomal recessive renal tubule acidosis (carbonic anhydrase 2) and autosomal dominant or autosomal recessive nephrogenic diabetes insipidus (aquaporin-2; AQP2) (Pisitkun et al. 2004). In the present study, we have demonstrated for the first time, at least to our knowledge, the presence of Oat5 in rat urinary exosomes. These results allowed us to propose urinary exosomal pathway as a way of Oat5 urinary excretion. In this connection, preliminary data obtained in this work showed an important increase in Oat5 abundance in urinary exosomes from cisplatin-treated animals as compared to control group. This could be due to an alteration in the exosome secretory process, caused by intracellular modifications triggered by cisplatin in PTC.

An ideal biomarker for the detection of AKI is one that is easily obtained, sensitive to minor disturbances in kidney function, easily and rapidly measured, correlated with the degree of injury, site specific, and indicative of injury progression. A single biomarker is rarely sufficient to clearly identify a particular pathologic state. Given inherent renal heterogeneity and the disparate settings under which kidney injury occurs, a panel of carefully selected biomarkers may prove to be most appropriate to accurately define a kidney disease (Rifai et al. 2006; Ferguson et al. 2008).

Recently, novel urinary biomarkers have been assessed in different models of cisplatin-induced AKI, such as Fetuin-A (Zhou et al. 2006) and Vanin-1 (Hosohata et al. 2012). In addition, McDuffie et al. have found that urinary Kim-1, α-GST and albumin showed a good concordance with cisplatin-induced renal injury progression and reversal and they were more sensitive than traditional serum biomarkers in detecting early renal tubular damage (McDuffie et al. 2013). Regardless of the major progress in AKI biomarker

discovery, further investigation is needed to validate their clinical utility. It is important to be aware of a biomarker that detects inflammation effectively may not be as sensitive in detecting early proximal tubule toxicity in the absence of inflammation, and a biomarker of injury might not detect a functional defect. Besides, a biomarker useful in an animal model may or may not be useful in the humans. More studies are necessary to validate the temporal pattern of various urinary biomarkers for early detection of AKI, how to combine them, and how their temporal course relates to the onset, severity and outcome of AKI (Han et al. 2008; Bonventre et al. 2010; Slocum et al. 2012).

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