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Toxicology Letters

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RENAL EXPRESSION OF ORGANIC ANION TRANSPORTERS IS MODIFIED AFTER MERCURIC CHLORIDE EXPOSURE: GENDER-RELATED DIFFERENCES.

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ABSTRACT

Mercuric ions (Hg^{+2}) gain access to proximal tubule cells primarily by the Organic Anion Transporter 1 (Oat1) and 3 (Oat3) in the basolateral plasma membrane. The removal process of Hg^{+2} ions from cells into the lumen involves an efflux process mainly mediated by the Multidrug Resistance-Associated Protein 2 (Mrp2). The aim of this study was to compare the sex-related differences in the renal expression of Oat1, Oat3, and Mrp2 after mercuric chloride ($HgCl_2$) treatment and analyze their relevance in the mercury-induced nephrotoxicity. Control and Hg-treated male and female Wistar rats were used. Animals received a dose of $HgCl_2$ (4 mg/kg bw, ip) 18 h before the experiments. Tubular injury was assessed by histopathological studies. The renal expression of Oat1, Oat3, and Mrp2 was analyzed by Western Blotting. Mercury levels were determined in urine by cold vapour atomic absorption spectroscopy. $HgCl_2$ treatment increased the expression of renal Oat1 and Mrp2 in both sexes, being more evident in females than in males. The Oat3 renal expression only increased in female rats. The higher expressions of Oat1, Oat3, and Mrp2 could explain the higher renal excretion of mercury and consequently, the lesser renal tubular damage in female rats than in male rats.

Keywords: sex differences; organic anion transporters; renal failure; mercuric chloride; nephrotoxicity.

1. INTRODUCTION

Mercury is a widespread environmental pollutant. Humans have been using mercury from ancient times to the present. Individuals are exposed to almost all forms of mercury mostly through fish consumption and mercury-containing dental amalgam fillings and vaccines. In addition, the sources of exposure to compounds containing mercury include different anthropological uses such as artisanal and small-scale gold mining operations, industrial processes and incineration of medicinal, chemical and municipal waste products. Within the various species of mercury, mercury salts have diverse applications in the industry as a common ingredient in soaps, in skin lightening beauty creams, in the chloralkali and caustic soda industries, in the manufacturing of electrical switches for automotive engineering and in the fluorescent lamp factories (Bjørklund et al., 2017; UNEP, 2013; Zalups, 2000).

Mercury can cause toxic effects in a variety of organs and tissues. Inorganic divalent mercury (Hg^{+2}) salts are the compounds with the greatest toxicological impact since the other forms of mercury (such as Hg^{0}, Hg^{+1} and organic mercury) can be converted to Hg^{+2} in the body. Present evidence indicates that all forms of mercury undergo a similar metabolism in humans and laboratory animals (Bjørklund et al., 2017; UNEP, 2013; Zalups, 2000). Inorganic species of mercury accumulate mainly in the renal proximal tubular cells causing acute kidney injury by oxidative stress mechanisms (Zalups, 2000). Hg^{+2} gain access from the peritubular blood into proximal tubule cells primarily across the Organic anion transporter 1 (Oat1, *Slc22a6*) and the Organic anion transporter 3 (Oat3, *Slc22a8*) in the basolateral plasma membrane. The removal process of Hg^{+2} ions from proximal tubular cells into the lumen involves a direct secretory process mainly mediated by the Multidrug resistance-associated protein 2 (Mrp2, *ABCC2*) (Bridges and Zalups, 2017; Zalups et al., 2014). Oat1 and Oat3 represent the principal organic anion/α-ketoglutarate exchangers located in the basolateral membranes from proximal tubule cells. Mrp2 belongs to the ATP-Binding Cassette (ABC) family and is expressed in the apical membrane of the proximal tubule cells. Mrp2 plays an important role in the elimination of several conjugated waste products into the urine (Nigam et al., 2015). It has been described sex-related differences in the renal expression of Oat1, Oat3, and Mrp2, where males exhibit higher protein levels of these transporters than females (Cerrutti et al., 2002; Ljubojevic et al., 2004; Wang et al., 2012).

In an experimental model of mercury-induced nephrotoxicity, we have demonstrated that female rats present a lower renal damage than male rats (Hazelhoff et al., 2012).

After the mercuric chloride ($HgCl_2$) administration, males showed a more important decrease in urine volume and creatinine clearance. Additionally, the urinary excretion of the Organic anion transporter 5 (a novel biomarker of nephrotoxicity) increased more in males than in females. Moreover, urinary alkaline phosphatase activity was modified only in male rats. In addition, in studies performed on both humans and experimental animals exposed to different forms of mercury, it has been reported that males have an increased systemic and renal retention of mercury than females and a lower mercury excretion rate. Besides, it has also been observed that males exposed to methyl mercury present a larger deleterious effect on development than females (Akesson et al., 1991; Ekstrand et al. 2010; Gimenez-Llort et al., 2001; Grandjean et al., 1998; Hultman and Nielsen, 2001).

Several studies have also described modifications in the renal expression of Oat1, Oat3, and Mrp2 in the presence of renal and extra-renal pathologies (Bulacio et al., 2012; Brandoni et al., 2012; Di Giusto et al., 2008, 2009; Tanaka et al., 2008). Moreover, in a recent work, we have observed sex-related differences in the liver toxicity caused by $HgCl_2$ administration (Hazelhoff and Torres, 2018). Male rats displayed a lower hepatic damage and a lesser accumulation of mercury in the liver than females. $HgCl_2$ treatment decreased Oat3 expression in the hepatocytes membranes only in males, limiting the uptake of mercury ions into the liver and protecting them from mercury hepatotoxicity.

Therefore, based on the evidence presented above, the purpose of this study was to compare the renal expression of Oat1, Oat3, and Mrp2 after $HgCl_2$ treatment between male and female rats in order to analyze their relevance in the gender-related differences in mercury-induced nephrotoxicity. Besides, there is currently little information about the mechanisms that differ between males and females in the renal handling of mercury. Identification and characterization of the potential changes in the expression of Oat1, Oat3, and Mrp2 after mercury administration are important both to a better understanding of the mechanisms of mercury toxicity and to identify potential therapeutic targets and/or novel therapeutic strategies. In order to accomplish the objective, male and female rats were treated with a single dose of $HgCl_2$ (4 mg/kg body weight (bw)) 18 hours before the experiments. Renal damage in the cortex and in the outer stripe of the outer medulla (OSOM) was quantified, and Oat1, Oat3, and Mrp2 expression on kidney total plasma membranes were assessed and compared in control and treated male and female rats.

2. EXPERIMENTAL PROCEDURES

2.1 Materials

Chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA) and were analytical grade. The antibodies against Oat1 and against human β-actin were purchased from Alpha Diagnostic International (San Antonio, TX, USA) and the polyclonal antibody against Mrp2 from Abcam (Cambridge, MA, USA). The non-commercial antibody against Oat3 was kindly provided by Prof. N. Anzai (Department of Pharmacology, Graduate School of Medicine, Chiba University, Japan). The molecular ruler was purchased from Bio Rad Laboratories (Hercules, CA, USA).

2.2 Experimental Animals

Three months old male and female Wistar rats were used. The animals had access to tap water and standard laboratory chow *ad libitum*, and a temperature and humidity- controlled environment on a 12:12 h light cycles was provided. All procedures were approved by the Faculty of Biochemical and Pharmaceutical Sciences Institutional Animal Care and Use Committee, National University of Rosario (N° 366/2016), were compliant to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and were in accordance to EC Directive 86/609/EEC.

2.3 Experimental Protocols

Animals were injected with a nephrotoxic intraperitoneal (ip) dose of $HgCl_2$ (4 mg/kg bw) and control animals received the vehicle (2 mL saline/kg bw). After 18 h of treatment, experiments were performed as reported before (Hazelhoff et al., 2012, 2015; Trebucobich et al., 2014; Torres et al., 2011; Zalups, 2000).

Four experimental groups were used: Control Males (CM), Control Females (CF), Hg-treated Males (Hg-M) and Hg-treated Females (Hg-F) (n=4, respectively).

After the $HgCl_2$ or vehicle injection, animals were placed in individual metabolic cages for 18 hours. The volume of urine was estimated by gravimetry. Urinary flow (Uf) was calculated and expressed as mL/min/100 g bw.

For experimental procedures, animals were anesthetized with an intraperitoneal dose (70 mg/kg bw) of sodium thiopental, and they were euthanized with an anaesthetic overdose and thoracotomy, as previously reported (Bulacio et al., 2012; Bulacio and Torres 2015; Cerrutti et al., 2002; Di Giusto et al., 2008, 2009; Dudek et al., 2016; Hazelhoff et al., 2012, 2015; Torres et al., 2011; Trebucobich et al., 2014). Depending on the study to be performed, distinct methods of obtaining and processing the renal tissue samples were used.

188 **2.4 Histopathological studies**

189 Histopathological studies of kidneys were performed, as previously described (Bulacio et
190 al., 2012; Bulacio and Torres 2015; Hazelhoff et al., 2012, 2015). Besides, renal injury was
191 evaluated in renal sections. The severity of tubular injury was considered as percent of
192 tubules of the section showing a given tubular alteration (tubular dilatation/flattening, loss of
193 brush border, vacuolated cells, cellular detachment, focal necrosis, intraluminal nuclei, and
194 debris), and was graded as follows: 0, less than 5 %; 1, 5-33 %; 2, 34-66 % and 3, over 66%,
195 as previously described (Hazelhoff et al., 2015). In order to achieve it, ten high-power
196 fields/section (at×400) were examined by a blinded observer unaware of the experimental
197 groups.

198 **2.5 Mercury content determination**

199 Total mercury concentration [Hg_u] in urine samples was assessed by cold vapor atomic
200 absorption, employing an Atomic Absorption Spectrophotometer Perkin Elmer AAnalyst
201 300, Flow Injection Analysis System (FIAS) 100–Perkin Elmer as previously described
202 (Trebucobich et al., 2014). Urine excreted load of mercury was calculated as Uf x [Hg_u].

203 **2.6 Preparation of total plasma membranes from kidneys**

204 Total plasma membranes from kidneys of controls and Hg-treated rats were obtained
205 according to previously described by our laboratory (Hazelhoff et al., 2012, 2015;
206 Trebucobich et al., 2014).

207 Kidney tissue was homogenized with a 250 mM sucrose, 5 mM Hepes-Tris and 0.1 mg /
208 mL phenylmethylsulfonylfluoride (PMSF), pH = 7.40 buffer at 4° C, in a ratio of 50 mL of
209 buffer per 4 g of renal tissue and then centrifuged for 15 min at 1200 g at 4° C. The
210 supernatant was centrifuged at 22,000 g for 15 min at 4° C. The pellet that is formed is
211 covered by a layer called "fluffy", which consists of the total plasma membranes. This layer
212 was resuspended in sucrose buffer.

213 **2.7 Electrophoresis and Immunoblotting studies**

214 The electrophoresis and immunoblotting studies were performed as previously described
215 by Hazelhoff et al. (2012). Proteins from total plasma membranes samples (18 µg) were
216 separated through 8.5% (for Oat1 and Oat3) or 5 % (for Mrp2) SDS-polyacrylamide gel
217 electrophoresis (SDS-PAGE) and then electroblotted to a pure membrane of nitrocellulose.
218 Kaleidoscope Prestained Standards of molecular mass were employed (Bio Rad Laboratories,
219 Hercules, CA, USA). Ponceau Red and antibody against ß-actin were used to verify equal
220 protein loading and transfer between lanes as previously described (Bulacio and Torres, 2015;
221 Hazelhoff et al., 2015). The membranes were incubated overnight at 4 °C with rabbit

polyclonal antibodies against rat Oat1 or Oat3, or with a mouse polyclonal antibody against rat Mrp2 or with a mouse monoclonal antibody against human β actin, as described before (Hazelhoff et al., 2012, 2015).

A commercial chemiluminescent detection system (ECL Plus Western Blotting Detection Reagents; Amersham, Buckinghamshire, UK) was used. The densitometric quantification of the Western blotting signal intensity was performed using analyzer software (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD, USA). The abundance of Oat1, Oat3, and Mrp2 in the samples of each experimental group were normalized to ß-actin.

2.8 Statistical Analysis

The unpaired Student´s t-test was employed for statistical analysis and a Welch´s correction was employed when variances were not uniform. For multiple comparisons, one way ANOVA followed by the Newman-Keuls test was used to evaluate statistical differences between groups. $p < 0.05$ was considered statistically significant. The values were expressed as the means ± standard error (SEM).

3. RESULTS

It has been previously documented (Hazelhoff et al., 2012, 2015; Trebucobich et al., 2014) that the dose of $HgCl_2$ employed in this work causes renal damage in rats. Moreover, male rats showed more renal injury than female rats (Hazelhoff et al., 2012). To characterize and to deepen in the gender-related differences in the $HgCl_2$-induced renal tubular toxicity, the histopathological damage in the renal cortex and in the OSOM was evaluated and compared in both sexes (Figure 1). Control groups showed normal tubules. Hg-M revealed alterations in proximal tubules integrity both in the cortex and the OSOM, such as vacuolated cells, cellular detachment, focal necrosis, intraluminal nuclei, and debris (Figure 1 A and C). The changes of tubules integrity in Hg-F were less significant than those observed in Hg-M as indicated by tubular injury scores (Figure 1 B and D).

Mercury levels were measured in urine from male and female rats after exposure to $HgCl_2$. As it is shown in Figure 2, the excretion of mercury in urine was greater in female rats than in male rats.

We evaluated the impact of the $HgCl_2$-treatment on Oat1, Oat3, and Mrp2 protein abundance in renal plasma membranes from female rats compared with male rats. Plasma membranes from CM, Hg-M, CF, and Hg-F animals were simultaneously subjected to Western blotting analyses for Oat1, Oat3, and Mrp2 proteins. Figure 3A shows an increase in Oat1 expression in plasma membranes following $HgCl_2$ treatment. However, the increase

observed in Hg-F rats was higher than the increase in Hg-M rats. Besides, in the plasma membranes, the Hg-F group showed a significant increase in the Oat3 abundance compared with the CF group (Figure 3B), but no significant modifications were observed in the Oat3 plasma membranes abundance between the CM and Hg-M groups.

In addition, as shown in Figure 4, the Mrp2 protein abundance was increased following $HgCl_2$ treatment in the plasma membranes in both sexes. This increase was higher in Hg-F rats than that observed in Hg-M rats.

Figure 3 and Figure 4 also show that the abundance of β-actin, as expected, was not modified with the $HgCl_2$ treatment.

4. DISCUSSION

The experimental model of $HgCl_2$-induced nephrotoxicity is characterized by structural and functional tubular abnormalities in both the renal cortex and OSOM since mercury affect convoluted S1-S2 and straight S3 segments of proximal tubules (Stacchiotti et al., 2003; Zalups, 2000). It is well established that the S3 region of the proximal tubule (the pars recta or straight segment) is the most vulnerable portion to $HgCl_2$ toxicity, especially the cortico-medullary junction. A selective damage of S3 segment was observed with doses of less than 2 mg/kg bw of $HgCl_2$ (Dobyan and Bulger, 1984; Eknoyan et al., 1982). Nevertheless, there is a clear dose-response relationship in the toxicity of $HgCl_2$ in the proximal tubule, and convoluted portions (S2 and even S1 segments) of proximal tubules can be affected at higher doses (Di Giusto et al., 2009; Hazelhoff et al., 2012, 2015; Stacchiotti et al., 2003; Zalups 2000). Moreover, most of the Hg^{+2} ions are detected in the cortex and in the OSOM when a non-toxic dose of $HgCl_2$ (0.135 mg/kg bw) is administered to rats (Bridges et al., 2011). In the present study, we quantified the tubular injury induced by $HgCl_2$ (4 mg/kg bw) in female and male rats. Female rats presented a lesser damage at the level of the tubular epithelium both in the cortex and in the OSOM, and consequently, females preserved a greater number of entire proximal tubules than males after $HgCl_2$ injury.

Mercuric ions conjugated with endogenous sulphydryl groups are the main form in which mercury is uptaken in the kidney by Oat1 and Oat3 in the basolateral membranes (Bridges and Zalups, 2017). Oat1 interacts with endogenous monocarboxylates, short chain fatty acids, urate, and acidic neurotransmitter metabolites. Oat3 transports the second messengers cAMP and cGMP, cholate and taurocholate, prostaglandins, cortisol, and urate. Moreover, Oat1 and Oat3 mediate the pharmacokinetics of a wide variety of drugs such as angiotensin II receptor blockers, angiotensin-converting-enzyme inhibitors, diuretics, β-

lactam antibiotics, antiviral agents, antineoplastic drugs, and non-steroidal anti-inflammatory drugs (Nigam et al., 2015). Mrp2 removes mercuric ions from inside the tubular cells to the lumen (Bridges and Zalups, 2017; Zalups et al., 2014). Mrp2 has a broad variety of physiologic and xenobiotic metabolites as substrates and is one of the main apical efflux transporters of several of the substrates taken up by Oat1 and Oat3 in the basolateral membrane. At present, it is believed that these transporters (Oat1, Oat3, and Mrp2) work together in concert to control the excretion of compounds of both toxicological and pharmacological importance (Nigam et al., 2015).

In our experimental model of mercury-induced nephrotoxicity, Oat1 protein expression significantly increased in renal plasma membranes after $HgCl_2$ treatment in both male and female rats. However, the increase was higher in females than in males (2271% in females vs 290% in males). Oat3 protein expression was not modified following treatment with $HgCl_2$ in male rats. However, it was of relevance the important increase of 120% in renal Oat3 protein expression observed in $HgCl_2$-treated females as compared with control female rats.

An increase in the protein renal expression of Mrp2 was observed after the treatment with $HgCl_2$ in both sexes. Nevertheless, the percentage of increase of Mrp2 protein expression in $HgCl_2$-treated rats in relation to renal Mrp2 protein abundance in control rats was higher in females than in males (1283 % in females vs 233% in males). In this sense, the induced up-regulation of renal Mrp2 (both protein expression and excretory activity) by oxidative stress mechanisms was previously reported following exposure to xenobiotics (such as cisplatin, cadmium, arsenic, and rifampicin).

The abundance of β-actin was not modified with the $HgCl_2$ treatment. Actins are highly conserved proteins that are involved in cell motility, structure, integrity, and intercellular signalling. β-Actin is a major constituent of the contractile apparatus and one of the two nonmuscle cytoskeletal actins that are ubiquitously expressed. As it has been assumed comparable expression between different cellular samples, β-Actin has been worldwide used as internal standard allowing normalization of signals so that expression of proteins between different samples can be compared (Ferguson et al., 2005). In this work, β-Actin was employed as internal standard and its expression was not affected by the treatment in both sexes. Moreover, the protein levels of Oat3 were not modified in Hg-treated males. Dissimilar modifications in the abundance of the renal transporters observed in $HgCl_2$-treated rats is probably due to specific regulatory signaling pathways instead of the large plasma membrane integrity disruption in response to $HgCl_2$ treatment. The heterogeneous changes in

the abundance of Oat1, Oat3, Mrp2 and β-actin exhibited after $HgCl_2$ administration emphasize the selectivity of the response.

Oat1 is expressed in S2>S1=S3 segments of proximal tubules and Oat3 is expressed in S1=S2>>S3 segments (Ljubojevic et al., 2004; Lungkaphin et al., 2006). Mrp2 is localized in all segments of proximal tubules (Schaub et al., 1997). It has been proposed that Mrp2 secretes intracellular mercuric ions into the lumen in S1 segments and then these secreted ions are reabsorbed by S2 and S3 segments of the proximal tubule, leading to cellular injury and/or death in those segments (George et al., 2017; Zalups et al., 2014). Hence, since female rats had lesser renal expression of Oat1, Oat3, and Mrp2 than had male rats in physiological conditions (prior to $HgCl_2$ administration) (Cerrutti et al., 2002; Ljubojevic et al., 2004; Wang et al., 2012), a smaller amount of mercuric ions would enter and be secreted in the early portions of proximal tubule causing lesser cellular poisoning in the more distal segments of proximal tubule in females than in males. Therefore, at early times after $HgCl_2$ administration, the cellular tubular machinery would be less affected in female rats than in male rats and more effective adaptive mechanisms would be triggered in order to increase the renal detoxification through Oat1, Oat3, and Mrp2 in the plasma membranes of tubular cells. The latter would explain the largest increase in both Oat1 and Mrp2 abundances and the increase in Oat3 renal expression after mercury administration in females than in males, the consequently greater excretion of mercuric ions into the lumen and the lower tubular damage observed in females.

There is some previous evidence suggesting that Oat1, Oat3, and Mrp2 could work together as part of a coordinated network in the nephrotoxicity induced by $HgCl_2$. In this regard, Torres et al. (2011) have reported the important role of Oat1 in the $HgCl_2$-induced nephrotoxicity, since Oat1 knock-out mice are protected from $HgCl_2$-induced renal damage. In addition, it has been described that the renal expression of Oat1 and Oat3 is low in neonatal and increases to reach its higher expression in the adult animal (Nigam et al., 2015). In this connection, the sensitivity to $HgCl_2$ has been shown to increase along with the age in rats (Daston et al., 1983). Finally, the importance of Mrp2 expression in the early portions of proximal tubules in the mercuric chloride-induced renal damage has been recently described (George et al., 2017; Zalups et al., 2014). Hence, the present work, where a direct relationship has been established between Oat1, Oat3, and Mrp2 and the sex-related differences in the mercuric chloride-induced nephrotoxicity, contribute to strengthening the idea of Oat1, Oat3, and Mrp2 working together in the mechanisms that underlay $HgCl_2$-induced nephrotoxicity.

The liver and the kidneys are the main organs involved in the clearance of metabolites, drugs, and toxins. The $HgCl_2$ induced alterations in the expressions of mercury transporters, both in the liver and in the kidney, were gender-related. Those alterations could explain, at least in part, the sex-related differences in both nephro- and hepatotoxicity induced by $HgCl_2$, where females are more sensitive to liver injury and males are more sensitive to kidney injury, as described in this work and as previously reported (Hazelhoff et al., 2012; Hazelhoff and Torres, 2018). The gender-related differences in the expression of kidney and liver transporters after mercury exposure could be also part of an interorgan small communication network in response to the injury in order to help and coordinate the restoration of homeostasis, known as "*Remote Sensing and Signalling Hypothesis*" (Nigam, 2015; Nigam et al., 2015; Nigam, 2018). Overall, these results could open a novel and relevant sex-related angle on remote sensing to be studied.

The results of the present work also highlight the clinical significance of the up-regulation of the renal expression of Oat1, Oat3, and Mrp2 in patients intoxicated with mercury since those patients could have significant alterations in the pharmacokinetics of different drugs that are substrates of these transporters.

Nevertheless, one of the limitations of this work is that we study an acute model of nephrotoxicity induced by a high single dose of mercury, and a chronic exposure to lower amounts of mercury in humans is generally found. Upcoming studies should be aimed at the analysis of renal expression of Oat1, Oat3, and Mrp2 in a model of chronic exposure to mercury.

On the other hand, further research should be directed toward delineation of the molecular basis for transcriptional, translational and post-translational regulation of mercury transporters in the kidney in both female and male rats, in order to a better understanding of the mechanisms of cellular adaptation in response to the mercury-induced nephrotoxicity.

It is important to consider that gender-related differences in the tubular injury induced by mercury are a toxicological issue and are essential for evaluation of possible therapeutic actions by health professionals. In addition, at the present, there are few studies regarding gender differences in metabolism and toxic effects of mercury. Male animals have been almost exclusively employed in experimental toxicological studies making possible that sex-related differences in mechanisms of toxicity were not detected.

5. CONCLUSIONS

Mercury chloride increases renal expression of Oat1, Oat3, and Mrp2 and these mercury-induced alterations are markedly greater in females than in males. Consequently, female rats present a higher renal excretion of mercury and a lesser tubular damage than male rats (see graphical scheme in Figure 5).

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CONFLICTS OF INTEREST

None.

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FIGURE CAPTIONS

Figure 1. Histopathological Studies. (A) and **(C):** Representative micrographs of hematoxylin/eosin stained sections of Control Males (**CM**), Hg-treated Males (**Hg-M**), Control Females (**CF**), and Hg-treated Females (**Hg-F**) renal cortex and outer stripe of outer medulla (OSOM), respectively. Arrows: focal necrosis; curved arrow: cellular detachment and disrupted brush border membranes; arrowhead: vacuolated cells. These pictures are representatives of samples obtained from four different rats from each experimental group. Bars: 40 μm **(B)** and **(D):** Tubular injury score of renal cortex and renal outer stripe of outer medulla, respectively. The score varies from 0 for completely normal histology to 3 for maximal and widespread injury. Anova plus Newman Keuls test: $p < 0.05$. (a) *vs* CM, (b) *vs* Hg-M, (c) *vs* CF, (d) *vs* Hg-F. Tubular Alterations in the renal cortex and the outer stripe of the outer medulla (OSOM) (e.g. tubular dilatation/flattening, loss of brush border, vacuolar degeneration, desquamation, and acute tubular necrosis) were graded as follows: 0, less than 5 %; 1, 5-33 %; 2, 34-66 % and 3, over 66%.

Figure 2. Mercury content in urine. Urine excreted load of mercury in Hg-treated males (**Hg-M)** and Hg-treated females (**Hg-F)** rats. Results are expressed as mean values ± SEM from experiments carried out in four animals for each experimental group. Student´s t-test (*) $p < 0.05$.

Figure 3. Oat1 and Oat3 renal expression. Western blotting for (a) Oat1 and (b) Oat3 in plasma membranes (16 μg proteins/lane) from kidneys of Control Males (**CM**), Hg-treated Males (**Hg-M**), Control Females (**CF**) and Hg-treated Females (**Hg-F**). Proteins are separated by SDS-PAGE and blotted to nitrocellulose membranes. The results are expressed as percentages. The mean of CM levels was set as 100%. Results are expressed as mean values ± SEM from experiments carried out in four animals for each experimental group. Anova plus Newman Keuls test: $p < 0.05$. (a) vs CM, (b) vs Hg-M, (c) vs CF, (d) vs Hg-F. Student´s t-test :(*) $p < 0.05$ CM vs CF. Kaleidoscope Prestained Standards of molecular mass corresponding to bovine serum albumin (89.4 kDa) and to carbonic anhydrase (38.9 kDa) are indicated in the right of the figure.

Figure 4. Mrp2 renal expression. Western blotting for Mrp2 in plasma membranes (16 μg proteins/lane) from kidneys of Control Males (**CM**), Hg-treated Males (**Hg-M**), Control Females (**CF**), and Hg-treated Females (**Hg-F**). Proteins are separated by SDS-PAGE and blotted to nitrocellulose membranes. The results are expressed as percentages. The mean of

564 CM levels was set as 100%. Results are expressed as mean values ± SEM from experiments
565 carried out in four animals for each experimental group. Anova plus Newman Keuls test: p
566 <0.05. (a) vs CM, (b) vs Hg-M, (c) vs CF, (d) vs Hg-F. Student´s t-test: (*) p <0.05 CM vs
567 CF. Kaleidoscope Prestained Standards of molecular mass corresponding to myosin (206.4
568 kDa), β-Galactosidase (127.5 kDa), and to carbonic anhydrase (38.9 kDa) are indicated in the
569 right of the figure.

570 **Figure 5**. Scheme illustrating the mechanisms involved in the lesser renal tubular
571 damage induced by mercury in female rats as compared with male rats.