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**AMELIORATION OF MERCURY NEPHROTOXICITY AFTER  
PHARMACOLOGICAL MANIPULATION OF ORGANIC ANION TRANSPORTER 1  
(OAT1) AND MULTIDRUG ASSOCIATED RESISTANCE PROTEIN 2 (MRP2) WITH  
FUROSEMIDE**

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***Running Headline: Furosemide improves mercury toxicity***

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**ABSTRACT**

Inorganic mercury is a major environmental contaminant. The primary site of mercury-induced injury is the kidney due to the uptake of  $\text{Hg}^{(2+)}$ -conjugated organic anions in the proximal tubule, primary across the organic anion transporter 1 (Oat1) at the basolateral membrane. At the luminal side, mercuric ions are eliminated by the multidrug resistance-associated protein 2 (Mrp2). It was described that furosemide treatment induces up-regulation of Oat1 renal expression. As novel preventive and therapeutic strategies based in pharmacological manipulation of drug transporters are emerging, this study was designed to evaluate the impact of furosemide modulation of Oat1 on the nephrotoxicity induced by  $\text{HgCl}_2$ . Wistar rats were treated with furosemide (6 mg/100 g/ day, s.c.) during 4 days or with  $\text{HgCl}_2$  (4 mg/kg, i.p.) 18 h before the experiments or with furosemide during 4 days before the  $\text{HgCl}_2$  injection. Furosemide treatment improved  $\text{HgCl}_2$ -induced tubular injury as assessed by urinary alkaline phosphatase activity, urinary glucose, Oat5 urinary excretion and histopathological studies. Besides, administration of furosemide enhanced mercury urinary excretion, reduced mercury total renal accumulation and increased Mrp2 renal expression. In summary, furosemide improves  $\text{HgCl}_2$ -induced proximal tubule injury up-regulating mercury transporters and thus, increasing renal elimination of the mercuric ions. Hence, pharmacological manipulation of mercury transporters with furosemide might be a preventive strategy to reduce mercury toxicity.

**Keywords:** Oat1, Mrp2, acute kidney injury, mercuric chloride, furosemide

## 1.-Introduction

Mercury is a highly toxic metal that is widely distributed in the environment. Somehow, the entire population is exposed to some form of mercury primarily through drinking water, food or occupational exposure<sup>1,2</sup>. It has been reported that high levels of mercury exposure may occur via the use of skin lightening beauty creams, soaps and herbal drugs<sup>3,4</sup>. The toxic effects of mercury vary with the different chemical forms of mercury, the dose and the rate of exposure<sup>2,5</sup>. Due to the significant presence of mercury and its compounds in the environment, the challenge is to take advantage of their usefulness while reducing the impact of their adverse health effects. To achieve this, it is necessary to know and understand not only the molecular and cellular mechanisms by which mercury causes its toxicity, but also how these mechanisms are regulated.

Inorganic species of mercury are, by far, the more nephrotoxic. Inorganic mercury accumulates mainly in the kidneys causing acute kidney injury (AKI)<sup>6,7</sup>. The renal proximal tubule cells are the primary target site where highly reactive mercuric ions ( $\text{Hg}^{2+}$ ) rapidly accumulate and induce cell injury by binding and interacting with complexes of protein- and non-protein thiols<sup>7</sup>. At the basolateral plasma membrane of renal cells,  $\text{Hg}^{2+}$  gains access from the peritubular blood into the intracellular compartment primarily across the organic anion transporter 1 (Oat1)<sup>8</sup>. Oat1 mediates the transport of many compounds including endogenous substances and exogenous substances such as various anionic drugs (as diuretics, antiviral agents and  $\beta$ -lactamic antibiotics) and environmental compounds<sup>9</sup>. Bridges et al.<sup>10</sup> have described that multidrug resistance-associated protein 2 (Mrp2) plays an important role in the renal cellular elimination and secretion of mercuric ions in rats.

Torres et al.<sup>11</sup> have demonstrated, using Oat1 knock-out mice, that  $\text{HgCl}_2$  induced AKI is mediated mainly by Oat1. In addition, since female rats express lower levels of renal cortical Oat1 than males<sup>12-14</sup>, Hazelhoff et al.<sup>15</sup> have recently observed that mercury-induced renal damage is reduced in female rats as compared with males. Currently, pharmacological modulation of drug transporters are emerging as preventive and therapeutic strategies<sup>16,17</sup>. Based on these, Oat1 down-regulation could be a therapeutic strategy to reduce  $\text{HgCl}_2$ -induced nephropathy. Nevertheless, the down-regulation of Oat1 would involve a decrease in renal elimination of mercuric ions causing their accumulation in the organism and the metal toxicity increase in other tissues. One alternative could be the up-regulation of Oat1 to increase renal

uptake of mercuric ions and hence, their urinary elimination. In this regard, a rise in Oat1 protein abundance has been described in rat kidney following furosemide treatment, suggesting that Oat1 may be up-regulated in vivo by substrate stimulation<sup>18,19</sup>. Therefore, the present study was designed to evaluate the impact of furosemide modulation of Oat1 on the nephrotoxicity induced by HgCl<sub>2</sub>.

## 2.-Results and Discussion

As shown in Figure 1, Oat1 abundance in renal plasma membranes increased after treatment with furosemide during 4 days as previously described<sup>18</sup>.

As a consequence of the diuretic action of furosemide, the urinary volume was enhanced in rats from FS group when compared with rats from C group ( $7.5 \pm 0.6 \mu\text{L}/\text{min}/100 \text{ g}$  vs  $2.6 \pm 0.4 \mu\text{L}/\text{min}/100 \text{ g}$ ,  $P < 0.001$ ). Moreover, HgCl<sub>2</sub> induced a decrease in urinary volume as previously described<sup>15</sup> ( $1.3 \pm 0.3 \mu\text{L}/\text{min}/100 \text{ g}$ ,  $P < 0.05$  vs C group) and the pretreatment with furosemide was able to preserve urinary volume within control values ( $3.5 \pm 0.7 \mu\text{L}/\text{min}/100 \text{ g}$ ).

In Hg rats, plasma urea, creatinine levels and proteinuria were markedly increased with respect to control rats (Figure 2). Moreover, creatinine clearance was lower in HgCl<sub>2</sub>-treated rats than in control rats, indicating a reduction in the glomerular filtration rate. These results corroborate the glomerular damage induced by the dose of HgCl<sub>2</sub> employed. The pretreatment with furosemide was not able to reverse HgCl<sub>2</sub>-induced glomerular damage.

Traditional parameters of tubular injury as urinary alkaline phosphatase activity and glucose urine concentration were measured and related to urinary creatinine levels. As shown in Figure 3A and Figure 3B, the alkaline phosphatase activity and the urinary glucose were only significantly increased in Hg rats. Pretreatment with furosemide returned these tubular parameters to control values.

The concentrations of urinary parameters were related to urinary concentration of creatinine in order to correct variations in urine production as previously described<sup>15,20-24</sup>. Individual measurements of urinary parameters are insufficient because normal physiological variations in urinary water excretion can dilute or concentrate urinary proteins, enzymes and transporters. Creatinine is excreted in urine at relatively constant rates; therefore it can be used to normalize urinary excretion of a particular parameter. Although creatinine had been described as a substrate for a number of transporters in the solute carrier superfamily expressed in renal proximal tubules,

the predominant pathway mediating creatinine secretion have been recently elucidated<sup>25,26</sup>. Vallon et al.<sup>25</sup> found that mouse Oat1 and mouse organic anion transporter 3 (Oat3) can transport creatinine in vitro, but with a relatively low affinity, and that Oat3-knockout mice had blunted creatinine secretion in vivo, postulating a contribution of Oat3 to the renal secretion of creatinine in mice. Lepist et al.<sup>26</sup> have recently demonstrated that organic anion transporter 2 (Oat2), organic cation transporter 2 (Oct2) and organic cation transporter 3 (Oct3) transport creatinine and that at physiologic creatinine concentrations, the specific activity of Oat2 transport was over twofold higher than Oct2 or Oct3, establishing Oat2 as a likely relevant creatinine transporter. Lepist et al.<sup>26</sup> did not observed Oat3 and Oat1 dependent transport of creatinine. Considering the results previously described, the increase in Oat1 protein expression induced by furosemide, should not alter creatinine renal handling. In this connection, we observed that there was no statistically difference between excreted load of creatinine (ug/min/100 g b.w.) in Control (C) and in furosemide treated (FS) rats ( $3.0 \pm 0.1$  vs  $3.3 \pm 0.2$ , respectively), indicating that the up-regulation of Oat1 protein expression induced by furosemide does not modify urinary creatinine excretion and that creatinine can be used as an appropriate marker for normalization of urinary parameters also in our experimental groups.

We have been pioneers in detecting the organic anion transporter 5 (Oat5) in urine and we have recently postulated that the urinary excretion of Oat5 is an early biomarker of proximal tubular damage in several models of renal and extra-renal injury<sup>20-24</sup>. Oat5 is located in the apical membranes of proximal tubule S3 segment where it functions as a dicarboxylate/organic anion transporter and is excreted in urine by the exosomal pathway<sup>24</sup>. Following treatment with HgCl<sub>2</sub>, an increase of urinary Oat5 abundance was observed in Hg and FS-Hg rats (Figure 3C), but this increase was significantly attenuated by pretreatment with furosemide.

Histopathological studies (Figure 4A) revealed that kidneys from animals treated with a single dose of HgCl<sub>2</sub> presented vacuolated cells, cellular detachment, disrupted brush border membranes, focal tubular dilatation and necrosis as previously described<sup>15,22,27-29</sup>. The microscopic changes in renal tubules from rats pretreated with furosemide were less significant than those observed in rats that received only the nephrotoxic dose of HgCl<sub>2</sub> as indicated by cortical tubular injury scores obtained (0.56 time below Hg rats,  $P < 0.001$ ) (Figure 4B).

The pretreatment with furosemide significantly reduced the renal accumulation of mercury after HgCl<sub>2</sub> administration (Figure 5A), and the excretion of mercury in urine was markedly greater in FS-Hg rats than in Hg rats (Figure 5B).

After observing these results, and considering that Mrp2 plays an important role in the renal cellular elimination and secretion of mercuric ions in rats<sup>10</sup>, we also decided to evaluate Mrp2 protein expression in renal plasma membranes following the 4 days of furosemide treatment. As shown in Figure 6, Mrp2 renal abundance was increased in FS 4d rats.

Eventhough mercury has been recognized as a hazardous pollutant, various forms of this metal continue to be added to the pool of environmental mercury. Primary anthropogenic sources of mercury have been: fossil fuels combustion, cement production, industrial processes, incineration of chemical or medical wastes and mining operations<sup>1</sup>. The major effect of exposure to inorganic mercury compounds is renal damage, because kidneys are the primary targets of mercuric ions accumulation. More specifically, mercuric ions accumulate almost exclusively along the S1, S2 and S3 segments of the proximal tubule<sup>7,30,31</sup>. To our knowledge there are no specific preventive and therapeutic strategies for the treatment of mercury nephrotoxicity.

The renal proximal tubules have a wide variety of transporters with overlapping substrate specificities that cooperate in basolateral uptake and luminal excretion. Frequently, unexpected changes in plasma metabolite levels and/or nephrotoxicity are consequences of clinically significant interactions that involve these multiple carriers<sup>30,31,32,33</sup>. At present, novel preventive and therapeutic strategies based in pharmacological manipulation of drug transporters are emerging<sup>16,17</sup>.

In rats, the administration of HgCl<sub>2</sub> is an established model of nephrotoxicity where it dose-dependently affects the epithelial cells lining the pars recta (S3 segment) of the proximal tubules<sup>7,30,31</sup>. The uptake of mercuric ions by these cells is primarily across Oat1 in the basolateral plasma membrane and via amino acid transporters in the luminal plasma membrane<sup>7,8</sup>. Oat1 is involved in the transport of organic anions of pharmacological and physiological significance at the basolateral membrane of renal tubular cells. Oat1 supports organic anion/ $\alpha$ -ketoglutarate exchange<sup>34,35</sup>. Several recent studies have demonstrated that Mrp2 plays an important role in the cellular elimination and secretion of certain mercuric species<sup>10,31,36</sup>. Mrp2 is localized exclusively in the apical plasma membrane of proximal tubule epithelial cells. Mrp2 is not present in renal exosomes<sup>37</sup> and it is involved in the organic anions transport of

a wide variety of potentially toxic endo- and xenobiotics in the form of amphiphilic anionic conjugates<sup>38</sup>.

We have recently demonstrated that most of the renal injury induced by HgCl<sub>2</sub> was abolished following HgCl<sub>2</sub> treatment of Oat1 knock-out mice<sup>11</sup>. Thus, HgCl<sub>2</sub> induced AKI was found to be mediated mainly by Oat1. Our assumption was that reducing the renal tissue levels of mercury by pharmacological manipulation of Oat1 expression could be a beneficial and specific preventive modality for mercury nephrotoxicity that would alleviate the renal damage. In this sense, it has been described that repeated administration of furosemide causes increase in Oat1 protein abundance in rat kidney<sup>18,19</sup>. The authors proposed that furosemide up-regulates Oat1 protein expression by *in vivo* substrate stimulation<sup>18,19</sup>. The diuretic administration may have a direct stimulatory effect on Oat1 protein synthesis since sucrose water loading, which also increases urine volume, failed to increase Oat1 protein abundance. Besides, furosemide selectively increased Oat1 protein but not Na-K-ATPase protein, suggesting that furosemide may have a direct stimulatory effect on Oat1 protein synthesis<sup>18,19</sup>.

In the present study, we corroborated that following treatment with furosemide, the Oat1 protein expression significantly increased in renal plasma membranes.

In our experimental model of HgCl<sub>2</sub>-induced nephrotoxicity, renal damage was assessed in part by both urea and creatinine plasma levels, creatinine clearance and urinary proteins<sup>39</sup>. As part of HgCl<sub>2</sub>-induced nephropathy, a decrease in glomerular filtration rate is observed probably by vasoconstriction of the afferent and /or efferent arterioles. However, the exact mechanisms are unknown, they are likely complex and involve a number of factors<sup>40</sup>. In this regard, the concentration of both creatinine and urea in plasma increases due to the significant decrease in the glomerular filtration rate. Therefore, the assay of plasma creatinine and plasma urea may be used as indicators of mercury-induced impaired glomerular function<sup>7,41</sup>. After treatment with 4 mg/kg b.w., i.p of HgCl<sub>2</sub> a significant decrease in the glomerular filtration rate and high values of creatinine in plasma, urea in plasma and urinary proteins were observed. The pretreatment with furosemide (6 mg/100 g b.w./day, s.c, 4 days) did not improve the glomerular damage induced by the dose of HgCl<sub>2</sub>.

When tubular injury is induced by mercury, cells along the proximal tubule undergo several degenerative modifications and lose some of their brush border membrane. In addition, the

capacity for the reabsorption of filtered plasma solutes and water is largely reduced after several proximal tubules have become functionally compromised by the toxic effects of mercury<sup>1,38-40</sup>.

In this study, different parameters were used as markers of renal proximal tubules injury, as urinary alkaline phosphatase activity, urinary glucose concentration and Oat5 urinary excretion. Following treatment with the nephrotoxic dose of HgCl<sub>2</sub>, impairment in the function of renal proximal tubules was observed, as previously described<sup>7,15,23,37,40</sup>. Treatment with furosemide before the administration of the nephrotoxic dose of HgCl<sub>2</sub> was able to improve proximal tubules damage as assessed by alkaline phosphatase activity in urine, urinary glucose and urinary excretion of Oat5 and histopathological studies.

In animals pretreated with furosemide, the total mercury accumulation in kidney (µg/g tissue) was lower and the urinary excretion of mercury was higher, respectively, as compared with animals that received only the dose of HgCl<sub>2</sub>. Because of the important role of Mrp2 in the transport of mercuric ions and the greater urinary excretion of mercury observed in rats pretreated with furosemide, we decided to evaluate Mrp2 protein expression. We demonstrated that treatment with furosemide induces Mrp2 renal expression.

The unchanged drug is the main pathway of renal elimination of furosemide but its metabolic clearance via glucuronidation is also very significant in the kidney<sup>40-42-44</sup>. It has been suggested that furosemide is actively transported by Mrp2<sup>42,44</sup>. Accordingly, we propose that the potential increase mechanism in Mrp2 protein expression by furosemide is via direct substrate stimulation, similar to the proposed mechanism by Kim et al.<sup>18</sup> for the furosemide-induced up-regulation of Oat1 protein. In addition, and to our knowledge, this is the first report regarding *in vivo* modulation of Mrp2 renal expression by furosemide administration.

The results from this work indicate that in rats pretreated with furosemide, the enhanced Oat1 expression increases the uptake of mercuric ions into the tubular cells, whereas the highest expression of Mrp2 increases excretion of mercuric ions into the tubular lumen, thus decreasing total mercury accumulation in kidney, and hence its renal tubular toxicity. The protection afforded by furosemide is due to the increase in the tubule secretion of mercury, due to the up-regulation of Oat1 and Mrp2 protein expression. In this connection, it has been described that approximately 95-99 % of the mercury in plasma is bound to proteins and consequently its glomerular filtration is very low. So, the urinary mercury represents a pool of mercury that has been secreted from the blood into the tubular lumen by a transepithelial mechanism<sup>7,8</sup>. On the

other hand, furosemide was not able to prevent the decrease in the glomerular filtration rate observed in HgCl<sub>2</sub> treated rats as shown through the evaluation of creatinine clearance (Figure 2C), so it is not expect any change in the low mercury glomerular filtration between both experimental groups.

Since pathological changes induced by mercury would be hard to revert, the acute mercury renal toxicity model is useful to study possible strategies aimed at preventing the onset of mercury toxicity. This study is a pioneering report to try to prevent mercury nephrotoxicity. Therefore, further studies, are warranted in the future to explore several uncertain and unresolved issues, for instance, drugs that up-regulate renal Oat1 and Mrp2 much more potently than furosemide could become a new clinical tools to prevent nephrotoxic AKI induced by mercury. Additionally, the dose of furosemide used in this study is equivalent to a dose of 9.8 mg/kg b.w./day<sup>45</sup> in humans, that is included in the furosemide dosage range (0.3-114 mg/kg b.w./day) currently employed in humans<sup>46,47</sup>. So, the results in the present work also remark the clinical relevance of considering the up-regulation of Oat1 and Mrp2 in patients receiving chronic administration of furosemide when other therapeutic drugs, that are substrates of these transporters, are concomitantly administered.

### **3.-Experimental**

#### **3.1.-Experimental Animals**

Adult male Wistar rats (aged 110–130 days) were used throughout the study. All animals were allowed free access to a standard laboratory chow and housed at constant temperature and humidity with regular light cycles (12 h) during the experiment. All experiments were conducted according to National Institutes of Health (NIH), Guide for the Care and Use of Laboratory. All experimental procedures were approved by the Faculty of Biochemical and Pharmaceutical Sciences (UNR) Institutional Animal Care and Use Committee.

#### **3.2.-Experimental Protocols**

Treatments: Animals were treated with furosemide (6 mg/100 g b.w./ day, s.c.) as previously described by Kim et al.<sup>18</sup> for four consecutive days and/or with a single injection of HgCl<sub>2</sub> (4

mg/kg b.w., i.p.) on the 4<sup>th</sup> day. Experiments were performed after 18 h of HgCl<sub>2</sub> injection as previously described<sup>7,11,15,27</sup>.

Experimental groups: The animals were randomly divided into four experimental groups of four animals each. Control animals (*Control group, C*), rats treated with furosemide (*furosemide group, FS*), rats receiving HgCl<sub>2</sub> (*HgCl<sub>2</sub> group, Hg*), and animals treated with furosemide and with HgCl<sub>2</sub> (*furosemide + HgCl<sub>2</sub> group, FS-Hg*).

C and Hg groups also received the furosemide vehicle (600 µL/100 g b.w, s.c.) for four consecutive days. C and FS groups also received HgCl<sub>2</sub> vehicle (100 µL saline/ 100 g b.w.), 18 h before the experiments.

Rats were provided with two separate bottles of drinking water, one containing 0.8% NaCl and 0.1 % KCl, and the other containing tap water. All the animals were placed in metabolic cages in order to collect the urine 18 h before the experiments. Urinary volume was determined by gravimetry.

Different sets of experimental animals were used for: biochemical determinations, histopathological studies and preparation of plasma membranes from kidneys for Western blotting studies.

The day of the experiment, all the animals were anesthetized with sodium thiopental (70 mg/kg b.w., i.p.). The collection and processing of renal tissue samples was different depending on the type of study performed.

Besides, two different experimental groups were used for the evaluation of Oat1 and Mrp2 protein expression in total renal plasma membranes on the 4<sup>th</sup> day (~~previously~~ prior to the administration of HgCl<sub>2</sub>) by immunoblotting technique: *control* (control 4d, n=4): rats that received furosemide vehicle (600 µL saline/100 g b.w./day, s.c) for 4 days; *furosemide* (FS 4d, n=4): rats that received furosemide (6 mg/100 g b.w. /day, s.c.) during 4 days. On the 4<sup>th</sup> day, these animals were anesthetized and the renal tissue samples were collected and processed.

### 3.3.-Biochemical Determinations

On the day of the experiments, blood samples were obtained by cardiac puncture and blood plasma was separated by centrifugation (1,000 x g for 10 min). Urine samples were centrifuged at 1,000 x g for 10 min to remove cell debris. Plasma samples were used to measure urea and creatinine levels [Cr]p. The urine samples were used for analyses of alkaline phosphatase

activity, creatinine [Cr]<sub>u</sub>, protein and glucose concentrations and Organic anion transporter 5 (Oat5) abundance. Plasma urea and creatinine levels, as well as urine creatinine, protein and glucose concentrations and alkaline phosphatase activity were determined employing commercial kits (Wiener Laboratory, Rosario, Argentina). Creatinine clearance was calculated employing the following formula:  $[\text{Cr}]_u \times \text{Urine Volume} / [\text{Cr}]_p$ . Urine Volume is expressed in mL/min/100 g b.w. Total mercury determination in kidney and urine samples was performed by cold vapor atomic absorption as previously described by Trebucovich et al.<sup>25,27</sup>.

### 3.4.-Preparation of Total Plasma Membranes from Kidneys

The preparation of total plasma membranes obtained from entire kidneys of each experimental group were performed by differential centrifugation according to the method described by Jensen and Berndt<sup>48</sup>, with the modifications previously reported by our laboratory<sup>12,15</sup>.

Aliquots of the membranes were stored immediately at  $-80\text{ }^{\circ}\text{C}$  for 2 weeks. Each preparation represented renal tissues from four animals.

### 3.5.-Electrophoresis and Immunoblotting

The electrophoresis and immunoblotting studies were performed as previously described by Hazelhoff et al.<sup>15</sup>. Total plasma membranes (18  $\mu\text{g}$  of protein/lane) and urine (10 $\mu\text{L}$ /lane) samples were boiled for 3 min in the presence of 1% 2-mercaptoethanol/ 2% sodium dodecyl sulphate (SDS). Proteins were separated through 8.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then electroblotted to a pure nitrocellulose membrane (NC membrane) (Trans-Blot<sup>®</sup> Transfer Medium, Bio Rad Laboratories, Hercules, CA, USA). To verify equal protein loading and transfer between lanes, Ponceau Red and antibody against human  $\beta$ -actin ~~was~~ were used as previously reported<sup>15,20,21,25,27</sup>. The membranes were incubated overnight at  $4\text{ }^{\circ}\text{C}$  with a commercial rabbit polyclonal antibody against rat Oat1 or a rabbit polyclonal antibody against rat Oat5 as previously described<sup>15,20</sup>, or a commercial mouse polyclonal antibody against rat Mrp2 or a commercial mouse monoclonal antibody against human  $\beta$ -actin. Blots were processed for detection using a commercial kit (ECL Plus Western Blotting Detection Reagents; Amersham, Buckinghamshire, UK). Kaleidoscope Prestained Standards of molecular mass were employed (Bio Rad Laboratories, Hercules, CA, USA). A densitometric quantification of the

Western blotting signal intensity of membranes was performed using the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA) software. The abundances of Oat1 and Mrp2 were normalized to  $\beta$ -actin and considered as percentage of the mean control value for each gel.

### **3.6.-Histopathological Studies**

Histopathology of kidneys was performed with hematoxylin-eosin as previously described<sup>22</sup>, after perfusing the kidneys 0.01 M NaIO<sub>4</sub>, 0.075 M lysine, 0.0375 M phosphate buffer, with 2 % paraformaldehyde, pH 6.20. Ten cortical high-power fields ( $\times 400$ ) were examined at random by a blinded observer. The tubular injury (e.g. tubular dilatation/flattening, tubular degeneration/vacuolization and acute tubular necrosis) was evaluated in hematoxylin-eosin sections. Alterations in affected tubules were graded as follows: 0, less than 5%; 1, 5–33%; 2, 34–66% and 3, over 66% as previously described<sup>49,50</sup>. Images were taken with an Olympus Coolpix-micro digital camera fitted on a CX-35 microscope (Olympus, Japan).

### **3.7.-Materials**

Chemicals were purchased from Sigma (St. Louis, MO, USA), analytical grade pure. The polyclonal antibody against Oat1 and the monoclonal antibody against human  $\beta$ -actin were purchased from Alpha Diagnostic International (San Antonio, TX, USA) and the polyclonal antibody against Mrp2 from Abcam (Cambridge, MA, USA). The polyclonal antibody against Oat5 was kindly supplied by Prof. N. Anzai (Department of Pharmacology and Toxicology, Dokkyo Medical University School of Medicine, Japan). The Kaleidoscope Prestained Standards of molecular mass were purchased from Bio Rad Laboratories (Hercules, CA, USA).

### **3.8.-Statistical Analysis**

Statistical differences between groups were evaluated using the unpaired Student's *t*-test or multiple comparisons with one way ANOVA followed by the Newman-Keuls test. For densitometry of immunoblots, samples from kidneys of FS, Hg and FS-Hg rats were run on each gel with corresponding control kidneys. Besides, renal membranes from FS 4d were run on each gel with the corresponding control 4d ones.

#### 4.-Conclusion

The up-regulation of the renal expression of Oat1 and Mrp2 by repeated administration of furosemide improves the proximal tubule damage induced by HgCl<sub>2</sub>. These results provide new evidence that achieving pharmacological manipulation of mercury transporters may be a preventive strategy to reduce its toxicity. Because furosemide has been used for a long time with a high safety and tolerability profile, induction of Oat1 and Mrp2 in the kidney in patients with nephrotoxicity caused by mercury may be a safe and new preventive tool to excrete mercury and to reduce renal tubule injury.

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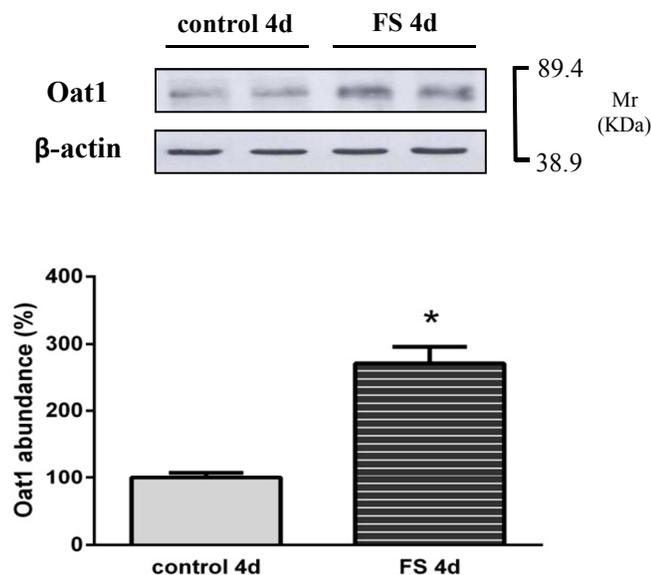
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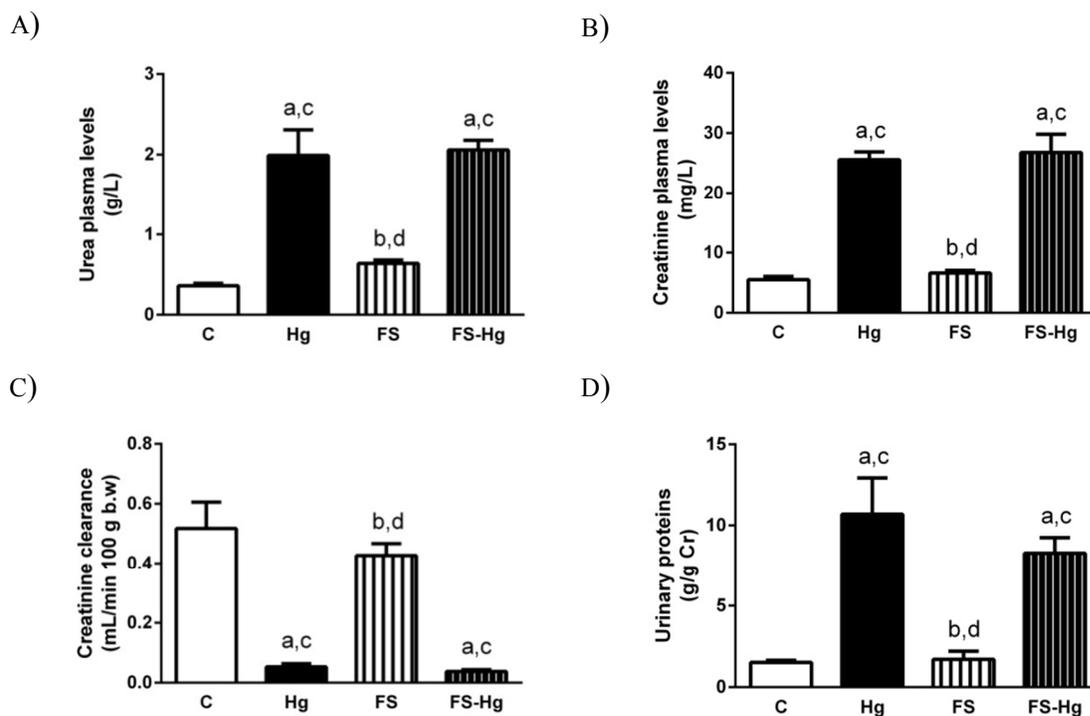
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## Figures

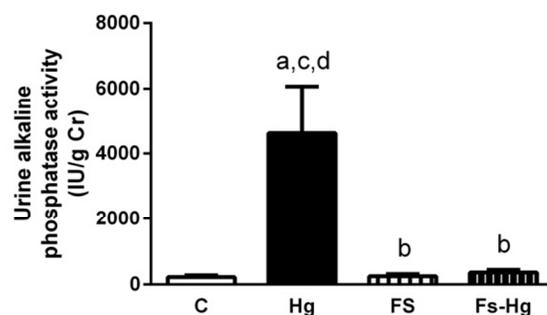


**Figure 1.** Western blotting for Oat1 in plasma membranes (18  $\mu\text{g}$  proteins) from kidneys of control 4d and FS 4d rats. The anti-Oat1 antibody labeled a specific protein band of 70-75 KDa. Proteins are separated by SDS-PAGE and blotted to nitrocellulose membranes. The results are expressed as percentages. The mean of control 4d levels were set as 100%. Results are expressed as mean values  $\pm$  SEM from experiments carried out in four different preparations for each experimental group. (\*)  $P < 0.05$ . Kaleidoscope Prestained Standards of molecular mass corresponding to bovine serum albumin (89.4 kDa) and to carbonic anhydrase (38.9 kDa) are indicated on the right of the figure.

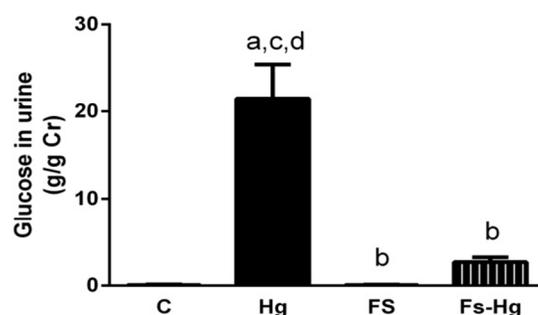


**Figure 2.** Urea (A) and creatinine (B) plasma levels, creatinine clearance (C) and urinary proteins (D) in C, Hg, FS and FS-Hg rats. Results are expressed as mean values  $\pm$  SEM from experiments carried out in four different preparations for each experimental group.  $P < 0.05$ . (a) versus C, (b) versus Hg, (c) versus FS, (d) versus FS-Hg.

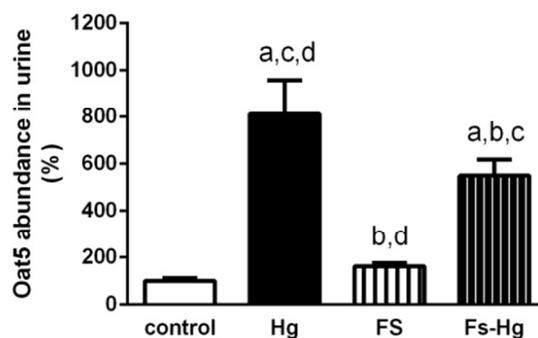
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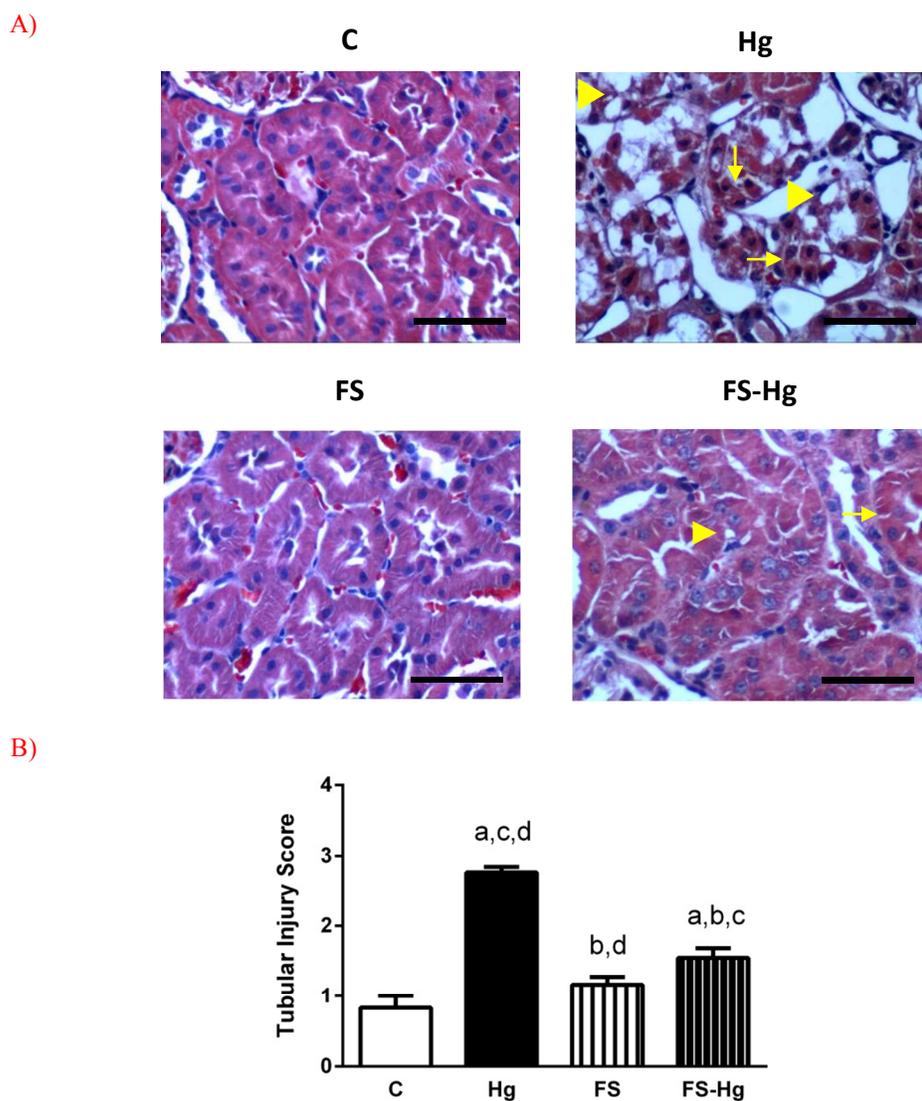
B)



C)

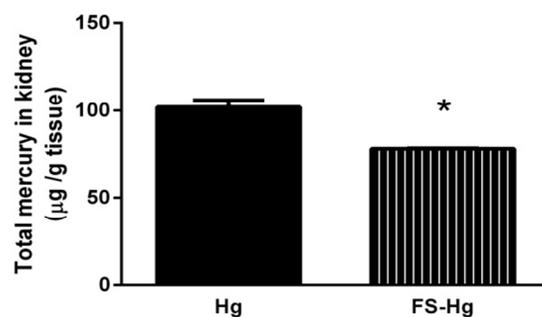


**Figure 3.** Alkaline phosphatase urinary activity (A), urinary glucose (B) and Oat5 abundance in urine (C) in C, Hg, FS and FS-Hg rats. Results are expressed as mean values  $\pm$  SEM from experiments carried out in four different preparations for each experimental group. In figure 3C, the results are expressed as percentages and the mean of C levels was set as 100%.  $P < 0.05$ . (a) versus C, (b) versus Hg, (c) versus FS, (d) versus FS-Hg.

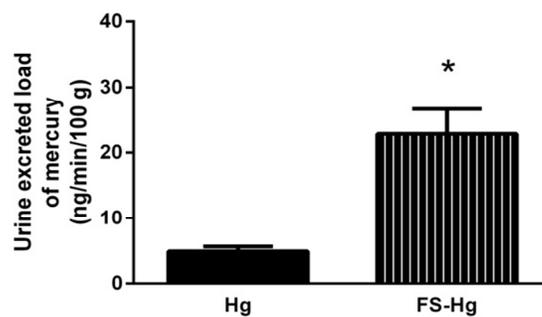


**Figure 4.** A) Representative micrographs of hematoxylin/eosin-stained sections of C, Hg, FS and FS-Hg rat kidneys. Photos are representative of samples obtained from four rats from each experimental group. In group Hg: vacuolated cells (arrow head) cellular detachment and disrupted brush border membranes were observed (arrows). Group FS-Hg showed lesser microscopic damage than group Hg. Bars: 40  $\mu$ m. B) Tubular injury score; the scores ranges from 0 for completely normal histology to 3 for maximal and widespread injury.  $P < 0.001$ . (a) versus C, (b) versus Hg, (c) versus FS, (d) versus FS-Hg.

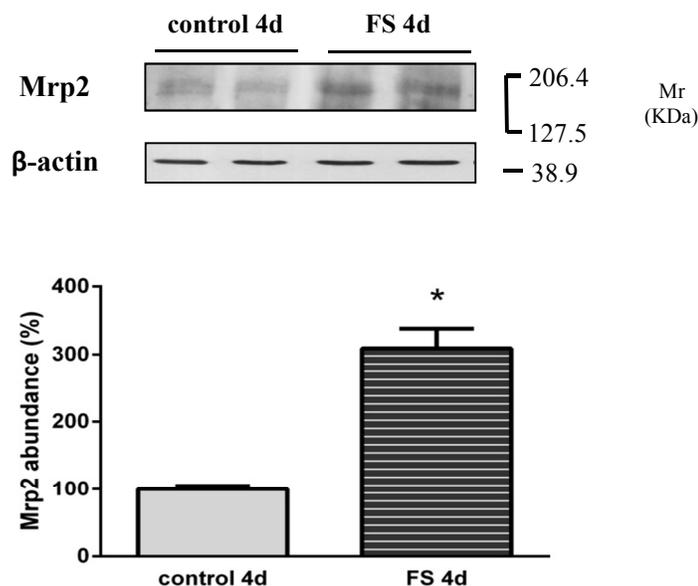
A)



B)



**Figure 5.** Total mercury in kidney (A) and urine excreted load of mercury (B) in Hg and FS-Hg rats. Results are expressed as mean values  $\pm$  SEM from experiments carried out in four different preparations for each experimental group. (\*)  $P < 0.05$ .



**Figure 6.** Western blotting for Mrp2 in plasma membranes (18  $\mu$ g proteins) from kidneys of control 4d and FS 4d rats. The anti-Mrp2 antibody recognized a specific protein band of 175 KDa. Proteins are separated by SDS-PAGE and blotted to nitrocellulose membranes. The results are expressed as percentages. The mean of control 4d levels ~~were~~ was set as 100%. Results are expressed as mean values  $\pm$  SEM from experiments carried out in four different preparations for each experimental group. (\*)  $P < 0.05$ . Kaleidoscope Prestained Standards of molecular mass corresponding to myosin (206.4 kDa),  $\beta$ -Galactosidase (127.5 kDa) and to carbonic anhydrase (38.9 kDa) are indicated on the right of the figure.

Furosemide improves  $\text{HgCl}_2$ -induced tubule injury up-regulating Oat1 and Mrp2 and thus, increasing renal elimination of mercuric ions.

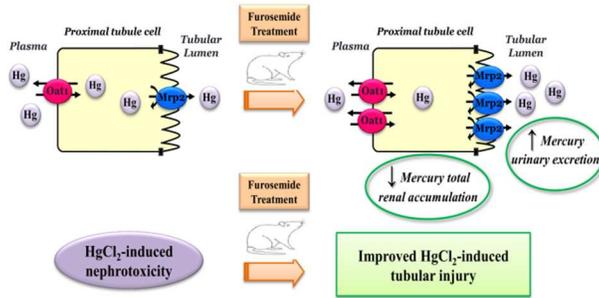


Figure 1

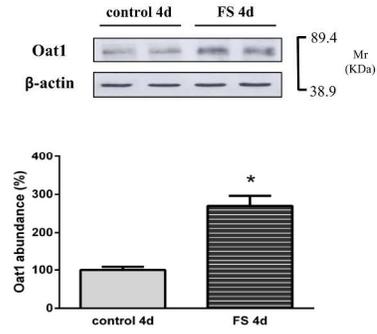


Figure 1  
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Figure 2

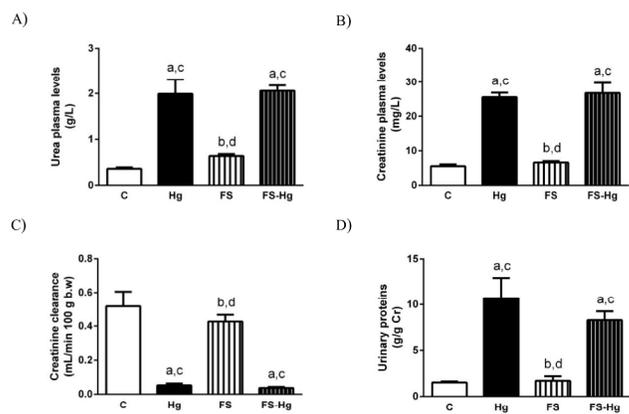
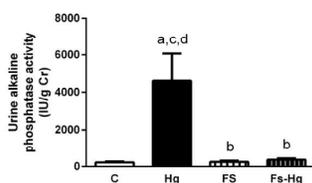


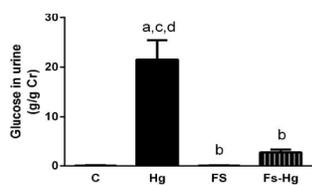
Figure 2  
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Figure 3

A)



B)



C)

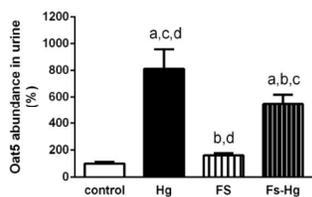


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Figure 4

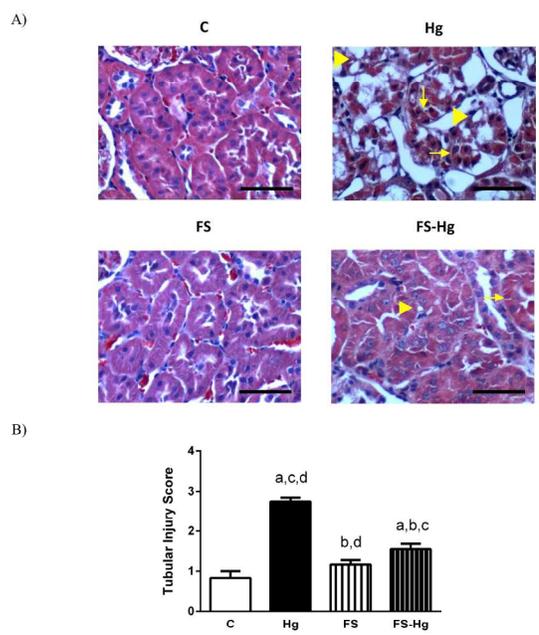
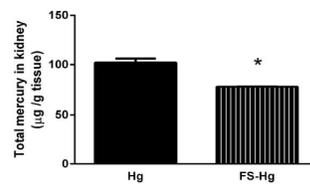


Figure 4  
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Figure 5

A)



B)

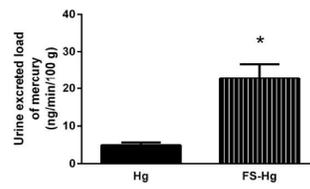


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Figure 6

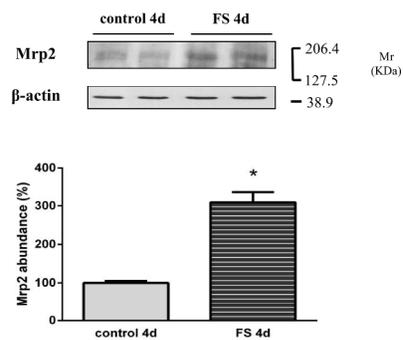


Figure 6  
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