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Octylguanidine ameliorates the damaging effect of mercury on renal functions

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Mercurials are known to induce morphological and functional modifications in kidney. The protective effect of octylguanidine on the injury induced by Hg^{2+} on renal functions was studied. Octylguanidine administered at a dose of 10 mg/kg body weight prevented the damage induced by Hg^{2+} administration at a dose of 3 mg/kg body weight. The findings indicate that octylguanidine spared mitochondria from Hg^{2+}-poisoning by preserving their ability to retain matrix content, such as accumulated Ca^{2+} and pyridine nucleotides. The hydrophobic amine also protected mitochondria from the Hg^{2+}-induced loss of the transmembrane potential, and from the oxidative injury of mitochondrial DNA. In addition, octylguanidine maintained renal functions, such as normal values of creatinine clearance and blood urea nitrogen (BUN), and serum creatinine after Hg^{2+} administration. It is proposed that octylguanidine protects kidney by inhibiting Hg^{2+} uptake to kidney tissue, and in consequence its binding to mitochondrial membrane through a screening phenomenon, in addition to its known action as inhibitor of permeability transition.

Keywords: kidney/mercury/mitochondria/octylguanidine/oxidative stress/renal functions.

Abbreviations: BUN, blood urea nitrogen; CSA, cyclosporin A; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; OG, octylguanidine.

Mercury accumulation in kidney tissue induces severe renal failure, among other functional disorders. Functional disorders in renal failure include diminished renal creatinine clearance, increased blood urea nitrogen (BUN) and serum creatinine (*1*–*3*). At the cellular level, Hg^{2+}-induced nephrotoxicity comprises massive Ca^{2+} accumulation, morphological changes, i.e. bleb formation and swelling of the endoplasmic reticulum and mitochondria (*4*). Furthermore, binding of Hg^{2+} to mitochondrial membrane causes loss of oxidative phosphorylation and an increased permeability to Ca^{2+} (*4*–*7*). It is known that Ca^{2+} plays a primary role in amplifying the process of mitochondrial injury induced by Hg^{2+}, i.e. Ca^{2+} overload switches selective membrane permeability from specific to non-specific (*8*, *9*). The so-called permeability transition is characterized by the opening of a non-specific transmembrane pore with a diameter of ~3 nm (*10*). Such a pore opening brings about the collapse of the electric transmembrane gradient and the release of ions and metabolites contained within the matrix (*11*, *12*). In an earlier work, we demonstrated that captopril, an inhibitor of the angiotensin converting enzyme, preserves mitochondria from the deleterious effect of Hg^{2+} on membrane permeability, oxidative phosphorylation and transmembrane potential (*13*). Therapy for mercury poisoning involves the use of a wide variety of drugs: D-penicillamine (*14*), chemicals such as EGTA and DTT (*4*), and microspheres for chelating Hg^{2+} (*15*). In *in vitro* experiments, we demonstrated that the immunosuppressor cyclosporin A (CSA), at a concentration of 0.5 μM, protects kidney mitochondrial functions, such as selective permeability and oxidative phosphorylation, from mercury-induced damage (*16*). We ascribed the protective effect of CSA to its well known property to promote closure of the non-specific transmembrane pore (*17*, *18*). Regarding the latter, in a previous work, we reported that the hydrophobic amine octylguanidine (OG) inhibits permeability transition in kidney mitochondria (*19*). Moreover, OG protects rat heart from the oxidative stress after ischaemia/reperfusion (*20*). Considering these antecedents, in this work we explore whether OG could protect the kidney from the detrimental effect of mercury on renal functions. The results revealed that OG administered *in vivo* prevents the deleterious effects of the xenobiotic agent on mitochondrial Ca^{2+} accumulation, the transmembrane electric gradient, and on oxidative stress. In addition, OG administration preserves renal functions by avoiding the increase in serum creatinine, BUN, as well as by maintaining renal creatinine clearance within control values. Furthermore, OG prevents the Hg^{2+}-mediated morphological changes produced in kidney tissue.

Materials and Methods

$HgCl_2$ was injected intraperitonealy (i.p.) at a dose of 3 mg/kg body weight, followed immediately by an injection of octylguanidine (10 mg/kg body weight). Toxic effects were investigated 17 h after

mercury administration. Kidney mitochondria from treated and non-treated Wistar rats, weighing ~300g, were prepared in 0.25 M sucrose and 1 mM EDTA, adjusted with Tris base to pH 7.3, following the standard centrifugation pattern. The last washing was carried out in EDTA-free sucrose medium. Protein was determined by the method of Lowry *et al*. (*21*). Mitochondrial Ca^{2+} movements were followed spectrophotometrically at 675–685 nm, using the metallochromic indicator Arsenazo III. Changes in the transmembrane electric gradient were analysed spectrophotometrically at 525–575 nm using the dye safranine. Aconitase activity was measured according to Hausladen and Fridovich (*22*). Briefly, mitochondrial protein was solubilized by adding 0.05% Triton X-100 containing 25 mM phosphate, pH 7.2, followed by the addition of 0.6 mM magnesium sulphate, 1 mM citrate and 0.1 mM NADP. The formed *cis*-aconitate was measured spectrophotometrically at 240 nm. The amount of membrane-free thiol groups was measured by using the Ellman's reagent 5′5-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, as previously described (*23*). Thiobarbituric acid reactive species (TBARS) were determined in blood serum according to Mihara and Uchiyama (*24*). Mitochondrial NAD was measured after perchloric acid extraction of 10 mg mitochondrial protein; the activity of lactate dehydrogenase was analysed spectrophotometrically at 340 nm as described by Correa *et al*. (*25*). Cytochrome c content was analysed in mitochondria as follows: protein (15 µg) was loaded onto 15% acrylamide SDS–PAGE gels and transferred to a PVDF membrane for immunodetection, using a primary monoclonal antibody against cytochrome c (1 : 1000 dilution), and an alkaline-phosphate conjugated secondary antibody. Mitochondrial DNA was isolated as described by García *et al*. (*26*). The genetic material was analysed in 0.7% agarose gel and visualized by adding ethidium bromide. Mercury quantification in whole kidney tissue and mitochondria was carried out digesting 30 mg protein by boiling in 0.5 ml of 99% H_2SO_4 plus 2.5 ml of 70% HNO_3 during 2 h; then, the volume of samples was adjusted to 5 ml. The content of mercury was determined using an atomic absorption spectrophotometer. In order to determine creatinine clearance, rats were placed in metabolic cages with free access to water for 12-h urine collection. A blood sample was taken immediately before removing the kidneys to determine plasma creatinine and BUN, according to Tenorio-Velázquez *et al*. (*27*). Samples were analysed using the creatinine and BUN analyser (Clinical Chemistry System ILab300 Plus, Instrumentation Laboratory). For the light microscopy studies, kidneys were fixed by vascular perfusion with 4% formaldehyde and 1% glutaraldehyde.

Results

Previous reports have established that the accumulation of Hg^{2+} in kidney tissue induces mitochondrial membrane leakage (*13*, *16*). In agreement, Fig. 1A, trace a, shows Ca^{2+} movements in mitochondria isolated from Hg^{2+}-treated rats. As observed, after a fast uptake reaction, efflux of the cation followed. However, the response of mitochondria isolated from Hg^{2+} plus OG-treated rats to Ca^{2+} accumulation was drastically different. As shown in trace c, OG effectively protected mitochondria from the deleterious effect of Hg^{2+}, and Ca^{2+} remained accumulated. Trace b illustrates that the addition of CSA partially inhibited Ca^{2+} release in mitochondria from Hg^{2+}-treated rats. Control mitochondria are shown in trace d. The change in mitochondrial volume was also evaluated to follow the increased permeability transition. Fig. 1B, trace a, illustrates that Hg^{2+}-treated mitochondria undergo a large osmotic swelling after the addition of Ca^{2+}. Trace b shows that mitochondria from Hg^{2+} plus OG-treated rats swell to a lesser extent. Fig. 1C, trace a, shows that mitochondria from rats injected with mercury do not build up a $\Delta\psi$. In contrast, mitochondria from OG-mercury-injected rats (trace b) preserved their ability to form

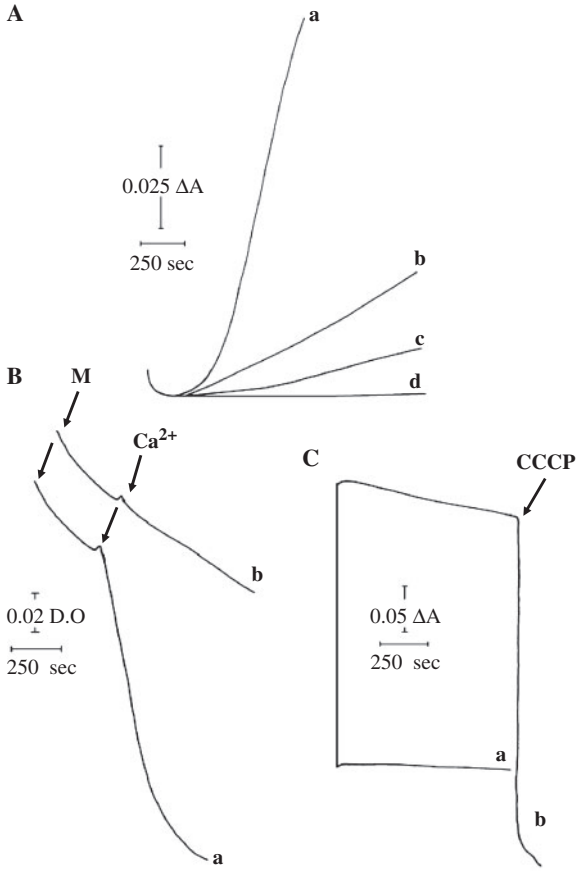

Fig. 1 The effect of octylguanidine on the deleterious effect of mercury on mitochondrial functions. (A) Ca^{2+} movements in mitochondria isolated from Hg^{2+}-treated rats (trace a), after the addition of 1 µM CSA (trace b). Trace c shows Ca^{2+} retention by mitochondria isolated from Hg^{2+} plus OG treated rats. Trace d shows control mitochondria. Mitochondrial protein (2 mg) was incubated in 3 ml of a medium containing 125 mM KCl, 10 mM malate, 10 mM glutamate, 10 mM HEPES, pH 7.3 and 5 mM phosphate, pH 7.3. In addition, the medium contained 50 µM Ca^{2+} and 50 µM Arsenazo III. (B) mitochondrial swelling. Mitochondrial protein (2 mg) was incubated under similar conditions, as described for (A), except that Arsenazo III was not added. Trace a indicates mitochondria isolated from Hg^{2+}-treated rats; trace b mitochondria isolated from Hg^{2+} plus OG-treated rats. Where indicated, 50 µM Ca^{2+} was added. (C) illustrates mitochondrial membrane potential; mitochondria (2 mg protein) were incubated under similar conditions, as in (A), except that 10 µM safranine was added instead of Arsenazo III. Trace a indicates mitochondria isolated from Hg^{2+}-treated rats; trace b illustrates mitochondria isolated from Hg^{2+} plus OG treated rats. Where indicated, 1 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added. Incubation temperature 25°C.

a transmembrane electric gradient, similar to that of the controls (not shown).

Mitochondrial permeability transition occurs through the opening of a non-specific pore that allows the efflux of matrix content, among them NAD^+ (*28*). To explore the latter, the experiment whose results are shown in Fig. 2 was carried out. As illustrated, OG successfully protected mitochondria from the deleterious effect of mercury; thus, roughly 80% of NAD^+ remained within the matrix, as compared to the NAD^+ content (20%) in mitochondria isolated from Hg-treated rats.

Since NAD^+ efflux indicates inner membrane permeability, next we explored the possibility that OG would also preserve outer membrane selective permeability from Hg^{2+} damage. To achieve this objective, the release of cytochrome c from mitochondria isolated from Hg^{2+} and Hg^{2+} plus OG treated rats was studied. The data shown in Fig. 3 clearly indicate that OG was inefficient to avoid the Hg^{2+}-induced increased permeability of the external mitochondrial membrane.

The damage by oxidative stress as induced by Hg^{2+} was also evaluated. To accomplish this objective, the activity of the aconitase enzyme was measured. The intactness, or not, of the activity of this enzyme can be a selective marker to evaluate a possible oxidative injury (*22*). Table I shows that Hg^{2+}-treatment inhibited by ~75% the activity of the enzyme. Interestingly, in mitochondria from Hg^{2+}-OG treated rats this inhibition was reduced to 33%, indicating a clear and defined protection exerted by the hydrophobic amine.

Mitochondrial DNA is also a target for oxidative insult (*26*); hence, mitochondrial DNA integrity was measured. As shown in Fig. 4, mitochondrial DNA from Hg^{2+}-treated rats was considerably disrupted by the action of the heavy metal. The disruption was almost completely avoided after OG treatment.

Lipid peroxidation also mediates the damaging effect of oxidative stress on membrane mitochondria. As a result of such peroxidation there is a tight correlation with an increase in the amount of TBARS formed.

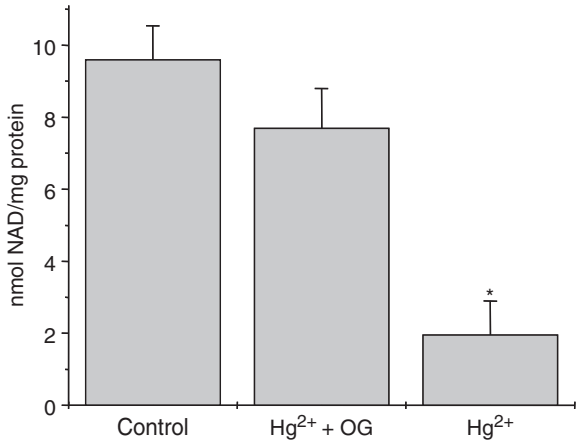

Fig. 2 NAD content in mitochondria isolated from Hg^{2+} and Hg^{2+} plus OG treated rats. Experimental conditions are as described under 'Materials and Methods' section. Values represent mean ± SD of three different preparations. $*P > 0.05$ Hg^{2+} plus OG versus Hg^{2+}.

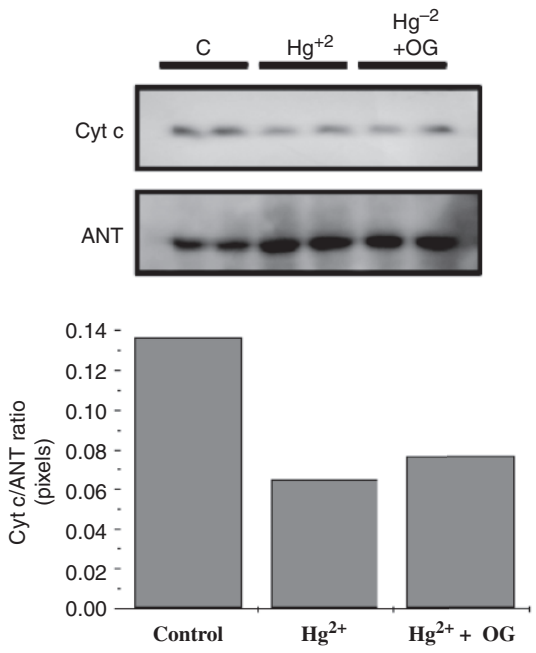

Fig. 3 Effect of mercury and mercury plus octylguanidine on mitochondrial cytochrome c content. Experimental conditions were as described in 'Materials and Methods' section. Immunodetection against adenine nucleotide translocase (ANT) was used as control of charge. The bars represent the mean of relative intensity ±SD, in pixels of the bands observed in four different experiments. $P < 0.05$ versus control.

Table I. The protective effect of octylguanidine on mercury-induced oxidative damage of aconitase.

Condition	nmol *cis*-aconitate/min/mg
Control	520 ± 120
+mercury	137 ± 35**
+mercury + OG	334 ± 42*

Aconitase activity was determined as described under 'Materials and Methods' section. Values are mean ± SD of four different mitochondrial preparations. $**P < 0.05$ versus control. $*P < 0.005$ versus OG.

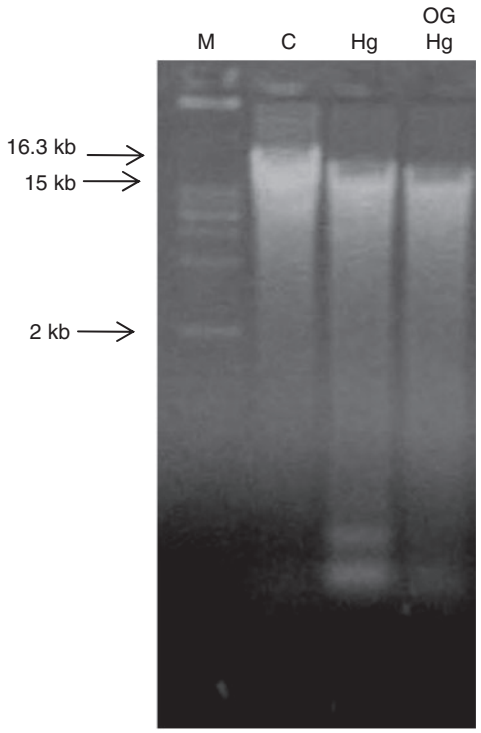

Fig. 4 Protection by octylguanidine on mercury-induced mitochondrial DNA damage. Approximately 2 µg of mitochondrial DNA was placed in 0.7% agarose gel. The lanes show molecular weight standards (M); DNA from control mitochondria (C); DNA from mitochondria isolated from Hg^{2+}-treated rats (Hg), and DNA from mitochondria isolated from Hg^{2+} plus OG treated rats (OG Hg).

Our experimental results indicate an increase, in blood serum, of these species from 14.14 ± 5 nmol/mg in Hg^{2+} untreated rats to 68 ± 26 in Hg^{2+}-injected rats. Interestingly, in the serum of Hg^{2+} plus OG treated rats the values diminished to 8.42 ± 2 nmol/mg. These data together with those showing DNA disruption and inhibition of *cis*-aconitase activity confirmed the protective role of OG on Hg^{2+}-induced oxidative damage.

The magnitude of the oxidative stress is also mirrored in the extent of the oxidation of membrane thiol groups (*29*, *30*). Thus, this process was evaluated by measuring free thiol groups; it was found that in mitochondria isolated from Hg^{2+}-treated rats the amount of free thiols diminished from 35.1 ± 3.4 to 10.65 ± 1.9; however, in mitochondrial isolated from Hg^{2+} plus OG-treated rats, the value increased to 17.37 ± 0.3. These values represent the average of four different determinations ±SD; $P < 0.001$, considering the difference between Hg^{2+} and Hg^{2+} plus OG-treated rats.

We recognize that the diminution in free thiol groups would also be dependent on the binding of Hg^{2+}, and not necessarily due to thiol cross-linking. Therefore, to obtain additional insight into the mechanism by which OG protects from Hg^{2+}-induced damage, the amount of Hg^{2+} content in mitochondria isolated from mercury- and mercury plus OG-treated rats was evaluated. The results shown in Fig. 5 indicate that, indeed, the addition of OG diminished by ~50% the binding of Hg^{2+}. However, the observed diminution would be due to an inhibitory action of OG on Hg^{2+} accumulation in the whole kidney tissue.

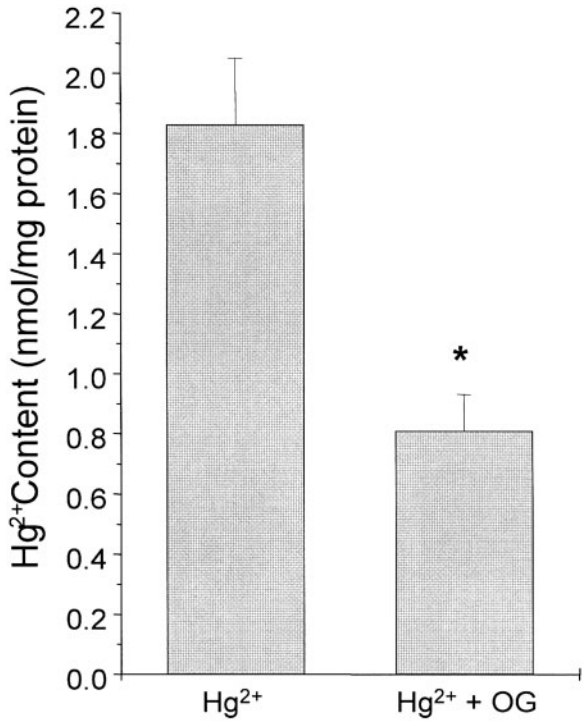

Fig. 5 Inhibition by octylguanidine of mercury binding on mitochondrial membrane. Experimental conditions are as described under 'Materials and Methods' section. Values are mean ± SD of 7 and 6 different mitochondrial preparations for Hg^{2+} and Hg^{2+} plus OG treated rats, respectively. *$P < 0.01$.

Considering the latter the amount of mercury was quantified in a total homogenate of kidney cortex. The results, an average of two different experiments, indicate that control kidney homogenate contained 0.03 nmol Hg^{2+}/mg, while those from rats injected with Hg^{2+} had 0.7 nmol/mg. A diminution to 0.42 nmol/mg was observed in kidney homogenate from rats injected with Hg^{2+} plus OG.

The protection by OG on Hg^{2+}-induced damage in renal function was explored. To this regard the results presented in Table II show that the values of BUN, serum creatinine, and creatinine renal clearance resulted severely affected by Hg^{2+} treatment. However, remarkably, administration of OG avoided kidney dysfunction. At this stage of the experimental work it was decide to know whether or not OG would induce, *per se*, kidney injury. As observed, administration of OG did not induce failure of renal function.

Previous reports (*4*, *13*, *31*) indicate that mercury induces histological and ultra-structural changes in kidney tissue. In accordance with these observations, we found that kidneys of Hg^{2+}-treated rats showed acute tubular necrosis (Fig. 6). These alterations were not observed in control animals. In addition, OG prevented most of the alterations produced by mercury; however, some focal areas of necrosis were observed.

Discussion

The morphological, histochemical and biochemical characteristics of Hg^{2+}-induced nephrotoxicity *in vivo* and *in vitro* systems have been extensively studied (*8*, *16*, *32–34*). Therapy for mercury poisoning involves the use of a wide array of drugs with more or less efficient effects (*4*, *14*, *15*, *35*). In a previous work, we demonstrated that captopril, an inhibitor of the angiotensin converting enzyme, prevents Hg^{2+}-induced renal failure (*13*). The present work shows that the hydrophobic amine octylguanidine, when administered *in vivo*, successfully prevented nephrotoxicity.

Kidney mitochondrial dysfunction as induced by mercury appears to be associated with the binding of the heavy metal on membrane thiols, with an affinity constant of 1.5×10^8 (*5*). The role of sulphhydryl groups of membrane proteins in cation permeability in mitochondria has been extensively studied. There is a large body of evidence in favour of the participation of these groups in the control of mitochondrial

Table II. Protective effect of octylguanidine on renal functions.

Condition	Serum urea nitrogen (mg/100 ml)	Serum creatinine (mg/100 ml)	Creatinine clearance (ml/min)
Control	17.25 ± 5.44	0.59 ± 0.07	1.51 ± 0.26
+OG	17.20 ± 6.72	0.53 ± 0.037	1.013 ± 0.17
+mercury	48.2 ± 16.4	2.07 ± 0.72	0.39 ± 0.16
+mercury + OG	21.57 ± 5.02	0.89 ± 0.28	1.22 ± 0.24

Experimental conditions as described under 'Materials and Methods' section. Values are mean ± SD of at least seven different preparations. $P < 0.001$.

Ca^{2+} transport (*29*, *36*). In a previous work, we demonstrated that the binding of Hg^{2+} to proteins with molecular weights between the ranges of 20–30 kDa induces non-specific permeability (*5*). Besides, mercury has been involved also in triggering oxidative stress that leads to increased permeability (*37*, *38*). The experiments carried out in this work demonstrate that mitochondria isolated from Hg^{2+}-treated rats were unable to retain Ca^{2+} and to build up an electric transmembrane gradient. These processes are characteristic of membrane permeability transition. In fact, as shown in Fig. 1, CSA, a specific inhibitor of non-specific pore opening, inhibited the release of accumulated Ca^{2+}. This is in close agreement with an earlier work in which we showed that, *in vitro*, CSA inhibited permeability transition promoted by the addition of Hg^{2+} (*16*). The clear-cut protection exerted by OG on mercury-induced permeability transition was demonstrated by the results indicating that administration of this chemical avoids mitochondrial Ca^{2+} release. In addition, as was shown, OG preserved the ability of mitochondria to form a transmembrane potential, and was able to hinder the release of matrix NAD. This is in agreement with the previously described property of OG on closing the non-specific transmembrane pore, in kidney (*19*), and yeast mitochondria (*39*).

From the results, it can also be inferred that OG protects from oxidative stress considering that the amine arrests mtDNA injury. Furthermore, it was also shown that OG preserves aconitase activity from mercury-induced oxidative injury. In addition, OG inhibited oxidation of membrane thiol groups. Regarding the above, it should be mentioned that OG provides protection to the rat heart against the oxidative stress induced by ischaemia/reperfusion (*20*). A notable feature of the experiments was also that OG ameliorates mercury-triggering acute renal failure, maintaining nitrogen balance within normal values, as shown in Table II. Renal failure would be produced by mercury-induced oxidative stress. Remarkably, OG shows also a protective effect against the damage induced by oxidative stress on mitochondrial DNA and on the aconitase enzyme.

Certainly, the explanation for the protective effect of OG on mercury-induced oxidative stress cannot be circumscribed merely to its ability to protect permeability transition, as previously demonstrated (*19*). Neither can the protective effect be ascribed to a scavenger action on oxygen derived free radicals, since OG was unable to inhibit the generation of these anions when the kinetics of the xanthine–xanthine oxidase system was analysed *in vitro* (data not shown). For a plausible explanation of the protective effect of this amphipathic compound on Hg^{2+}-induced poisoning, the diminution of Hg^{2+} uptake into kidney tissue must be taken into account. This process would be due to the solubilization of the hydrophobic moiety of the amine in the lipid milieu of the membrane. This allows the positive charge of the guanidine group to become exposed to the exterior of the membrane, causing a positive shift in the external surface of the membrane. Such a positive screen avoids the binding of mercury and, hence, kidney injury. Finally, the results in this work might serve to introduce OG as a new therapeutic agent for the prevention of kidney failure generated by Hg^{2+} poisoning.

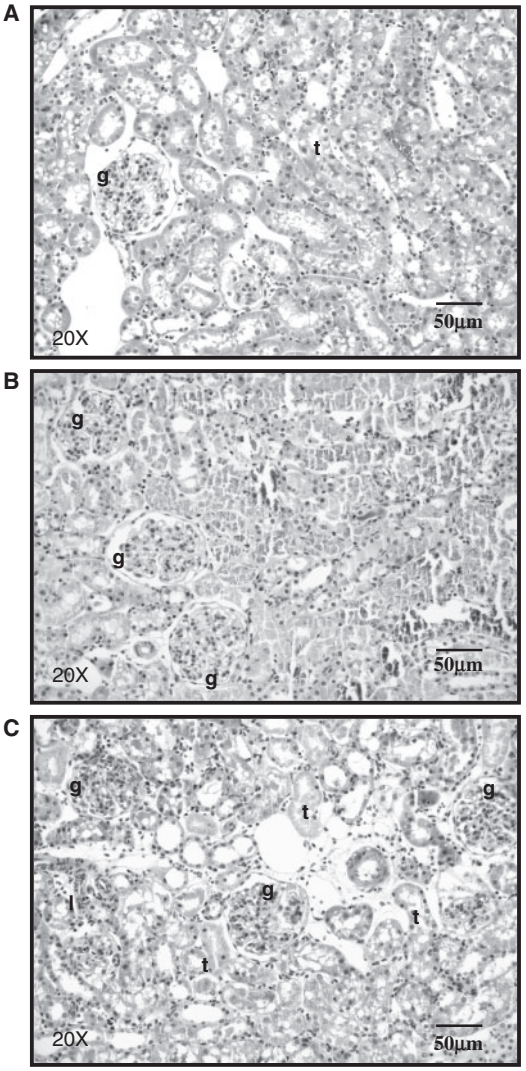

Fig. 6 Histological image of kidney tissue. (A) kidneys from control animals. As shown, the glomerulus (g) is in good shape as well as the renal tubules, although there is a minor vacuolization. (B) histological section from rat kidney after mercury injection. As illustrated the glomerulus is well preserved but tubules (t) show great vacuolization and there are wide areas of necrosis with lymphocyte infiltration (l). As shown in (C), OG prevented most of the damage and only focal areas of cell necrosis are observed.

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Conflict of interest

None declared.

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