Effect of Salviae Radix on renal tubular reabsorption in rabbits with mercury-induced acute renal failure

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I. Introduction

Mercury (Hg) is a well-known human and animal nephrotoxicant. Acute oral or parenteral exposure induces extensive kidney damage, especially in the proximal tubules.

Since renal proximal tubules are principal sites for active reabsorption of organic substances filtered from glomeruli, the injury to proximal tubules would cause impairment in reabsorption of organic compounds such as glucose, amino acid, and dicarboxylates. Indeed, mercury poisoning causes glucosuria and aminoaciduria.

Studies in vivo and in vitro have demonstrated that mercury induces lipid peroxidation, suggesting the involvement of oxidative stress in its cytotoxicity. Lund et al. reported that mercury enhances renal mitochondrial hydrogen peroxide formation in vivo and in vitro. However, causative correlation between mercury-induced lipid peroxidation and cellular toxicity remains controversial. Some authors reported that lipid peroxidation plays a critical role in cell injury induced by mercury in renal cells, whereas other investigators reported that lipid peroxidation is not directly responsible for mercury-induced cell injury in hepatocytes and renal cells.

Salviae Radix (SR) is a drug promoting blood circulation to remove blood stasis, removing heat from the blood and relieving restlessness. It is
used to subdue kidney failure, hypertension, coronary disorder, cerebrovascular disorder\textsuperscript{81}.

Our previous studies have shown that SR has a strong antioxidant action in rabbit kidneys\textsuperscript{81}. Therefore, the present study was undertaken to determine whether (1) lipid peroxidation is involved in Hg-induced acute renal failure, and (2) SR prevents mercury (Hg)-induced renal tubular reabsorption.

\section*{II. Materials and Methods}

\subsection*{1. Salviae Radix (SR) extract preparation}

2 kg of crushed crude drug was extracted with methyl alcohol under reflux for 4 hr three times and the total extractive was evaporated under reduced pressure to give 168 g.

\subsection*{2. Nephrotoxicity studies}

New Zealand White rabbits weighing 1.5-2.5 kg were housed in metabolic cages to collect urine. The animals were allowed 2 days to acclimate to the cages, and followed by a 24-hr basal period, during which urine and blood samples were collected. They received a single subcutaneous dose of mercury chloride (10 mg/kg body wt.). In order to test the effect of SR, rabbits were pretreated with SR (0.3 g/day/kg body wt., orally) for 7 days before mercury chloride administration. The other animals were pretreated with an equal volume of saline instead of SR. Individual 24-hr urine samples were collected for 24 hr after the mercury chloride injection and blood samples were taken from ear vein. In experiments for the antioxidant effect, DPPD (0.5 g/kg in corn oil) was given intraperitoneally 24 hr before Hg administration.

\subsection*{3. Urine and blood analyses}

Urine samples were analyzed for creatinine (Iatron Lab., Japan), glucose (Iatron Lab., Japan), phosphate\textsuperscript{82}. Blood samples were analyzed for creatinine, glucose, and phosphate.

Glomerular filtration rate (GFR) was estimated from the creatinine clearance and fractional excretion of solutes was calculated in the standard fashion.

\subsection*{4. Accumulation of organic ions in renal cortical slices}

The uptake of organic ions in cortical slices was performed as previously described\textsuperscript{83}.

Animals were sacrificed 24 hr following the administration of mercury or saline. The kidneys were quickly removed and the renal artery was immediately perfused with an ice-cold isotonic saline solution containing 140 mM NaCl, 10 mM KCl and 1.5 mM CaCl\textsubscript{2}, to remove as much blood as possible. Thin (0.4-0.5 mm thick) slices of renal cortex were prepared using a Stadie-Riggs microtome and were stored in an ice-cold modified Cross-Taggart medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM CaCl\textsubscript{2}, 5 mM Na acetate and 20 mM Tris/HCl (pH 7.8). Approximately 50 mg (wet wt.) of slices were then transferred into a 20 ml beaker containing 4 ml of the modified Cross-Taggart medium, and incubated with 14C-labeled substrates (Amersham, Arlington heights, IL). The concentrations of substrates used were 75 M for p-aminophippurate (PAH) and 10 M for tetraethylammonium (TEA). The incubation was carried out for 60 min in a Dubnoff metabolic shaker at 25°C under a 100% oxygen atmosphere. After incubation, the slices were quickly removed from the beaker, blotted, weighed and solubilized in 1 N NaOH.

Aliquots of the incubation medium and the solubilized tissue were pipetted into a scintillation vial containing Aquasol (New England Nuclear) and the radioactivity was determined using a liquid scintillation counter (Packard Tricarb 300C). The uptake of organic ions by renal slices was expressed as the slice to medium (S/M) ratio: the concentration of the compound in the tissue (mole/g wet tissue) divided by that in the medium (mole/ml medium).

\subsection*{5. Na\textsuperscript+-K\textsuperscript+-ATPase activity measurement}

The microsomal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was measured as described previously\textsuperscript{84}. The microsomal fraction was prepared from cortex and medulla of kidneys of control and Hg-treated rabbits. The ATPase activity of the microsomal fraction was determined by measuring inorganic phosphate (Pi) released by ATP hydrolysis during incubation of microsome with an
appropriate medium containing 3 mM ATP (Sigma) as the substrate.

The total ATPase activity was determined in the presence of 100 mM Na⁺, 20 mM K⁺, 3 mM Mg²⁺, 2 mM EDTA, and 40 mM imidazole (pH 7.4). The Mg²⁺-ATPase activity was determined in the absence of K⁺ and in the presence of 1 mM ouabain. The difference between the total and the Mg²⁺-ATPase activities was taken as a measure of the Na⁺-K⁺-ATPase activity. After a 5-min preincubation at 37°C, the reaction was initiated with the addition of the microsomal fraction. At the end of a 10-min incubation, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3500 g, and Pi in the supernatant was determined by the method of Fiske and SubbaRow[16].

6. Preparation of plasma membrane vesicles

Brush-border membrane vesicles (BBMV) were isolated by the Percoll-density gradient centrifugation and Mg²⁺-precipitation method, as previously described[12,20]. The vesicles were suspended in the vesicle buffer, adjusted to yield a protein concentration of 6 mg/ml and stored at -70°C until use. The composition of vesicle buffer is given in figure legends. Protein was determined according to Bradford[11] using γ-globulin as a standard.

7. Transport studies in membrane vesicles

The uptake of substrates by vesicles was measured by a rapid filtration technique. Briefly, the reaction was initiated by adding membrane vesicles to the incubation medium (a 1:10 dilution of membrane vesicle suspension) containing 50 μM of [14C]-labeled substrate at 25°C. The composition of the incubation medium is given in figure legends. Following 1 min of incubation, 100 μl aliquots were taken and quickly filtered under vacuum through Millipore filters (HAWP, 0.45 m pore size) which had been soaked overnight in distilled water. The filters were then washed with 5 ml of ice-cold stop solution comprising the identical composition to the incubation medium but without substrate, and dissolved in 1.0 ml of methoxyethanol. After addition of 10 ml of scintillation cocktail, the amount of radioactivity taken up by vesicles was determined by liquid scintillation spectrometry (Packard Tricarb 300C). Nonspecific binding of radioactive substrate to the plasma membrane was determined by incubating vesicles in transport buffer containing 0.1% deoxycholate and radiolabeled substrates. All uptake data were corrected for nonspecific binding. All the radioactive compounds were purchased from the Amersham International (Amersham, UK).

8. Lipid peroxidation measurement

Lipid peroxidation was estimated by measuring the renal cortical content of malondialdehyde (MDA) according to the method of Uchiyama and Miura[21]. Slices were homogenized in ice-cold 1.15% KCl (5% wt/vol). A 0.5 ml aliquot of homogenate was mixed with 3 ml of 1% phosphoric acid and 1 ml of 0.6% thiobarbituric acid. The mixture was heated for 45 min on a boiling water bath. After addition of 4 ml of n-butanol the contents were vigorously vortexed and centrifuged at 2,000 g for 20 min. The absorbance of the upper, organic layer was measured at 535 and 520 nm with a diode array spectrophotometer (Hewlett Packard, 8452A), and compared with freshly prepared malondialdehyde tetraethylylacetal standards. MDA values were expressed as pmol/kg mg protein. Protein was measured by the method of Bradford[11].

9. Reagents

[¹⁴C]-D-glucose, [¹⁴C]-glycine, [¹⁴C]-PAH, and [¹⁴C]-TEA were purchased from the Amersham International (Amersham, UK). Catalase, superoxide dismutase (SOD), and malondialdehyde tetraethylylacetal were purchased from Sigma Chemical (St. Louis, MO). N,N'-diphenyl-p-phenylenediamine (DPPD) was purchased from Aldrich Chemical (Milwaukee WI). All other chemicals were of the highest commercial grade available.

10. Statistical analysis

The data are expressed as mean ± SE and the difference between two groups was evaluated using Student’s t-test. A
probability level of 0.05 was used to establish significance.

III. Results

1. Clearance studies

A subcutaneous injection of Hg resulted in reduction of GFR to 4.9% of the basal value (0.05 ± 0.01 vs. 1.017 ± 0.194 l/day/kg for the basal period), which was accompanied by an increase in serum creatinine levels (Figs. 1 and 2). Such changes were significantly prevented by pretreatment of SR. GFR in SR-treated animals was 15% of the basal value and its value was significantly higher than that in SR-untreated animals (4.9%). Serum creatinine level increased from 0.89 ± 0.08 to 7.68 ± 0.72 mg/dl in animals treated with Hg alone, whereas these levels increased from 1.03 ± 0.07 to 4.52 ± 0.60 mg/dl in SR-pretreated animals.

In order to examine whether proximal tubular function is impaired by Hg treatment, the urinary fractional excretion of glucose and inorganic phosphate (Pi), substances which are reabsorbed in the proximal tubule, was measured. As shown in Fig. 3, the fractional excretion of both substances was markedly increased compared with the respective basal value. When animals were pretreated with SR, fractional excretion of these substances were not different between the values before and after Hg injection.

2. D-glucose and glycine uptake by BBMV

In order to determine whether the administration of Hg produced a direct impairment in proximal tubular transporters for organic compounds, uptakes of D-glucose and glycine were measured in BBMV isolated from renal cortex of control and Hg-treated animals. The results are depicted in Fig. 4. Uptakes of these compounds were significantly decreased in Hg-treated animals. These results suggest that glucosuria observed in Hg-treated animals was attributed to an impairment of glucose transport systems located at brush-border membranes. Such changes were prevented by SR pretreatment, suggesting SR appears a direct protective effect against an impairment in membrane transport function.
3. Renal cortical slice studies

Renal cortical accumulation of organic ions has been used as a sensitive indicator in the assessment of nephrotoxicity. We, therefore, measured in the present study the uptake of organic anion PAH and organic cation TEA by renal cortical slices as a biochemical index for an in vitro evaluation of proximal tubular cell injury. As shown in Fig. 5, the ability of cortical slices to accumulate organic ions was markedly decreased by Hg treatment. However, pretreatment of SR attenuated reduction in organic ion uptake induced by Hg.

4. Na+-K+-ATPase activity in microsomal fraction

In order to examine whether Hg impaired Na+-pump activity, we measured Na+-K+-ATPase activity in microsomes prepared from cortex and medulla of kidneys 24 hr after administration of Hg. As shown in Fig. 6, the Na+-K+-ATPase activities in cortex and medulla from kidneys of Hg-treated rabbits were significantly lower than control, but those in SR-pretreated animals were not different from control.

5. Lipid peroxidation in kidneys intoxicated with Hg

Lipid peroxidation is one of the well-known manifestations of oxidative cell injury, although the role that lipid peroxidation plays in the pathogenesis of irreversible cell injury with an acute oxidative stress has been a matter of continued debate. In this study, we measured changes in lipid peroxidation in cortical and medulla of kidneys from Hg-treated animals. As shown in Fig. 7, Hg increased lipid peroxidation in both tissues, suggesting that oxidative stress...
plays critical role in Hg-induced renal failure. By contrast, when animals were treated with Hg after SR pretreatment, lipid peroxidation reduced to the control levels. These results suggest that SR prevents Hg-induced acute renal failure by antioxidant action. In order to further confirm that lipid peroxidation plays an important role in Hg-induced cell injury, the effect of a potent antioxidant DPPD on Hg-induced renal failure was examined. As expected, DPPD prevented reduction in GFR and an increase in fraction Na⁺ excretion induced by Hg (Fig. 8).

Indeed, the present study showed that fractional excretion of glucose and phosphate significantly increased in Hg-treated animals. Such effects were resulted from intrinsic alterations of transporter activity at the membrane level, as evidenced by the inhibition of uptakes of glucose and glycine by renal brush-border membrane isolated from Hg-treated animals and the inhibition of phosphate uptake in Ok cells. Hg treatment also inhibited PAH and TEA uptake by renal cortical slices. These results indicate that proximal tubular secretory function is impaired by Hg as well.

Pretreatment of SR for 7 days prior to the administration of Hg provided protection against Hg-induced acute renal failure. Rabbits pretreated with SR were able to maintain their urine volume and GFR significantly higher than rabbits given Hg alone. The extent of increase in serum creatinine levels were also attenuated by SR pretreatment. Impaired proximal tubular reabsorption was significantly prevented by SR pretreatment. Numerous studies in vivo and in vitro have demonstrated that renal proximal tubular cell mitochondria are a principal target of Hg effects, as indicated by mitochondrial swelling\(^{17}\), impairment of oxidative phosphorylation\(^{26,27}\), and ATP depletion\(^{10}\). The mitochondrial electron transport chain is the principal site of cellular production of reactive oxygen species (ROS) such as superoxide and H₂O₂, with approximately 2-5% of the O₂ consumed in

**IV. Discussion**

The present study demonstrated that at 24 hr following the subcutaneous injection of mercury chloride there is a decrease in GFR along with an increase in serum creatinine level, indicating that Hg injection induces acute renal failure.

Morphological studies have shown that Hg causes the cell injury to all three portions of the proximal tubule, with the pars recta showing the greatest extent of damage\(^{26}\). Since proximal tubules are main sites for reabsorption of organic and inorganic substances such as glucose, phosphate, and amino acids, Hg treatment would induce impairment in reabsorption of these substances.
state 4 respiration resulting in H₂O₂ formation. Previous studies have demonstrated that the principal toxic effect of Hg is resulted from alterations in the structural integrity of the mitochondria inner membrane. This effect is accompanied by depletion of mitochondrial reduced glutathione content and increased formation H₂O₂ by the mitochondrial electron transport chain in vitro and in vivo, leading to increased lipid peroxidation. These results suggest that increased production of ROS may be involved in the pathogenesis of Hg-induced nephrotoxicity. In the present study, lipid peroxidation increases in kidneys of Hg-treated animals compared with the control, and an antioxidant DPPD pretreatment prevented Hg-induced acute renal failure. These results support the hypothesis that oxidative stress is responsible for Hg-induced nephrotoxicity. Therefore, protective effect of SR against Hg-induced acute renal failure may be resulted from antioxidant action.

V. Summary

This study was undertaken to determine if Salviae Radix (SR) exerts beneficial effect against Hg-induced acute renal failure in rabbits. Acute renal failure was induced by subcutaneous injection of mercury chloride (Hg. 10 mg/kg), and SR was pretreated for 7 days prior to the injection of Hg. Urine and blood samples were collected for 24 hr before (the basal period) and after the administration of Hg. GFR in Hg-injected animals were decreased as compared with the basal values, which was accompanied by the increase in serum creatinine levels, indicating that the administration of Hg produces acute renal failure. The fractional excretion of glucose and phosphate was markedly increased after Hg injection. Such changes were resulted from a direct impairment of brush-border membrane carriers and reduced Na⁺-pump activity. Uptakes of organic ions PAH and TEA by renal cortical slices were inhibited by Hg injection. Hg-induced acute renal failure was accompanied by an increase in lipid peroxidation. The pretreatment of SR significantly attenuated reduced GFR and increased serum creatinine levels. The impaired tubular reabsorption of solutes was prevented by SR. The pretreatment of SR decreased Hg-induced lipid peroxidation. Hg-induced acute renal failure was ameliorated by potent antioxidant DPPD.

These results indicate that Hg-induced acute renal failure is associated with generation of reactive oxygen species, and lipid peroxidation is responsible for the cell injury induced by Hg injection. SR exerts the beneficial effect against Hg-induced acute renal failure and its effect may be due to an antioxidant action.

VI. References

12. Lund B. O., Miller D. M. and Woods J.


