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수은으로 유발된 토끼의 신장 기능 손상에 대한 丹參의 효과

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

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독성약물에 의한 급성신부전시 세뇨관세포의 물질 재흡수 장애에 대한 丹參 추출액의 효과를 조사하였다. 토끼에 수은 (HgCl2)을 10 mg/kg 되게 피하 주사하여 급성신부전을 유발하였고, 丹參 추출액의 효과는 수은을 주사하기 전 7일 동안 0.05% 液 0.3 g/kg 용량을 경구 투여하여 관찰하였다. 수은을 주사하기 전 24시간 동안 요와 혈액을 채취하여 신장기능을 측정하여 대조기간 (basal period)의 값으로 하였고, 수은을 주사한 후 24시간 동안 요와 혈액을 얻어 수은에 의한 신장기능 변화를 평가하였다.

수은을 처리한 후 사구체여과율이 대조값에 비해 감소하였고, 혈청내 creatinine 농도가 증가하였다. 이러한 결과들은 수은이 급성신부전을 유발하였음을 가리킨다. 수은을 처리한 동물에서 포도당 및 인산의 배설분율이 증가하였고, 이러한 변화는 brush-border membrane에서 물질의 이동장애와 Na-pump 활성의 감소에 기인하였다. 수은을 주사한 동물의 신장피질 절편에서 유기이온인 PAH와 TEA 이동이 억제되었다. 토끼의 신장조직에서 지질의 과산화가 수은을 주사한 후 증가하였다. 丹參 추출액을 전 처리한 후 수은을 주사한 경우 수은에 의해 유발된 사구체여과율의 감소와 혈청내 creatinine 농도 증가 현상이 유의하게 완화되었다. 수은에 의한 세뇨관에서 물질의 재흡수 장애가 丹參 추출액의 전처치에 의해 방지되었다. 丹參 추출액은 수은에 의한 지질의 과산화를 억제하였다. 수은에 의한 급성신부전은 항산화제로 잘 알려진 DPPD에 의해 방지되었다.

이상의 결과를 종합하면 생체실험결과 수은에 의한 급성신부전의 유발과정에 지질의 과산화가 중요한 역할을 할 가능성을 보이고 있고, 丹參 추출액은 수은에 의한 급성신부전을 방지하는 효과를 가지고 있으며, 그 효과는 丹參의 항산화작용에 起因할 가능성이 많다.

Key Word : Salviae Radix, HgCl2, acute renal failure, GFR, tubular reabsorption, lipid peroxidation

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[1-5].

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 dicar boxylates. Indeed, mercury poisoning causes glucosuria and aminoaciduria[6].

Studies in vivo and in vitro have demonstrated that mercury induces lipid peroxidation, suggesting the involvement of oxidative stress in its cytotoxicity[7-11]. Lund et al.[12] reported that mercury enhances renal mitochon drial 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[7,12], whereas other investigators reported that lipid peroxidation is not directly responsible for mercury-induced cell injury in hepatocytes and renal cells[11,13].

Salviae Radix (SR) is a drug pro moting blood circulation to remove blood stasis, removing heat from the blood and relieving restlessness. It is

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used to subdue kidney failure, hypertension, coronary disorder, cerebrovascular disorder[14].

Our previous studies have shown that SR has a strong antioxidant action in rabbit kidneys[15]. 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 reabsorp tion.

II. Materials and Methods

1. Salviae Radix (SR) extract preparation

2kg 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.

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.

3. Urine and blood analyses

Urine samples were analyzed for creatinine (Iatron Lab., Japan), glucose (Iatron Lab., Japan), phosphate[16]. 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.

4. Accumulation of organic ions in renal cortical slices

The uptake of organic ions in cortical slices was performed as previously described[17].

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 $CaCl_2$, 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 CaCl2, 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, Alrington heights, IL). The concentrations of substrates used were 75 M for p-aminohippurate (PAH) and 10 M for tetraethylammonium (TEA). The incubation was carried out for 60 min in a Dubnoff metabolic shaker at 25℃ 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).

5. Na^+-K^+-ATPase activity measurement

The microsomal Na^+-K^+-ATPase activity was measured as described previously[18]. 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 Mg^{2+}, 2 mM EDTA, and 40 mM imidazole (pH 7.4). The Mg^{2+}-ATPase activity was determined in the absence of K and in the presence of 1 mM ouabain. The difference between the total and the Mg^{2+}-ATPase activities was taken as a measure of the Na^+-K+-ATPase activity. After a 5-min preincubation at 37℃, 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 fraction 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 Mg^{2+}-precipitation method, as previously described[19,20]. The vesicles were suspended in the vesicle buffer, adjusted to yield a protein concentration of 6 mg/ml and stored at -70℃ until use. The composition of vesicle buffer is given in figure legends. Protein was determined according to Bradford[21] 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℃. The composition of the incubation medium is given in figure legends. Following 1 min of incubation, 100 l 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 Mihara[22]. Slices were homogenized in ice-cold 1.15% KCl (5% wt/vol). A 0.5 ml aliquat 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 tetraethy lacetal standards. MDA values were expressed as pmoles per mg protein. Protein was measured by the method of Bradford[21].

9. Reagents

[^{14}C]-D-glucose, [^{14}C]-glycine, [^{14}C]-PAH, and [^{14}C]-TEA were purchased from the Amersham International (Amersham, UK). Catalase, superoxide dismutase (SOD), and malondialde hyde tetraethylacetal 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

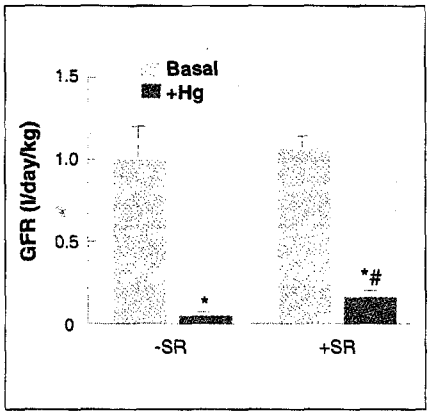

Fig 1. Effect of Salviae Radix (SR) pretreatment on changes in glomerular filtration rate (GFR) in Hg-induced acute renal failure. Data are mean± SE of five experiments. *p<0.05 compared with the respective basal period; #p<0.05 compared with Hg alone.

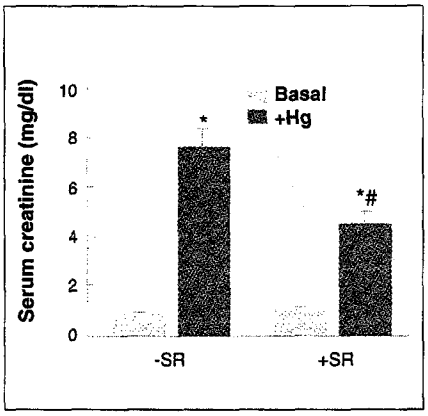

Fig 2. Effect of Salviae Radix (SR) pretreatment on changes in serum creatinine levels in Hg-induced acute renal failure. Data are mean±SE of five experiments. *p<0.05 compared with the respective basal period; #p<0.05 compared with Hg alone.

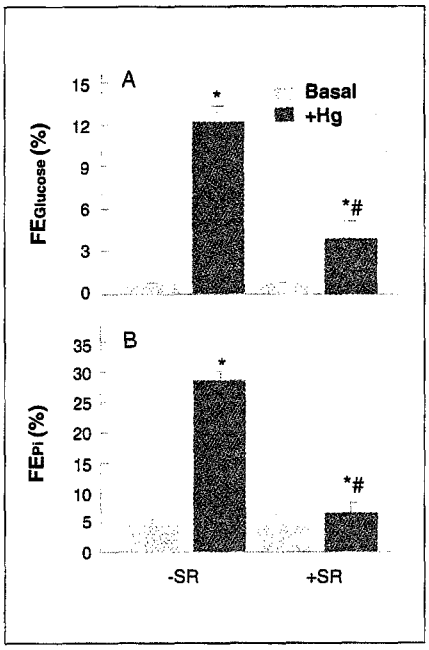

Fig 3. Effect of Salviae Radix (SR) pretreatment on changes in fractional excretion of glucose (FEglucose) and phosphate (FEPi) in Hg-induced acute renal failure. Data are mean± SE of five experiments. *p<0.05 compared with the respective basal period; #p<0.05 compared with Hg alone.

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.

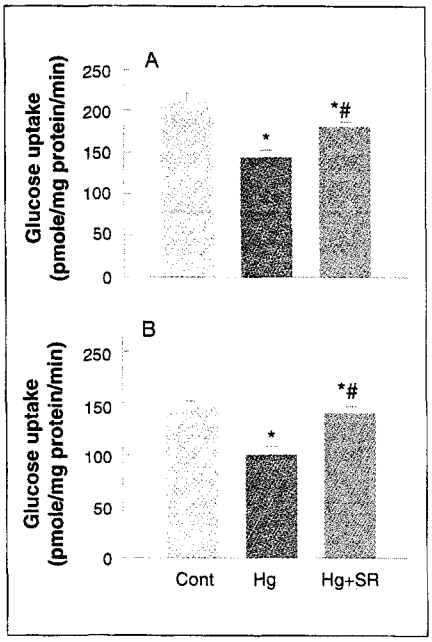

Fig 4. Effect of Salviae Radix (SR) pretreatment on changes in uptakes of glucose (A) and glycine (B) by brush-border membrane vesicles (BBMV) in Hg-induced acute renal failure.Membrane vesicles were loaded with a buffer containing 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.5) and were incubated in a buffer containing 50 M [^{14}C]-labeled substrate, 100 mM mannitol, 100 mM NaCl and 20 mM Hepes/Tris (pH 7.5) for 1 min at 25 ℃. Data are mean±SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with Hg alone.

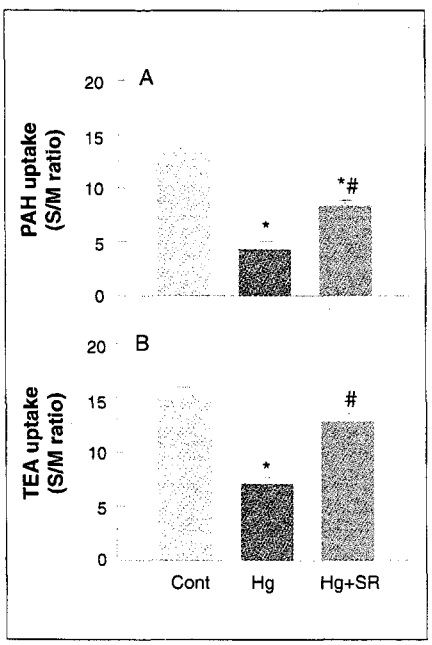

Fig 5. Effect of Salviae Radix (SR) pretreatment on changes in uptakes of PAH (A) and TEA (B) by renal cortical slices in Hg-induced acute renal failure.Data are mean±SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with Hg alone.

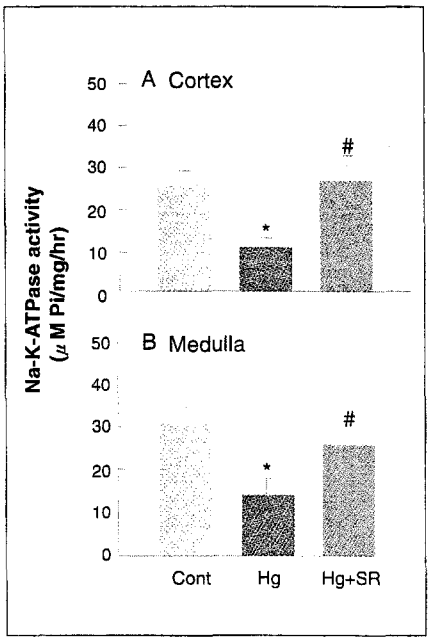

Fig 6. Effect of Salviae Radix (SR) pretreatment on changes in Na$^+$-K$^+$-ATPase activities of cortex and medulla of kidneys in Hg-induced acute renal failure. Data are mean±SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with Hg alone.

3. Renal cortical slice studies

Renal cortical accumulation of organic ions has been used as a sensitive indicator in the assessment of nephrotoxicity[23]. 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[24]. 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

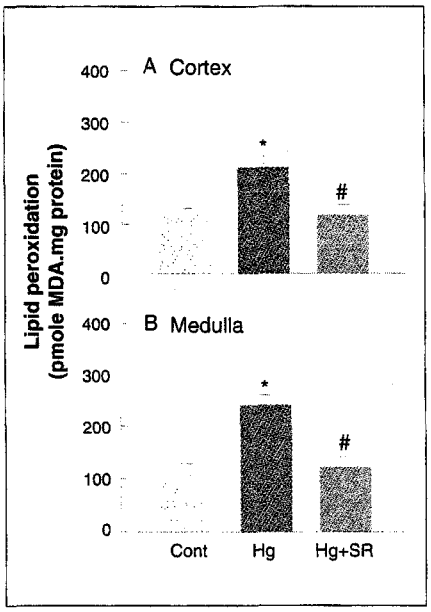

Fig 7. Effect of Salviae Radix (SR) pretreatment on changes in lipid peroxidation of cortex and medulla of kidneys in Hg-induced acute renal failure. Data are mean±SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with Hg alone.

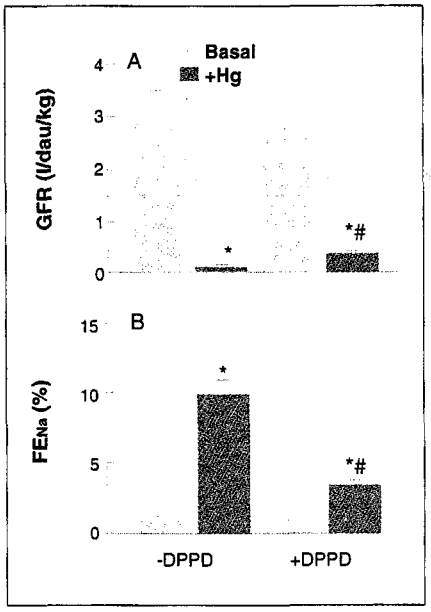

Fig 8. Effect of DPPD pretreatment on changes in glomerular filtration rate (GFR) and fractional Na^+ excretion ($FENa^+$) in Hg-induced acute renal failure. Data are mean±SE of five experiments. *p<0.05 compared with the respective basal period; #p<0.05 compared with Hg alone.

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).

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[25]. 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. 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 pretreat ment. 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[1,2], impairment of oxidative phosphorylation[26,27], and ATP depletion[28]. The mitochondrial electron transport chain is the principal site of cellular production of reactive oxygen species (ROS) such as superoxide and H_2O_2, with approximately 2-5% of the O2 consumed in

state 4 respiration resulting in H_2O_2 formation[29,30]. Previous studies have demonstrated that the principal toxic effect of Hg is resulted from alterations in the structural integrity of the mitochondria inner membrane[2,7,27]. This effect is accompanied by depletion of mitochondrial reduced glutathione content and increased formation H_2O_2 by the mitochondrial electron transport chain in vitro and in vivo, leading to increased lipid peroxidation[7,12]. 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 bene ficial effect against Hg-induced acute renal failure and its effect may be due to an antioxidant action.

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