

Changes in Various Hydroxyproline Fractions in Rat Kidneys after Mercuric Chloride Treatment

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Abstract: The present study was carried out to study the effect of mercuric chloride (HgCl_2) treatment on various hydroxyproline fractions in rat kidneys. The following groups were studied: (i) rats injected intraperitoneally (ip) with normal saline solution (Control group, $n = 5$ rats) (ii) rats were divided into four subgroups according to the dose of HgCl_2 and rats were injected with a single dose (ip) of 0.1, 0.5, 2 and 3 mg of HgCl_2/kg body weight/24 h (mercuric chloride treated group, $n = 5$ rats). The doses of HgCl_2 used caused no significant change ($p > 0.05$) in free and peptide bound hydroxyproline fractions in the kidney. Doses of HgCl_2 0.1, 0.5, 2.0 and 3.0 mg kg^{-1} body weight caused a decrease in protein bound hydroxyproline by 27% ($p < 0.01$), 37% ($p < 0.001$), 79% ($p < 0.01$) and 68% ($p < 0.001$), respectively, when compared to control rats. Although a dose of 0.1 mg and 0.5 mg kg^{-1} body weight of HgCl_2 caused a significant increase of 28 and 31% ($p < 0.01$) in total hydroxyproline when compared to control rats, other doses of HgCl_2 viz., 2.0 and 3.0 mg kg^{-1} body weight caused a decrease in total hydroxyproline by 33% ($p < 0.01$) and 60% ($p < 0.001$), respectively when compared to control rats. The changes in the total collagen in kidneys of HgCl_2 treated rats parallel the changes in total kidney hydroxyproline (Hyp). The effect of HgCl_2 treatment on serum and urinary total protein, albumin, potassium and sodium were also studied. In conclusion HgCl_2 treatment to rats caused a disturbance in connective tissue matrix in the kidneys.

Key words: Collagen, hydroxyproline, kidney, mercuric

INTRODUCTION

Within the earth's crust, cinnabar or mercuric sulfide is the principal ore of element mercury. Humans may be exposed to various species of mercury, which includes charged inorganic mercurous (Hg^{1+}) and mercuric salts (Hg^{2+}), neutral elemental metal (Hg^0) and organic molecules. Exposure to mercury vapor and organic mercurials specifically affect the central nervous system, while kidney is the target organ for inorganic mercury compounds (Aleo *et al.*, 2002). In general mercury toxicity derives from the fact that mercury binds to sulfide groups and disrupts the proper functioning of sulphydryl enzymes (Baum, 1999). Routes of exposure, toxicity, target organs and ultimately treatment strategies vary according to the species of mercury involved in the exposure. Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein. Connective tissue derives its prominent features such as mechanical strength from collagen. The Hydroxyproline (Hyp) is a post translational product of proline hydroxylation catalyzed by the enzyme prolylhydroxylase (EC 1.14.11.2) (Pihlajaniemi *et al.*, 1991). The occurrence of this amino acid is thought to be confined exclusively to collagen, where it is present in the Y position of the Gly-X-Y repeating tripeptide (Nemethy and Scheraga, 1986). Consequently, the presence of Hydroxyproline

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(Hyp) in tissues or serum can be used as a measure of collagen or collagen degradation products (Reddy and Enwemeka, 1996). In our previous studies we have reported the concentrations of various Hyp fractions in the plasma (Siddiqi and Alhomida, 2001; Siddiqi *et al.*, 2002), erythrocytes (Siddiqi and Alhomida, 2002) and tissues (Siddiqi, 2000; Siddiqi *et al.*, 2000, 2001; Siddiqi and Alhomida, 2003) of different mammals.

The kidneys excrete waste products of metabolism and play an important role in maintaining the homeostasis by regulating the body water and solute balance. In addition to the excretory function, the kidneys also have an endocrine function producing hormones like renin, erythropoietin etc. Inorganic mercury (HgCl_2) has been shown to accumulate in kidneys (Khan *et al.*, 2001) along with in other organs. In our previous studies (Siddiqi and Alhomida, 2005, 2006) we have shown that HgCl_2 treatment to rats damages the collagen which is reflected by increased levels of Hyp in serum and an increased excretion of Hyp in urine. The present study was carried out to study the effect of HgCl_2 treatment on various Hyp fractions in rat kidneys.

MATERIALS AND METHODS

Chemicals

Chloramine-T, p-dimethylaminobenzaldehyde (Ehrlich's reagent), L-hydroxyproline, sodium acetate, citric acid, perchloric acid, n-propanol, sodium hydroxide and acetic acid were purchased from Sigma Chemical Company, St Louis, MO, USA. Glass distilled water was used throughout the study.

Animal Care

Healthy adult male Wistar rats weighting 150-200 g were obtained from Breeding Laboratory, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The animals were labeled by identifying ear notches, housed in clean cages and placed in the animal care room.

Dose-Response of Mercury Chloride on Serum Hyp Fractions

Following a one-week acclimatization period, rats were randomly divided into different groups (5 rats group⁻¹) and individually housed in stainless-steel metabolic cages (Mini Mitter Co., Inc., Bend, Oregon, USA). Rats were allowed free access to food (Purina rodent chow) and tap water for one day. After one day control, the following groups were studied: (i) rats injected intraperitoneally (ip) normal saline solution (Control group, n = 5 rats) (ii) rats were divided into four subgroups according to the dose of HgCl_2 and rats were injected with a single dose ip of 0.1, 0.5, 2 and 3 mg of HgCl_2 kg⁻¹ body weight/24 h (mercuric chloride treated group, n = 5 rats). The rats were sacrificed 24 hours after the HgCl_2 treatment.

Sample Preparation

The animals were killed by cervical dislocation and the kidneys were dissected out, washed and frozen immediately in liquid nitrogen and stored at 80°C until processed. Tissues were homogenized in normal saline using a stainless steel Omni-Mixer homogenizer (Omni International, Inc, Gainesville, VA, USA). The homogenate was used for determination of Hyp concentrations. Further details about sample collections have been previously reported (Siddiqi *et al.*, 2001).

Extraction of Free, Peptide and Protein-Bound Hydroxyproline

Free and protein-bound Hyp was extracted by the method of Varghese *et al.* (1981) with slight modification. Briefly, 0.5 mL of homogenate was treated with 3×2 mL portion of re-rectified absolute alcohol and centrifuged at 600 g for 10 min. The supernatants were pooled and evaporated to dryness. The residue was dissolved in suitable amount of distilled water and an aliquot of the extract was used

for estimation of free Hyp. The peptide-bound Hyp was determined after alkaline hydrolysis of the ethanol extractable fraction. The pellets were dissolved in distilled water and an aliquot of the extract was used for determination of protein-bound Hyp. The free Hyp fraction was measured on an aliquot of the ethanol extracted residue without alkali hydrolysis, whereas the peptide-bound Hyp was measured after alkaline hydrolysis. The precipitate obtained on ethanol treatment of the homogenate was subjected to alkali hydrolysis to determine protein-bound Hyp. Further details about the extraction of Hyp fractions have been described previously (Siddiqi *et al.*, 2000).

Determination of Hydroxyproline Concentration

Hyp was measured after alkaline hydrolysis (Reddy and Enwemeka, 1996). Briefly, to an aliquot of the sample was added NaOH (2 N final concentration) and the aliquot was hydrolyzed by heating in a boiling water bath for about 3-4 h. Nine hundred microliters of 56 mM chloramine-T reagent was added to the hydrolyzed sample and oxidation was allowed to proceed at room temperature for 25 min. Then of 1000 μ L 1 M Ehrlich's reagent (p-dimethylaminobenzaldehyde) was added to the oxidized sample and the chromophore was developed by incubating the samples at 65°C for 20 min. The absorbance was read at 550 nm using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech Ltd., Science Park, Cambridge, England). The Hyp concentration in the samples was calculated from the standard curve of Hyp. Further details about the optimization, linearity, specificity, precision and reproducibility of the method were described previously (Siddiqi *et al.*, 2000).

Determination of Collagen Content

Total collagen content was calculated from the total Hyp concentration assuming that Hyp constitutes 12.5% collagen (Edwards and O'Brien, 1980).

Statistical Analysis

Each sample was run in duplicate. The Hyp content was expressed as mean \pm SD μ g g⁻¹ wet tissue, for n = 5 rats. Hyp levels between groups were compared using one way ANOVA analysis followed by Dunnett's or Tukey's test for multiple comparison test. Values were considered significant if p < 0.05. Statistical analysis was performed by means of InStat® package for personal computers (GraphPad™ Software, Inc., San Diego, USA).

RESULTS

All the doses of HgCl₂ used caused no significant alteration (p > 0.05) in total serum protein levels. Doses of HgCl₂ 0.5, 1.0, 2.0 and 3.0 mg kg⁻¹ body weight of rat caused a decrease in serum albumin concentration by 17 (p < 0.01), 33 (p < 0.001), 48 (p < 0.001) and 53% (p < 0.001), respectively when compared to control. Administration of 2 and 3 mg HgCl₂ kg⁻¹ body weight of rat caused an increase in serum potassium by 85% (p < 0.001) and 120% (p < 0.001), respectively when compared to control rats. The same dose i.e., 2 and 3 mg of HgCl₂ kg⁻¹ body weight of rats caused a decrease in serum sodium by 4 and 6% (p < 0.01), respectively when compared to control group (Table 1).

Various doses of HgCl₂ caused no significant change in the excretion of total urinary protein. However doses of HgCl₂ 0.5, 1.0, 2.0 and 3.0 mg kg⁻¹ body weight of rat caused a significant increase in the excretion of urinary albumin by 24% (p > 0.01), 101, 151 and 158% (p < 0.001), respectively as compared to control rats. Doses of HgCl₂ 2.0 and 3.0 mg kg⁻¹ body weight of rats caused a significant decrease in urinary potassium concentration by 56 and 67%, respectively (p < 0.001) when compared to control group. Doses of HgCl₂ 2.0 and 3.0 mg kg⁻¹ body weight caused increased excretion of sodium by 472% (p < 0.001) and 702% (p < 0.001), respectively when compared to control rats (Table 2).

Table 1: Effect of HgCl₂ administration on various serum biochemical parameters in rats

Parameters	Control	Placebo	HgCl ₂ treated				
			0.1	0.5	1.0	2.0	3.0
Total protein (g L ⁻¹)	65.86±5.11	65.57±3.31 ^{ns}	66.57±2.64 ^{ns}	64.43±3.99 ^{ns}	61.43±2.57 ^{ns}	53.01±4.12 ^{ns}	60.14±5.18 ^{ns}
Albumin (g L ⁻¹)	27.47±1.25	26.91±1.31 ^{ns}	25.66±1.27 ^{ns}	22.80±0.99*	18.40±1.05**	14.33±1.03**	12.81±0.27**
Potassium (mM)	4.53±0.44	4.74±0.5 ^{ns}	4.79±0.21 ^{ns}	5.01±0.16 ^{ns}	5.97±0.51 ^{ns}	8.37±1.83**	9.95±1.79**
Sodium (mM)	142.01±2.16	142.10±2.49 ^{ns}	141.11±1.22 ^{ns}	140.31±2.69 ^{ns}	144.70±0.76 ^{ns}	136.60±3.26**	133.12±3.91**

Data are expressed as the mean±SD for n = 5 rats per group per 24 h. Rats were treated with a single ip injection of HgCl₂ where as placebo group was injected with 0.9% saline solution intraperitoneally. ns: not significant as compared to control group (p>0.05, Dunnett's multiple comparison test); *Values significantly different as compared to control group (p<0.01, Dunnett's multiple comparison test); **Values significantly different as compared to control group (p<0.001, Dunnett's multiple comparison test)

Table 2: Effect of HgCl₂ administration on various urinary biochemical parameters in rats

Parameters	Control	Placebo	HgCl ₂ treated				
			0.1	0.5	1.0	2.0	3.0
Total protein (g/L/24 h)	12.43±1.07	12.43±1.16 ^{ns}	21.29±3.12 ^{ns}	29.06±2.66	37.33±2.23 ^{ns}	47.80±3.76 ^{ns}	56.30±3.62 ^{ns}
Albumin (g/L/24 h)	88.86±24.61	93.29±23.36 ^{ns}	90.43±18.04 ^{ns}	110.30±11.93*	178.90±16.28**	223.31±21.06**	229.10±18.74**
Potassium (mM/24 h)	71.43±5.20	76.59±4.07 ^{ns}	58.69±4.08 ^{ns}	53.14±4.90 ^{ns}	43.13±5.37 ^{ns}	31.50±3.06**	23.53±5.11**
Sodium (mM/24 h)	11.86±2.26	13.71±1.60±	17.08±2.52 ^{ns}	25.29±2.36 ^{ns}	34.01±67.86 ^{ns}	67.86±7.80**	95.14±12.39**

Data are expressed as the mean±SD for n = 5 rats per group per 24 h. Rats were treated with a single ip injection of HgCl₂ where as placebo group was injected with 0.9% saline solution intraperitoneally. ns: not significant as compared to control group (p>0.05, Dunnett's multiple comparison test); *Values significantly different as compared to control group (p<0.01, Dunnett's multiple comparison test); **Values significantly different as compared to control group (p<0.001, Dunnett's multiple comparison test)

Table 3: Effect of HgCl₂ administration on various Hydroxyproline fractions in rat kidney

Dose of HgCl ₂ (mg kg ⁻¹ body weight)	Hydroxyproline fractions µg g ⁻¹ tissue			
	Free	Peptide-bound Hyp	Protein-bound Hyp	Total Hyp
Control	331.4±170.5	648.5±113.5	860.2±41.25	1840.00±226.5
0.1	187.6±54.32 ^{ns}	1538.0±117.8 ^{ns}	629.0±4.48*	2355.00±133.8*
0.5	183.6±15.50 ^{ns}	168.0±12.12 ^{ns}	538.5±86.03**	2403.37±337.23*
2.0	156.8±20.52 ^{ns}	879.2±196.6 ^{ns}	181.6±25.32**	1218.00±208.3*
3.0	249.8±34.39 ^{ns}	263.8±63.59 ^{ns}	275.8±82.06**	744.10±83.06**

Data are expressed as the mean±SD for n = 5 rats per group per 24 h. Rats were treated with a single ip injection of HgCl₂. ns: not significant as compared to control group (p>0.05, Dunnett's multiple comparison test); *Values significantly different as compared to control group (p<0.01, Dunnett's multiple comparison test); **Values significantly different as compared to control group (p<0.001, Dunnett's multiple comparison test)

All the doses of HgCl₂ used viz., 0.1, 0.5, 2.0 and 3.0 mg kg⁻¹ body weight caused no significant change (p>0.05) in free and peptide bound Hyp fractions in the kidney. Doses of HgCl₂ 0.1, 0.5, 2.0 and 3.0 mg kg⁻¹ body weight caused a decrease in protein bound Hyp by 27 (p<0.01), 37% (p<0.001), 79% (p<0.01) and 68% (p<0.001), respectively when compared to control rats. Although doses of 0.1 and 0.5 mg kg⁻¹ body weight of HgCl₂ caused significant increases of 28 and 31%, respectively (p<0.01) in total Hyp content when compared to control rats, other doses of HgCl₂ viz., 2.0 and 3.0 mg kg⁻¹ body weight caused a decrease in total Hyp by 33% (p<0.01) and 60% (p<0.001), respectively when compared to control rats (Table 3).

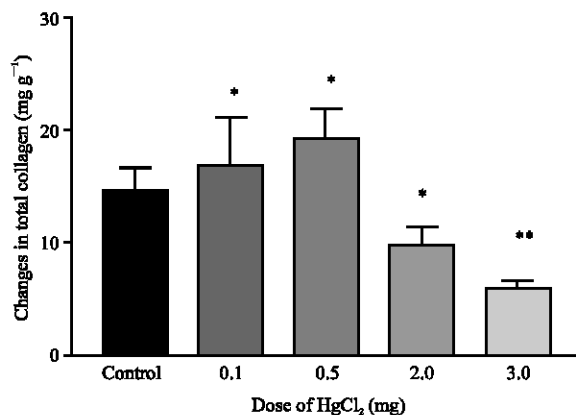


Fig. 1: Changes in total collagen in the kidneys of mercuric chloride treated rats. Collagen content of the kidneys is expressed as mg of collagen g⁻¹ fresh tissue. Rats were treated with a single ip injection of HgCl₂ where as placebo group was injected with 0.9% saline solution intraperitoneally. NS: not significant as compared to control group ($p > 0.05$), *Values significantly different as compared to control group ($p < 0.01$), Dunnett's multiple comparison test. **Values significantly different as compared to control group ($p < 0.001$), Dunnett's multiple comparison test

Figure 1 shows the effect of various doses of HgCl₂ on total collagen in rat kidney. A dose of 0.1 and 0.5 mg kg⁻¹ body weight of HgCl₂ caused a significant increase of 28 and 31%, respectively ($p < 0.01$) in total collagen when compared to control rats. Other doses of HgCl₂ viz., 2.0 and 3.0 mg kg⁻¹ body weight caused a decrease in total collagen by 33% ($p < 0.01$) and 60% ($p < 0.001$), respectively when compared to control rats.

DISCUSSION

Mercury is a highly toxic metal to animals. Exposure to high levels of heavy metals causes overt clinical disease. Furthermore, there is growing evidence that chronic exposure to low concentration of metals like mercury causes tissue or organ damage (Sug *et al.*, 1997). Other manifestations of mercury toxicity include impairment of electrolyte, water and nonelectrolyte transport in variety of cells and tissues, the principal target organ being the kidneys (Girardi and Elias, 1991). Inorganic mercury (HgCl₂) has been shown to accumulate in the renal cortex and affect the morphology and function of the proximal tubules (Kyle *et al.*, 1983). In our previous studies (Siddiqi and Alhomida, 2005) we have showed that HgCl₂ treatment to rats causes an increase in serum creatinine and serum urea nitrogen indicating an impaired renal function. In the present study HgCl₂ treatment caused a decrease in serum albumin which was accompanied by increased excretion of albumin in the urine. Albuminuria may be due to nephrotic syndrome caused by accumulation of HgCl₂ in the proximal renal tubules (Kyle *et al.*, 1983). Proteinuria indicates renal disease which may be the first reaction of the kidney to HgCl₂ assault which is followed by decline in glomerular filtration rate (Remuzzi and Bertani, 1998). The hyperkalemia and hyponatremia seen after HgCl₂ administration may be due to renal glomerular dysfunction caused by HgCl₂.

The kidneys are paired bean-shaped organs located on either side of the spinal column. The kidneys perform a variety of functions for the body, the most important being removal of unwanted substances (waste and surplus) from the plasma, homeostasis of the body's water, electrolyte and acid/base status and participation in endocrine regulation. The amount of collagen in the kidney depends on factors like the species of the animal, its age and the presence of disease. In general collagen

forms only a small proportion of the renal mass about 2% of the dry weight of renal cortex of adult rats (Weiss and Jayson, 1982) and this may be due to the presence of an active collagenolytic mechanism in the kidney (Weiss and Jayson, 1982). Nevertheless, the collagen is of great physiological importance as a support for the renal parenchyma and as a component of the basement membrane. In our earlier studies with rat tissues (Siddiqi, 2007) we found the collagen content of the kidneys to be lowest among the tissues studied i.e., heart, spleen, skeletal muscle, brain, testes, lungs. Liver was the only tissue which contained collagen less than the kidneys. Earlier studies of Kucharz and Olczyk (1994) have demonstrated that rats intoxicated with HgCl_2 (1 mg kg^{-1} body weight) daily for 12 weeks have increased total collagen in the kidneys. This increase was mainly from elevated soluble collagen. In the present study an initial dose of 0.1 mg kg^{-1} body weight caused an increase in total collagen but higher doses caused a decrease in total collagen. This may be due to the fact that rats were sacrificed 12 h after HgCl_2 treatment which may be a short time for the body to react. Previous studies of Kucharz and Olczyk (1994) showed that HgCl_2 treatment to rats caused an increase in serum and urinary Hyp level which in accordance with our earlier studies (Siddiqi and Alhomida, 2005, 2006). The increased levels of Hyp in the serum and urine could be due to increased catabolism of collagen in the tissues and one such tissue could be the kidney.

Hyp is excreted by the kidneys as small peptides (Adams and Frank, 1986). In the present study HgCl_2 treatment caused no significant change in the peptide bound Hyp levels in the kidneys. This may indicate the fact that the kidneys were still viable 24 h after the treatment. Earlier studies of Duran *et al.* (1990) have shown that though the kidneys were seriously damaged 24 and 48 h after HgCl_2 injection, they still retained their viability. The increase in the protein-bound Hyp may be due to the fact that HgCl_2 is bound to some protein in the kidney which is rich in Hyp. Earlier studies of Abdulwajid and Sarkar (1983) have shown that nickel which also accumulates in the kidneys is present bound to a low molecular weight protein which has Hyp as one of the amino acids. Earlier studies of Nagelschmidt and Struck (1977) had shown that protein-bound Hyp does not mirror collagen turnover and therefore in the present study also protein-bound Hyp may not reflect collagen breakdown in the kidney.

In conclusion, HgCl_2 treatment to rats caused a disturbance in connective tissue matrix of the kidneys.

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