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Ruthenium red induced nephrotoxicity: Biochemical and oxidative stress study

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ABSTRACT

A single dose of ruthenium red induces serious functional and structural changes in the liver. However, few data exist with respect to its effects on kidney functionality and oxidative stress status. The time-course of a single intraperitoneal administration of ruthenium red on systemic oxido-reductive status and kidney functionality was assessed in rats. Ruthenium red in aqueous solution was administered intraperitoneally in the dose of 20 mg/kg. Control group was treated with saline solution only. Oxidative stress was assessed by measuring lipid peroxidation products and nitric oxide catabolites. Serum urea and creatinine were measured for determination of renal function. A significant increase in serum ruthenium red was observed at 30 minutes after the administration of ruthenium red and the peak response was detected at 60 minutes and serum ruthenium red decreased slowly afterwards. Creatinine and urea showed significant increases after ruthenium red administration. Lipid peroxidation products in serum and kidney tissue showed significant time-dependent increases after ruthenium red injection. Nitric oxide catabolites also exhibited time-dependent increases after ruthenium red injection. A single intraperitoneal injection of ruthenium red caused renal and systemic oxidative stress, and induces nephrotoxicity in rats.

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KEYWORDS

Nephrotoxicity;
Ruthenium red;
Oxidative stress;
Urea;
Creatinine;
Rat.

INTRODUCTION

Ruthenium red $[(\text{NH}_3)_5\text{Ru}-\text{O}-\text{Ru}(\text{NH}_3)_4-\text{O}-\text{Ru}(\text{NH}_3)_5]^{6+}\text{Cl}_6^- \cdot 4\text{H}_2\text{O}$, is a water-soluble synthetic crystalline and inorganic polycationic dye that has been used in histology and electron microscopy to stain certain complex polysaccharides. For instance, ruthenium red has been used in optic microscopy to stain pectines of plant cell walls and staining in electron microscopy to locate acidic polysaccharide like material. This use was described initially in detail by Luft^[1,2]. Additionally ruthenium red is a useful ligand for binding osmium tetroxide for examining uncoated soft biological material under the scanning electron microscope^[3].

Ruthenium red has been linked to specific biochemical events, such as inhibition of microtubule polymerization^[4], inhibition of Ca^{2+} uptake and release in cells and subcellular fractions^[5-7]. At is regard ruthenium red inhibits the mitochondrial calcium uniporter, the ryanodine receptor-mediated Ca^{2+} channel on the endoplasmic reticulum^[8] and inhibits multiple transient receptor potential channels^[9]. In addition, it possesses immunosuppressive^[10], anti-tumoral^[11], and antiapoptotic^[12] activities at micromolar or submicromolar concentrations. Additionally ruthenium red has been shown to be neurotoxic *in vivo* when injected intracerebrally at micromolar concentrations^[13].

A single intraperitoneal injection has been used to investigate possible acute toxic effects of ruthenium red in male rats and other mammals. At high dye concentration (10–20 mg/kg body weight) as a single dose, can produce from atypical motor activity to flaccid paralysis and seizures^[14,15], and serious functional and structural changes in the liver as well as clotting alterations^[16,17]. As reported previously, there are inconsistent data about the role of ruthenium red with regard to its protective or toxic effects. It has been reported that ruthenium red induces neuronal death *in vivo* and in primary cultures, due to intracellular Ca^{2+} dishomeostasis and disruption of mitochondrial oxidative function^[18]. Conversely, protective effects of ruthenium red have been described using an immortalized mouse hippocampal cell line (HT22) that lacks ionotropic glutamate receptors. HT22 cells exposed to 5 mM glutamate become rounded and form apoptotic bodies until after 7 h of glutamate exposure. Interestingly, ruthenium red pro-

tects the cells when added up to 6 h after glutamate, ie, this dye prevents the cellular increases in reactive oxygen species production elicited by glutamate^[19]. Additionally ruthenium red delays the onset of cell death during oxidative stress of rat hepatocytes^[20]. Therefore ruthenium red has acquired many applications in the study of oxidative stress.

Although there are several studies showing the neurotoxic and hepatotoxic effects of ruthenium red, systemic investigations on the oxidative stress status and kidney functionality under the treatment of this dye are still lacking. On the other hand, the kidney is the main route of the dye excretion. Hence, in the present study, an attempt has been made to assess the serum alterations in oxidative stress and renal dysfunction markers that accompany a single dose treatment of ruthenium red on Wistar rats.

EXPERIMENTAL

Adult Wistar rats (250–300 grs) were housed in groups of four animals per cage in an air-conditioned room at $22 \pm 1^\circ\text{C}$, and feed with standard diet and tap water *ad libitum* throughout the investigation. Rats were maintained on a 12/12 h dark/light cycle. The animal experiments conformed to local regulations and with the Principles of Laboratory Animal Care (NIH no. 85-23, revised 1985). Rats were randomly assigned to control and experimental groups. Eight rats receiving intraperitoneal saline injections were used for control purposes. The experimental group (n=32) received a single dose (20 mg/kg intraperitoneally) of ruthenium red (Polisciencias Inc.) in 3% aqueous solution. Rats were euthanized at 30, 60, 120, and 480 minutes after the injection and blood samples were drawn from the left ventricle. Serum was separated by centrifugation at 3000 *xg* for 10 min. Serum samples were immediately subjected to estimation of ruthenium red concentrations, urea, creatinine, nitric oxide catabolic products, and lipid peroxidation products. In some experiments, kidney tissues were homogenized in ice-cold 20 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) with a polytron-like stirrer to produce 1:10 homogenates. Homogenates were centrifuged at 3000 *xg* for 30 min at 4°C . The supernatant was collected and immediately assayed for products of lipid peroxidation.

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Serum ruthenium red concentration was measured spectrophotometrically at 533 nm by the method of Luft^[1]. A calibration curve with standard ruthenium red showed that, within the range of concentration used, there is a linear relationship between concentration and absorbance (not shown). Creatinine was measured by using a kinetic alkaline picrate assay^[21]. Urea nitrogen was measured by using the urease method as reported elsewhere^[22].

The level of end products of lipid peroxidation was measured as the sum of malondialdehyde and 4-hydroxyalkenals using the Lipid Peroxidation Assay Kit (Oxford Biomedical Research, Oxford MI, USA), by spectrophotometry at 586 nm after incubating the samples with N-methyl-2-phenylindole at 45 °C.

The nitrite/nitrate concentration in serum was used as an indicator of nitric oxide synthesis. In order to quantify nitric oxide metabolites, samples were deproteinized as established by Ghasemi et al^[23], and then we used the Nitric Oxide Assay kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions.

Data were statistically analyzed by one-way analysis of variance, followed by Dunnett's test for multiple comparisons or by Student's t test for comparisons between two groups. Statistical significance was defined as a P value of ≤ 0.05 . All values in text and Figures are expressed as means \pm SD of determinations for all rats in the group (N = 8).

RESULTS

Serum ruthenium red concentrations

As shown in Figure 1, 30 minutes after a single injection of ruthenium red, a significant increase in the serum ruthenium red concentration was observed (1.9 ± 0.76 mg/dl). The peak response was observed at 60 minutes (4.3 ± 1.0 mg/dl) and serum ruthenium red decreased slowly afterwards.

Renal functionality

Figure 2 displays concentrations of creatinine and urea in the sera of experimental animals during the course of the study. Low concentration in serum urea and creatinine were detected in controls. A significant increase in serum creatinine was detected as early as 30 minutes. It began to decrease by 60 minutes and

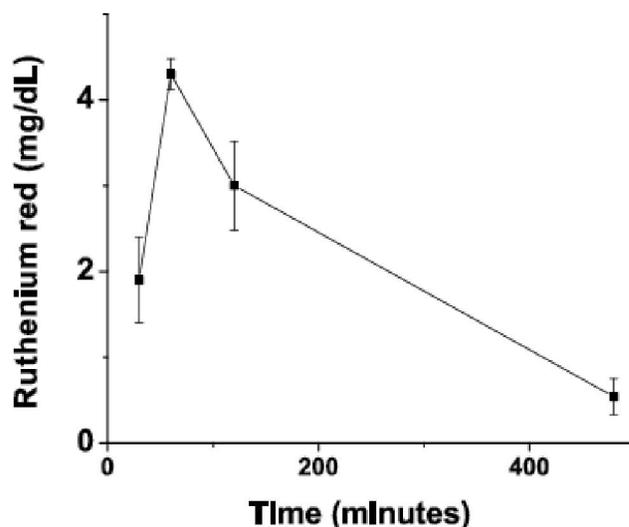


Figure 1 : Time course of serum ruthenium red concentrations after a single intraperitoneal injection of ruthenium red. The highest value occurred at 60 min. Error bars indicate the mean with s.d.

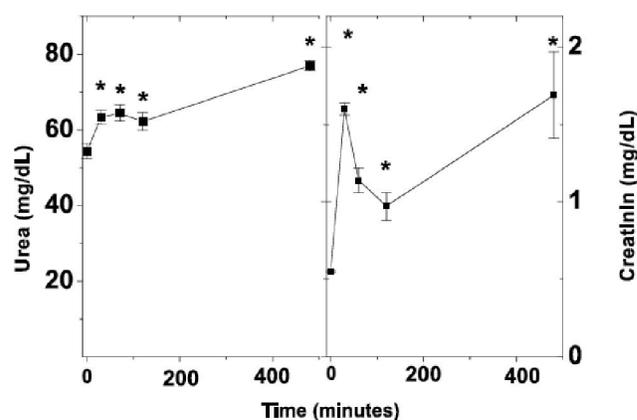


Figure 2 : Time course of serum urea and creatinine levels after a single intraperitoneal injection of ruthenium red. The serum concentrations of urea and creatinine were increased at 480 and 30 minutes, respectively. * $P < 0.05$ comparison of values at different times with respect to control group, before ruthenium red treatment. Error bars indicate the mean with s.d.

rose again at 480 minutes ($p < 0.001$). Serum urea concentrations increased significantly at 30 minutes and reached its maximum at 480 minutes.

Oxidative stress parameters

As shown in Figure 3 and Figure 4, basal levels of lipid peroxidation products (malondialdehyde and 4-hydroxyalkenals) and nitric oxide catabolites (nitrates/nitrites), respectively were detected in serum from control rats. In addition intraperitoneal injection of ruthenium red produced time-dependent increases in the lipid peroxidation products and nitric oxide catabolites.

DISCUSSION

These increases were statistically significant ($p < 0,001$).

The baseline level of lipid oxidation products in kidney homogenate was $0,42 \pm 0,08$ nmol/mg protein. After a single intraperitoneal injection of ruthenium red, the end products of lipid peroxidation in kidney homogenate rose to $0,92 \pm 0,09$ nmol/mg protein at 30 minutes, and steadily time-dependent increases similar to increased serum lipid peroxidation products were observed (not shown).

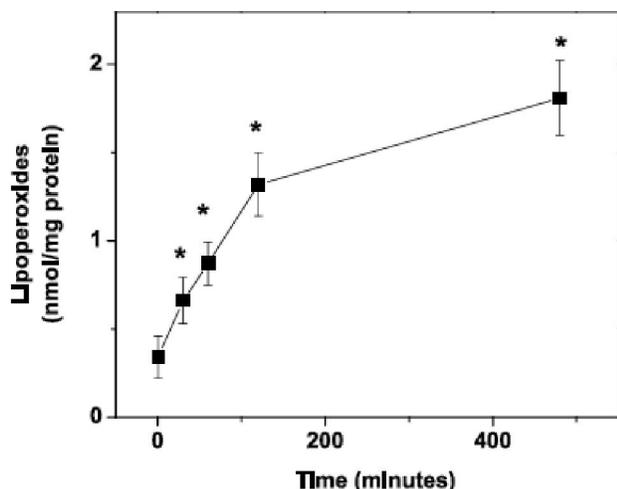


Figure 3 : Time course of serum lipid peroxidation products after a single intraperitoneal injection of ruthenium red. The highest concentration was detected at 480 minutes. * $P < 0.05$ comparison of values at different times with respect to control group, before ruthenium red treatment. Error bars indicate the mean with s.d.

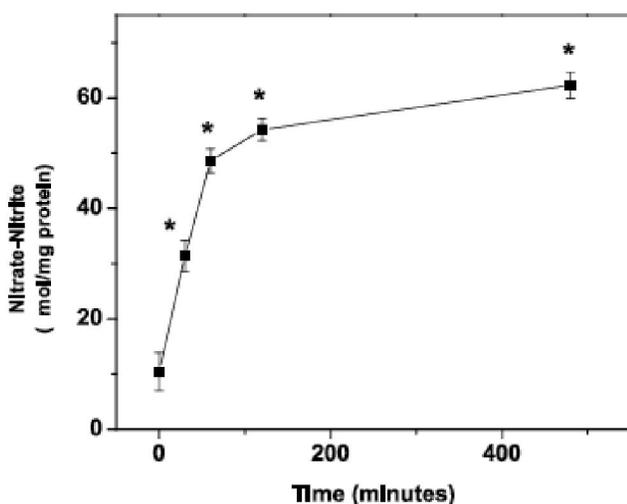


Figure 4 : Time course of serum nitric oxide metabolites after a single intraperitoneal injection of ruthenium red. Maximal concentration was detected at 480 minutes. * $P < 0.05$ comparison of values at different times with respect to control group, before ruthenium red treatment. Error bars indicate the mean with s.d.

The result of the present study indicated that ruthenium red administration in a single dose of 20 mg/kg body weight caused an enhanced renal injury and systemic oxidative stress status as evidenced by elevated levels creatinine, urea, lipid peroxidation products and nitric oxide catabolites. In consonance with these data is the observation that plasma malonyldialdehyde values are accompanied by increases in renal malonyldialdehyde levels in rats with renal mass reduction, suggesting that plasma reactive oxygen species levels could reflect local reactive oxygen species production in kidney^[24]. In addition high levels of nitric oxide catabolites have also been observed in several renal dysfunction models^[25]. The deleterious effect of increased nitric oxide in kidney might be greatly enhanced by a simultaneous increase in superoxide radicals. In the presence of superoxide, nitric oxide interacts with superoxide to form peroxynitrite radicals, an important agent that can cause oxidative damage.

On the other hand serum creatinine level is widely used as a parameter to evaluate both glomerular filtration and tubular reabsorption^[26]. Our results show that ruthenium red treatment significantly reduced renal function as demonstrated by an increase in serum creatinine and urea compared with the control group. In addition, serum urea was highly associated with the serum ruthenium red concentrations, as indicated linear coefficient correlation of $-0,94$. It has been reported that oxidative stress can promote the formation of a variety of vasoactive mediators that can cause kidney dysfunction and morphological damage^[27]. Interestingly, our results indicate that disturbances of redox balance are associated to disturbance in renal function.

Although no clear time-dependent correlations between oxidative stress and renal damage were found, the results suggest that the sustained increases of lipid oxidation products and nitric oxide metabolites could lead to renal injury. Although, we cannot rule out that these two phenomena are independent outcome by ruthenium red. Therefore we are developing experiments to clarify these data.

We hypothesized that the initial increase in serum ruthenium red elicits the changes in the biochemical parameters detected in this study. The mechanisms by

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which ruthenium red treatment induces nephrotoxicity are not yet fully understood. However, previously it has been reported that ruthenium red exerted inhibition of the mitochondrial calcium uniporter, the ryanodine receptor-channel on the endoplasmic reticulum^[8] and inhibits a newly-described group of membrane proteins that perform both as receptors and ion channels (the transient receptor potential family) involved in calcium homeostasis, especially in non-electrically active cells^[28]. These effects could exert a disruption of Ca²⁺ homeostasis and mitochondrial oxidative function^[18]. Consistent with this suggestion is that micromolar concentration of ruthenium red slightly reduced ATP content in tubules from rats^[29].

However, it is also possible to speculate on many other mechanisms, such as changes in the level of other substances that can increase oxidative stress but are yet to be detected in this role. At this regard, ruthenium red obtained from commercial sources contains impurities, mostly ruthenium brown (an oxidation product of ruthenium red) and ruthenium violet. Although Luft^[1] described a method to purify ruthenium red, no attempt was made to do so in our study. Therefore, these impurities could enhance the renal injury.

Ruthenium red has been extensively used as a model of epilepsy^[30,31]. However, caution should be exercised with the use of higher dose of i.p. ruthenium red currently being used in neurological studies. Our findings pointed out the risk of increased systemic lipid peroxidation and renal damage due ruthenium red use. In addition, it has been shown that ruthenium red has an agglutinating effect on the adult rat blood cells and induces serious functional and structural changes in the liver; such as increased activity of plasma aminotransferases and degeneration, respectively^[17].

In summary, our data suggests that a single intraperitoneal injection of ruthenium red induces systemic oxidative stress and kidney injury, as assessed by increased lipid peroxidation products, nitric oxide metabolites, creatinine and urea. Such effects must be taken into consideration, particularly with regard to neurological studies.

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