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ZINC ALLEVIATES POTASSIUM DICHROMATE-INDUCED HEPATOTOXICITY IN PREGNANT *WISTAR* RATS

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Abstract

The present study aimed to investigate the potential protective effects of zinc (Zn) against hexavalent chromium-induced hepatotoxicity in pregnant *Wistar* rats. Female rats were treated subcutaneously (s.c) on the 3rd day of pregnancy, with NaCl 0.9 % and served as control, $K_2Cr_2O_7$ (10 mg/kg bw) alone, or $K_2Cr_2O_7$ in association with $ZnCl_2$ (20 mg/kg bw). Hepatic biochemical parameters, oxidative stress biomarkers and DNA damage were monitored. Results revealed that $K_2Cr_2O_7$ disturbed plasma ALT, AST, ALP and GGT, induced hepatic oxidative stress and DNA fragmentation. The co-treatment with Zn has alleviated $K_2Cr_2O_7$- induced hepatotoxicity by exhibiting antioxidant and genoprotective effects in pregnant *Wistar* rats.

INTRODUCTION

The hazardous effects of hexavalent chromium (CrVI) have been a major concern because of its bioaccumulation in environmental media and living beings, subsequent to the growing use of its compounds in anthropogenic activities and the improper discarding of untreated industrial effluents [1, 2]. Because of its widespread presence, the exposure risk for humans and even animals has been raised in the past decades. Eventually, CrVI compounds can adversely affect the organism; previous studies reported the deleterious effects induced by CrVI compounds in several systems of the organism [3- 6]. Evenly, the toxicity of CrVI depends largely upon its potency of generating free radicals through its intracellular reduction into different reactive chromium intermediates, resulting in reactive oxygen species (ROS) overproduction and subsequent impairment of cellular functions due to the oxidative deterioration of biological molecules. Thus, it is suggested that oxidant/ antioxidant imbalance is among the pertinent biochemical pathways of CrVI- induced toxicity [7]. Moreover, it's known to induce tissue damage and exert genotoxic, carcinogenic, mutagenic and teratogenic effects [8- 10]. In addition to their genotoxic impact, CrVI compounds are known to disturb the hepato- renal integrity. Indeed, it was reported that CrVI induced oxidative damage and preneoplastic lesions in both kidney and liver in male rats [11]. The liver is among the prime target organs of CrVI compounds since it is implicated in this metal's metabolism and detoxification processes [12]. Multiple studies have revealed the hepatotoxic potential of CrVI *in vivo* characterized by hepatic oxidative stress, disturbance of hepatic function markers and genomic damage [3, 11- 13]. In this context, many natural elements were used for their possible genoprotective and antioxidant potential against CrVI- induced hepatotoxicity and genotoxicity [14- 17]. However, the protective effects of Zn against CrVI-induced hepatotoxicity and genotoxicity in pregnant *Wistar* rats have not been investigated.

Zn is an essential element and an integral component of a large number of proteins and enzymes that participate actively in a broad spectrum of biological processes [18]. In addition, it was reported that Zn is involved in reducing ROS overproduction and restoring the mitochondrial membrane potential. Moreover, it acts as an anti-apoptotic agent and displays antioxidant and chemopreventive effect

against Cr- induced cytotoxicity and genotoxicity [19]. Its multi-protective effects were efficient against heavy metals-induced toxicities [20- 22]. Thus, in the present study, we aimed to investigate the potential hepatoprotective, genoprotective and cytoprotective effects of Zn against $K_2Cr_2O_7$-induced toxicity in the liver of pregnant *Wistar albino* rats.

MATERIALS

Potassium dichromate ($K_2Cr_2O_7$) and zinc chloride ($ZnCl_2$) were obtained from Sigma Aldrich (Chemie Gmbh, Taufkirchen, Germany). All chemicals were dissolved in sterile saline (NaCl 0.9%) and the pH was adjusted when necessary to 7.5. All other chemical products used in the experiment were of analytical grade.

Animals

In the current study, eighteen female *Wistar albino* rats with an average weight of 180- 250 g were utilized. They were obtained from Pasteur Institute, Algiers, Algeria. All animals were housed in polypropylene cages (3 rats/ cage) in favorable breeding environments of temperature (23 ± 1 ˚C) and 12 h light/ dark cycles. They were fed with a standard pellets diet (ONAB; Bejaia, Algeria) *ad libitum* and allowed free access to water during the experiment. After two weeks of acclimatization, female rats were kept with fertile males overnight to conceive. On the following day, pregnancy was confirmed by the appearance of spermatozoa in the vaginal mating smear of selected rats and it was designated as day zero of gestation. All experimental procedures were conducted in compliance with the International Guidelines for Laboratory of Animal Care and Use [23] and were approved by the Institutional Ethics Committee at Batna University.

METHODS

Experimental Design

Pregnant *Wistar albino* rats were divided randomly into three groups of six each; they were housed separately from day zero of gestation and treated subcutaneously (sc) on their 3rd gestational day as follows:
The 1st group (control): rats received a single injection (sc) of saline solution 0.9%. The 2nd group: rats were treated by 10 mg/ kg, sc. bw of $K_2Cr_2O_7$ [24, 25]. The 3rd group: rats were co- treated simultaneously by $K_2Cr_2O_7$ and $ZnCl_2$ (20 mg/ kg, sc. bw) [26].

Animals were anaesthetized with diethyl ether and blood samples were taken from the jugular vein in tubes containing heparin on the 6th and 20th day of gestation. Then, the tubes were centrifuged at 1500 × g for 15 min at 4 °C; plasma was recovered and stored at - 20°C until used for the assessment of plasma activities of hepatic enzymes. After the sacrifice on the 20th gestational day, liver tissues were excised, rinsed in ice-cold physiological saline and stored at - 20 ˚C until used for the evaluation of oxidative stress markers and DNA fragmentation.

Hepatic Biochemical Parameters Quantification

Plasma aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were determined by atomic spectrophotometry using Roche Cobas Integra ® 400 plus analyzer and commercial kits (Roche Diagnostics ®, Germany), according to the manufacturer's instructions. Results were expressed as U/L.

Malondialdehyde Assay

Lipid peroxidation was evaluated by the spectrophotometric analysis of MDA at a wavelength of 546 nm according to the method described previously [27].

Protein Carbonyl Assay

Carbonyl proteins content in liver homogenates was determined based on the evaluation of the reactivity of carbonyl proteins with 2, 4- dinitrophenylhydrasine. The optical density was read at 340 nm and results were expressed as nmol/ mg of proteins [28].

Liver Enzymatic Antioxidant Activities

Superoxide dismutase (SOD) activity was estimated at 540 nm, referring to the method of Beauchamp and Fridovich [29]. Results were expressed as IU/ mg of proteins. Catalase (CAT) activity was assayed following the method of Clairbone [30]; values were expressed as millimole of decomposed H_2O_2/min/mg of proteins.

Glutathione peroxidase (GPx) activity was determined spectrophotometrically at 412 nm, referring to the method of Flohe and Gunzler [31]. GPx activity was expressed in micromole of oxidized glutathione/ min/ mg of protein.

Alkaline Comet Assay

To evaluate the genotoxic potency of $K_2Cr_2O_7$, the alkaline comet assay was performed to detect DNA breakage in liver cells. Briefly, cell suspensions of the liver were prepared by the mean of phosphate-buffered saline solution. Then, the obtained cell suspensions were mixed with 0.5 % low melting agar and 80 µl of each final cell- agarose suspension was put in a thin layer on a microscope slide previously covered with 1% normal- melting agar. After submerging the slides in lysis buffer overnight at 4 ˚C, they were washed with deionized water and electrophoresed in alkaline solution (pH > 13) for 15 min at 300 mA and 25 V (0.90 V/ cm) to allow the DNA to unfold. Then, the

neutralization was processed with Tris buffer solution. Afterward, slides were stained with ethidium bromide (20 µg/ ml) and analyzed with a fluorescence microscope (Nicom Eclipse TE 300, Tokyo, Japan). Comets were classified into five classes (0 to 4) depending on the level of occurring DNA damage which was represented by the intensity of fluorescence in the comet tail [32]. 100 comet were visualized and scored on each slide and the total score was calculated according to the following equation: (percentage of cells in class 0×0) + (percentage of cells in class 1×1) + (percentage of cells in class 2×2) + (percentage of cells in class 3×3) + (percentage of cells in class 4×4) and it ranged for 100 comets from 0 to 400 [33].

Statistical Analysis

All data were presented as mean ± S.D (n = 6), statistical comparisons were carried out by one-way analysis of variance (ANOVA) for hepatic biochemical parameters and two-way ANOVA for the other parameters, then followed by Tukey's test as a post hoc test. All statistical analysis was performed using GraphPad Prism 7. The differences were considered significant when $p < 0.05$.

RESULTS

Effects of $ZnCl_2$ on Plasma Levels of Liver Function Markers of $K_2Cr_2O_7$- Treated of Pregnant *Wistar* Rats

The quantification of plasma ALT, AST, ALP and GGT on the 6th and 20th day of pregnancy revealed that $K_2Cr_2O_7$ administration disturbed the hepatic function and increased the activities of these enzymes significantly when compared with the control group. Indeed, plasma ALT increased significantly on the 6th (33.08 %, $p < 0.01$) and 20th days (21.58 %, $p < 0.05$) of pregnancy. Moreover, AST values increased significantly by 17.92 % ($p < 0.05$) and 54.23 % ($p < 0.001$) on both gestational days respectively. In addition, a significant increase of plasma ALP was marked on the 6th day (131.93 %, $p < 0.001$) and 20th day (130.06 %, $p < 0.001$) of pregnancy. Regarding plasma GGT, the values increased significantly by 15.92 % ($p < 0.005$) and 25.91 % ($p < 0.01$) on the 6th and 20th days of gestation respectively (Table 1).

Whereas the simultaneous co-treatment with $ZnCl_2$ decreased the activities of the enzymes significantly on both gestational days to near control values compared with $K_2Cr_2O_7$- treated group (Table 1).

Table 1. Effects of $ZnCl_2$ on hepatic biochemical parameters in $K_2Cr_2O_7$- treated pregnant *Wistar* rats

Hepatic Biochemical parameters		Control	$K_2Cr_2O_7$	$K_2Cr_2O_7$+ $ZnCl_2$
ALT (U/ L)	Day 6	43.13±5.13	57.4±6.04**	43.35±9.08^{++}
	Day 20	49.43±2.42	60.1±3.95*	43.58±8.80^{+++}
AST (U/ L)	Day 6	79.33±2.784	93.55±11.21*	91.04±7.99
	Day 20	69.86±3.42	107.75±8.74***	93.17±11.96^{+}
ALP (U/ L)	Day 6	62.65±7.50	145.31±22.44***	107.06±16.15^{+++}
	Day 20	59.3±10.58	136.43±16.90***	106.90±18.20^{++}
GGT (U/ L)	Day 6	18.21±2.85	21.11±1.96*	19.15±1.61
	Day 20	16.32±0.95	20.55±1.50**	17.51±1.63^{+}

Values are mean ± SD, (n = 6). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: significant when compared to control group, + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$: significant when compared to $K_2Cr_2O_7$ group.

Indeed, ALT values decreased by -24.47 % ($p < 0.01$) and -27.48 % ($p < 0.001$) on both gestational days respectively, plasma AST decreased significantly on the 20th day of pregnancy by -13.53 % ($p < 0.05$). Furthermore, the plasma ALP was decreased in a significant way on the 6th and 20th days by -26.32 % ($p < 0.001$) and -21.64 % ($p < 0.01$) respectively. Moreover, plasma GGT decreased significantly on the 20th of pregnancy by -14.79 ($p < 0.005$).

Effects of $ZnCl_2$ on Lipid Peroxidation, Protein Carbonylation and DNA Fragmentation Levels in Liver of $K_2Cr_2O_7$- Treated Pregnant *Wistar* Rats

Results in Table 2 showed that $K_2Cr_2O_7$ is a potent peroxidative, oxidative and genotoxic toxicant. It increased significantly ($p < 0.001$) the hepatic levels of MDA by 2771.52 %, protein carbonyls by 341.08 % and DNA

fragmentation by 378.83 %, when compared to the control group. However, $ZnCl_2$ co-administration exhibited protective effects. It decreased significantly ($p < 0.001$) the lipid, protein and DNA oxidative damage in liver homogenates by -38.4 %, 42.81 % and 26.08 % respectively when compared to $K_2Cr_2O_7$- treated group.

Table 2. Effects of $ZnCl_2$ on oxidative stress biomarkers in the liver of $K_2Cr_2O_7$- treated pregnant *Wistar* rats

Oxidative stress biomarkers	Control	$K_2Cr_2O_7$	$K_2Cr_2O_7$ + $ZnCl_2$
MDA level (µmol/ mg of proteins)	0.425±0.223	12.204±2.52***	7.517±1.628^{+++}
Protein carbonyls level (nmol/ mg of proteins)	2.478±0.55	10.93±1.57***	6.25±0.893^{+++}
Total score of DNA damage (arbitrary unit)	57.5±10.173	275.33±16.908***	203.5±30.76^{+++}

Values are mean ± SD, (n = 6). *** $p < 0.001$: significant when compared to control group, +++ $p < 0.001$: significant when compared to $K_2Cr_2O_7$ group.

Effects of $ZnCl_2$ on SOD, CAT and GPx Activities in Liver of $K_2Cr_2O_7$- Treated Pregnant *Wistar* Rats

Antioxidant enzymes play a crucial role in maintaining cellular redox status and protecting cellular components against oxidative deterioration. Hence, SOD, CAT and GPx activities were evaluated in liver tissues. Results in Table 3 showed that antioxidant enzymes activities were increased significantly ($p < 0.001$) in liver of pregnant *Wistar* rats exposed to $K_2Cr_2O_7$ when compared to the control group by 234.22 %, 341.08 % and 234.2 % for CAT, SOD and GPx activities, respectively. However, $ZnCl_2$ co- treatment attenuated the increment in the activities of these enzymes when compared to $K_2Cr_2O_7$- treated group. Indeed, CAT; SOD and GPx activities were decreased by -33.37 % ($p < 0.001$), 42.81 % ($p < 0.001$) and 13.07 % ($p < 0.05$).

Table 3. Effects of $ZnCl_2$ on antioxidant enzymes activities in liver of $K_2Cr_2O_7$- treated pregnant *Wistar* rats

Antioxidant enzymes activities	Control	$K_2Cr_2O_7$	$K_2Cr_2O_7$ + $ZnCl_2$
CAT activity (µmol/min/mg of protein)	270.57±36.90	904.32±92.39***	602.54±160.71^{+++}
SOD activity (µmol/min/mg of protein)	2.478±0.55	10.93±1.57***	6.25±0.893^{+++}
GPx activity (µmol/min/mg of protein)	10.73±1.48	35.86±4.008***	31.17±2.63^{+}

Values are mean ± SD, (n = 6). *** $p < 0.001$: significant when compared to control group, + $p < 0.05$; +++ $p < 0.001$: significant when compared to $K_2Cr_2O_7$ group.

DISCUSSION

The results of the present study revealed that the subcutaneous exposure to $K_2Cr_2O_7$ in pregnant *Wistar* rats provoked liver dysfunction characterized by an elevation in plasma levels of ALT, AST, ALP and GGT. The increased activities of these enzymes may reflect the occurrence of hepatocellular lesions which led to an increase release of these enzymes in the blood stream. Our results are in agreement with previous findings of other authors [3, 12]. In addition, $K_2Cr_2O_7$ hepatotoxicity was manifested by an enhanced lipid peroxidation and protein carbonylation. In fact, lipid peroxidation is considered to be one of the toxic mechanisms involved in CrVI- induced liver injury [3]. Indeed, an increase in MDA level, a down- regulation of the nuclear factor erythroid- 2 related factors- 2 gene and an up- regulation of nibrin gene were detected in hepatic and renal tissues of $K_2Cr_2O_7$- exposed rats [11]. Moreover, increased lipid peroxidation level may be attributed to the accumulation of Cr in the hepatic tissue as reported previously [34]. Furthermore, proteins oxidation is one of the deleterious outcomes of CrVI- induced toxicity; they are prone to the toxic action of ROS which may result in an increased cellular content of oxidatively modified proteins and subsequent cellular functions disruption [35]. The occurrence of protein oxidative modification in liver tissues upon $K_2Cr_2O_7$ exposure was confirmed by the elevated protein carbonyls content as seen in the present experiment

and as reported before [12]. In order to counteract ROS-mediated cellular damage, biological systems have implicated an antioxidant enzymatic defense system that can scavenge ROS and protect cellular biomolecules. Therefore, an increment of the activities of SOD, CAT and GPx in liver tissue was detected in the current study. Since these enzymes are involved in the neutralization of ROS, the increase of their activities may be considered as a coping response to ROS overproduction. Accordingly, it has been reported by several authors the induction of antioxidant enzymatic activities upon CrVI- administration in hepatic, cerebral and thyroidal tissues [3, 12, 36, 37]. Indeed, CrVI- induced oxidative stress in hepatocytes may lead to mitochondrial dysfunction, genomic changes and subsequent cell death [38]. CrVI compounds are potent genotoxic agents, mutagens and chromosomal aberrations inducers [39]. DNA damage measured by the alkaline comet assay in the present study showed that the subcutaneous exposure to $K_2Cr_2O_7$ induced DNA fragmentation in liver of pregnant *Wistar* rats, which concords with earlier studies reporting the genotoxic potency of CrVI compounds [40, 41, 37]. CrVI- induced genotoxicity and cytotoxicity could be linked to its ability to induce genomic oxidative damage by the mean of generating ROS and reactive chromium reduction intermediates, such as CrV and CrIV, during its cellular reduction cascade. Subsequently, these reactive species are able to attack biological macromolecules leading to their structural and functional deterioration; their interaction with DNA and proteins are susceptible to cause chromosomal abnormalities, which may eventually alter cellular integrity and functions [42]. Multiple mechanisms are thought to be involved in CrVI- induced genotoxicity including DNA damage, chromosomal aberrations and micronuclei formation. Moreover, CrVI acts as a gene expression disruptor. It binds to DNA and affects genome stability by interacting with the base paring and stacking process, allowing mutations to occur. Furthermore, DNA modification caused by the covalent attachment of a chemical, DNA protein cross-links and DNA–DNA cross-links, abasic sites and oxidized DNA bases is implied in CrVI- induced mutagenicity in vivo and in vitro [43- 45]. Since the generation of oxidative stress is one of the key mechanisms behind CrVI- induced hepatotoxicity, genotoxicity and cytotoxicity, the antioxidant properties of Zn may enable this microelement to reduce oxidative stress and counteract DNA oxidative damage [46]. Besides its antioxidant potential, Zn is known to possess antigenotoxic and anti-carcinogenic effects *in vivo* and *in vitro* against genome damage generated upon exposure to genotoxins [47]. Also, Zn plays a role in DNA stability by regulating the expression of DNA repair genes via zinc-finger transcription factors [48]. Indeed, Zn has been shown to counteract the toxicity of heavy metals through several mechanisms [49]. Hence, the protective effect of Zn can be attributed mainly to its vital role as a free radicals scavenger. It was found to counteract nickel-induced neurotoxicity by mitigating glutathione and lipid peroxidation [50]. In addition, the enhancement of Zn intake prevented the oxidative/anti-oxidative imbalance and yielded protective effects against injury of macromolecules in the nervous system under cadmium exposure [51]. Evenly, it acts as a cofactor and a regulator of many antioxidant enzymes [52, 53]. It also reduces heavy metals toxicity by inducing metallothionein and preventing metal bioaccumulation in the organism [54, 55]. Furthermore, Zn has a marked impact on maintaining DNA integrity by preventing oxidative damage and promoting its repair [56].

CONCLUSION

In the current work, the subcutaneous exposure to $K_2Cr_2O_7$ in pregnant *Wistar* rats provoked hepatotoxicity by disturbing liver function markers, inducing hepatic oxidative stress and DNA fragmentation. These toxic effects induced by $K_2Cr_2O_7$ exposure were effectively mitigated by the simultaneous co-treatment of $ZnCl_2$. Indeed, Zn has exhibited hepatoprotective, antioxidant and genoprotective efficacy against $K_2Cr_2O_7$ toxicity in the liver of pregnant *Wistar* rats.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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