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Protective Effect of Melatonin Against Chromium-Induced Hepatotoxic and Genotoxic Effect in Albino Rats

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Abstract: Chromium is a widespread environmental waste. It is an industrial contaminant with teratogenic, mutagenic and carcinogenic effects on animals and human. This study was carried out to evaluate the potential protective effect of melatonin on the hepatotoxicity and genotoxicity generated by potassium dichromate ($K_2Cr_2O_7$) in albino rats. Rats were divided into four groups; control, melatonin (10 mg/kg b.wt.), melatonin pretreated with single S/C injection of $K_2Cr_2O_7$ (15 mg/kg b.wt.) and potassium dichromate treated group. Rats were sacrificed 24 h after $K_2Cr_2O_7$ treatment. $K_2Cr_2O_7$ treated rats showed significant ($P<0.05$) increase in the hepatic marker enzymes activity (aspartate aminotransferase-AST and alanine aminotransferase-ALT) and serum bilirubin (total and direct) was detected. Significant ($P<0.05$) decrease in the serum total protein and albumin was observed after $K_2Cr_2O_7$ treatment. Meanwhile, serum globulin, A/G ratio and indirect bilirubin showed no significant change. Hepatic DNA damage was observed using comet assay. The histological alterations confirmed the previous results. Melatonin pretreated rats showed an amelioration of the adverse effects of $K_2Cr_2O_7$ toxicity. An improvement in the serum hepatic enzymes (AST and ALT), proteinogram (total protein and albumin) and bilirubin (total and direct) was observed. Melatonin pre-treatment showed significant improvement in the hepatic DNA and the histopathological picture. It could be concluded that potassium dichromate is hepatotoxic and genotoxic. Melatonin has a potential protective effect to reverse the DNA damage and has the ability to improve the hepatic function associated with $K_2Cr_2O_7$ intoxication.

Key words: Chromium · Hepatotoxicity · Genotoxicity · Melatonin

INTRODUCTION

Chromium is a transition metal which can be found in many compounds of Earth's crust. It is ranked the 21st in elemental abundance. Chromium comes from several anthropogenic sources such as chemical, metallurgical and refractory industry [1]. It is responsible for various health hazards including cancers, dermatitis, damage to the liver and kidney, infertility in both males and females, defects in embryo and developmental problems in young children [2].

Melatonin (N-acetyl-5-methoxytryptamine) is the main product of the pineal gland in both animals and human. It plays a major role in the neuroimmuno-endocrine system. Melatonin participates in many vital physiological functions such as anti-inflammatory and a broad spectrum antioxidant. It decreases the level of free radical by stimulating the activities of enzymes involved in the antioxidative defense [3]. It protects the liver in several models of liver injury via inhibiting the oxidative and the nitrosative damage [4]. Its protection against the oxidative damage is enhanced by its amphiphilic nature. The melatonin molecule is readily access to all cell compartments including the nucleus [5]. Melatonin stimulates the immune response and exhibits immunomodulatory effects [6]. The immunomodulatory properties include the inhibition of the expression of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) to exert marked anti-inflammatory effect [7]. It also mitigates the oxidative stress and the vital organ damage induced by heavy metals and snake venom toxicity [8, 9].

Intensive research over the last two decades has reported the beneficial protective effect of melatonin in a multitude of pathological processes. It is considered as an immunostimulant for both humeral and cellular immunity

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[10]. No previous studies reported the protective effect of melatonin against chromium induced hepatoxicity. Therefore, in the present investigation, an attempt was made to evaluate the protective effect of melatonin on potassium dichromate mediated hepatotoxicity in order to study the role of melatonin in the amelioration of the chromium intoxication.

MATERIALS AND METHODS

Chemicals: Melatonin was obtained from Sigma-Aldrich (product number M5250). Melatonin was administered as single intraperitoneal injection 10 mg/kg b.wt., daily for 10 successive days [11] before induction of acute toxicity. Potassium dichromate ($K_2Cr_2O_7$) was obtained from Sigma-Aldrich (product number 207802). It was used to induce acute toxicity. Rats were given a single subcutaneous (S/C) injection of $K_2Cr_2O_7$ at a dose of 15 mg/kg b.wt. [12].

Experimental Animals: Thirty two female albino rats weighing 150-200 g were obtained from the Laboratory Animal House, Helwan, Egypt. Throughout the experiment, all animals were housed in clean hygienic metal cages inside environmentally controlled room and kept under a uniform laboratory condition. All rats were administered balanced ration and water free from medication. The food and drinking water were allowed *ad.libitum* throughout the experimental period. Rats were maintained under standardized hygienic conditions (12:12 h light/dark cycles, the temperature was 24±2 °C and a minimum relative humidity of 45%) throughout the experimental period. All rats were acclimatized for 2 weeks before starting the experiment. The study was complied with the Animal Welfare Act. The National Research Council Committee guidelines were followed.

Experimental Design: Rats were divided into four groups; Group I, served as control. Group II, melatonin was IP administered daily at a dose of 10 mg/kg b.wt for 10 days. Group III, melatonin pretreated for 10 days then subcutaneous (S/C) injected with a single dose of $K_2Cr_2O_7$ 15 mg/kg b.wt. Group IV, was S/C administered $K_2Cr_2O_7$, 15 mg/kg b.wt. dissolved in distilled water. The samples were collected 24 h post $K_2Cr_2O_7$ injection.

Blood and Liver Samples: Rats from all groups were autopsied under light ether anesthesia 24 h post $K_2Cr_2O_7$ injection. The blood was drawn from the retro-orbital venous sinus using capillary tubes. Blood was allowed to flow smoothly into the tubes, left at room temperature for 2 hours to clot then centrifuged at 3000 rpm for 15 min. The clear supernatant serum was collected using sterile Pasteur pipettes. Serum was transferred to sterile, dry labeled eppendorf tubes for chemical analysis. Liver from all groups was removed, washed with saline and cut into two parts, one part was preserved at -20°C for comet assay and the other part was preserved in 10% neutral-buffered formalin for the histological investigation.

Biochemical Analysis: All biochemical analysis was calorimetrically measured using commercial kits provided by bioMérieux, France. The analysis was done using a spectrophotometer (5010 v5C, RIELE GmbH, Berlin, Germany).

Serum Enzymes: Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) activity were determined [13].

Serum Proteins: Serum total protein was colometrically measured [14]. Serum albumin was colometrically measured [15]. Serum globulin level was determined by subtracting the albumin from the total proteins. Albumin/Globulin ratio (A/G ratio) was measured using albumin and globulin values.

Serum Bilirubin: Serum total and direct bilirubin were measured [16]. The indirect bilirubin was measured through subtracting the obtained direct bilirubin from the total bilirubin.

Comet Assay for Detection of Hepatic DNA Damage: The alkaline comet assay was done using single cell gel electrophoresis according to [17]. Briefly, liver tissue (1 g) was crushed and transferred to 1 mL ice-cold PBS, pH 7.4 and homogenized with a Potter-Elvehjem type-homogenizer (Ikemoto Scientific Technology, Tokyo, Japan). The suspension was centrifuged at 500 RPM/ 5 min; the supernatant was aspirated and repeat centrifugation for 3 times in PBS. The last suspension was centrifuged at 3000 RPM/10 min to sediment hepatic cells. An aliquot of the cell suspension was mixed with 0.5% low melting agarose then spread using a cover slide on pre-coated slides with 1% normal-melting agarose, freeze for 2min then remove the cover slide. Then slides were immersed in a lysis buffer over night. The slides were transferred to electrophoresis buffer in electrophoresis chamber. The current of electrophorsis was adjusted to 300mA for 30 min.

Slides were stained with with 1% ethidium bromide at 4 °C, then examined under a fluorescence microscope. About 50 to 100 random cells were selected and analyzed per each sample and analyzed by Comet Score™ software to calibrate the comet parameters. The software was used to evaluate the quantitative and qualitative DNA damage in the cells by measuring the tail length, tail DNA percentage and tail moment.

Histopathological Study: Rats were anesthetized with diethyl ether and sacrificed. A portion of liver tissue from all groups was collected and immediately prepared for hisopathological examination [18]. Simply, the specimen labeled and fixed in 10 % neutral buffered formalin solution overnight. Fixed tissues were processed after washing and preserved in 70 % ethanol, dehydrated in a series of graded concentrations of ethyl alcohol and cleared in xylene. Tissue were embedded in paraffin waxes (melted paraffin at 55–60 °C), casting and cutting at 4-5 µm thickness. These sections were placed on top of glass slides, routinely stained with Hematoxylin and Eosin (H and E) to be examined and visualized under the microscope.

Statistical Analysis: System software package was used to analyze the data by one-way analysis of variance, ANOVA [19]. All data showed a normal distribution and passed equal variance testing. The Data were expressed as the means ± standard error (SE). Significant differences between means were determined at a level of ($P<0.05$) by post-hoc Duncan's test for comparison between different experimental groups.

RESULTS

The Activity of the Hepatic Marker Enzymes (AST and ALT) of all Treated TM Rats was Evaluated: Group IV ($K_2Cr_2O_7$ treated rats) showed a significant ($P<0.05$) increase in the activities of the hepatic enzymes comparing with the control. Group II (melatonin treated) showed a non significant change in the activities of these enzymes comparing with the control. This indicates the safe use of melatonin as a natural product. Group III (melatonin pretreated) showed significant ($P<0.05$) decrease in the activity of these hepatic enzymes in the serum when compared to the group IV ($K_2Cr_2O_7$ treated). The ameliorative effect of the melatonin pre-treatment was pronounced (Figure 1).

Assessment of Proteinogram Level of all Treated Rats: Group IV ($K_2Cr_2O_7$ treated rats) showed a significant ($P<0.05$) decrease in the level of serum total protein and albumin comparing with the control. Group II (melatonin treated) showed a non significant change when compared with the control. Group III (melatonin pretreated) showed significant ($P<0.05$) increase in the level of serum total protein and albumin when compared to the group IV ($K_2Cr_2O_7$ treated). The serum globulin level and A/G ratio showed a non significant change in all treated groups (Figure 2).

The Level of Serum Bilirubin: Group IV ($K_2Cr_2O_7$ treated rats) showed a significant ($P<0.05$) increase in the level of serum bilirubin (total and indirect) comparing with the control. Groups II and III showed a non significant change in the serum level of total and direct bilirubin

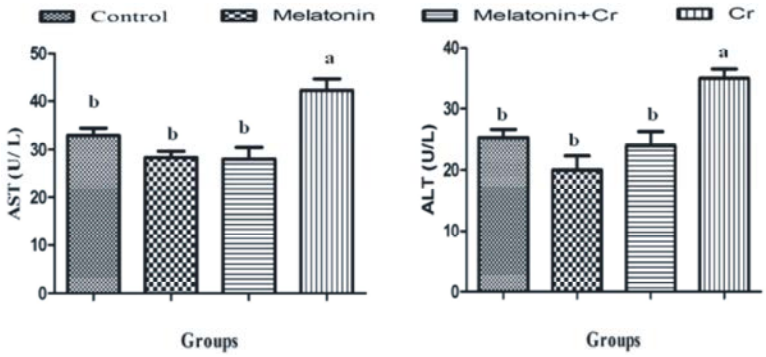

Fig. 1: Effect of pretreatment of melatonin on the hepatic serum enzymes (AST and ALT). Melatonin treated and pretreated rats, showed a non significant change comparing with the control. Group IV showed a significant ($P<0.05$) increase in the AST and ALT level comparing with the control. Bars with different letters are expressed at ($P < 0.05$) in means ± SE.

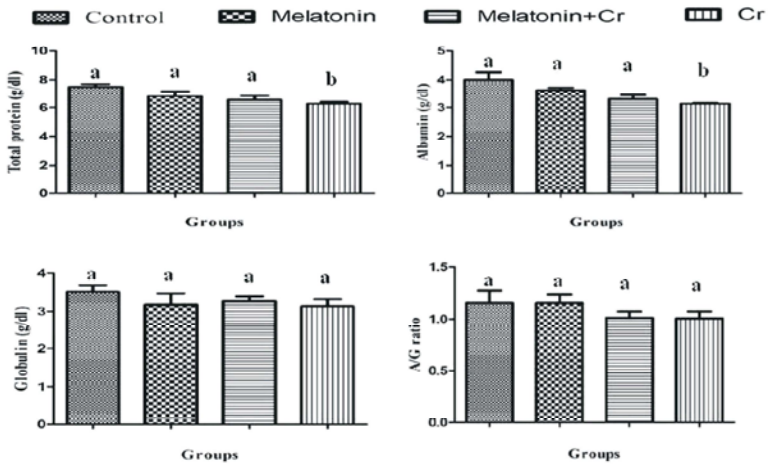

Fig. 2: Effect of pretreatment of melatonin on proteinogram. Melatonin treated and pretreated rats, showed a non significant change in the proteinogram comparing with the control. Group IV showed a significant (P<0.05) decrease in the level of total protein and albumin comparing with the control. Bars with different letters are expressed at (P < 0.05) in means ± SE.

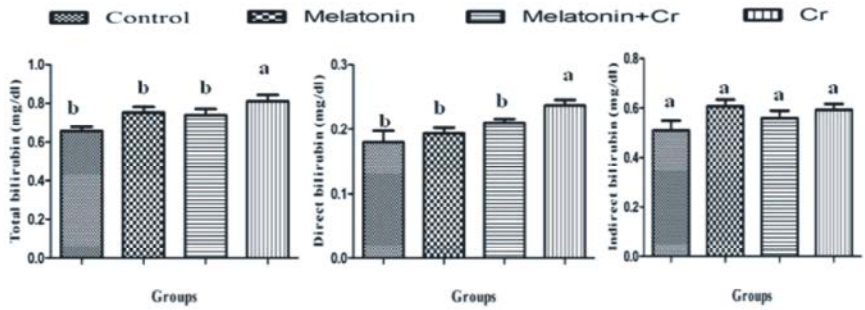

Fig. 3: Effect of pretreatment of melatonin on serum bilirubin level. Melatonin treated and pretreated rats, showed a non significant change in serum bilirubin (total, direct and indirect) comparing with the control. Group IV showed a significant (P<0.05) increase in the level of total and direct bilirubin comparing with the control. Bars with different letters are expressed at (P < 0.05) in means ± SE.

comparing with the control. The level of the serum indirect bilirubin showed a non significant change in all treated groups (Figure 3).

Effect of Melatonin on Cr-induced-DNA Damage in Liver Was Determined: Control and melatonin treated rats showed normal hepatic cells as no hepatic DNA damage was noticed (Figure 4 A and B). Meanwhile, melatonin pre-treated rats showed an improvement in the comet parameters (tail percentage and length, beside the DNA tail percentage and moment) (Figure 4C). Pre-treatment with melatonin restored the DNA damage parameters in the liver to near control. Cr induced an increase in DNA damage as indicated by a significant increase in the tail percentage and length beside an increase in the tail moment in the liver of the Cr-treated rats, group IV with marked damaging effect on the DNA (Figure 4D).

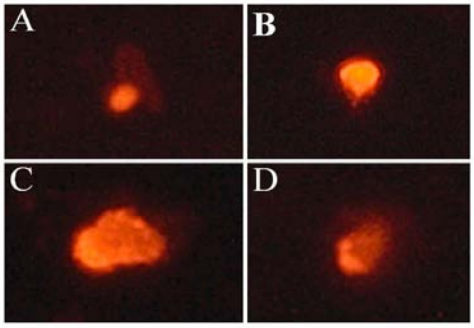

Fig. 4: Hepatic cell images of the comet assay. A) Control. B) Melatonin treated rats. C) Melatonin pretreated group. D) Chromium treated group.

Histopathological Examination of the Hepatic Tissue: Normal liver structure was observed in the control group (Figure 5A). Group II, melatonin treated, the hepatic tissue

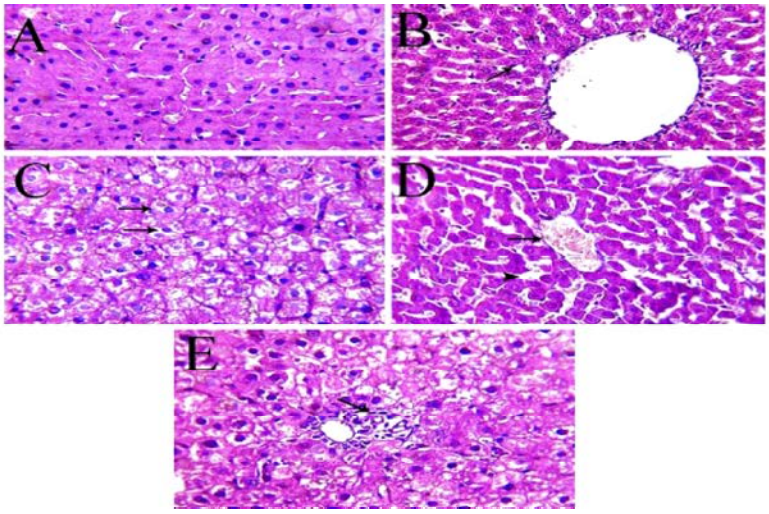

Fig. 5: Histopathological finding of the hepatic tissue, HE x400. A) Control group showed normal hepatic architecture. B) Melatonin treated, showing mild proliferation of the hepatic Kupffer cells (arrow). C) Melatonin pretreatment, showing swollen hepatocytes with intact nuclei (arrows). D) Chromium treated group showing congested central vein (arrow) and dilation of the hepatic sinusoids (arrow head). E) Chromium treated group, showing moderate mononuclear cells infiltration in the portal area (arrow).

appeared normal with mild proliferation of the hepatic Kupffer cells (Figure 5B). The liver in group III, melatonin-pretreated, showed nearly normal architecture with moderate degenerative changes of hepatocytes which appeared swollen with intact nuclei (Figure 5C). In addition, moderate congestion was seen in the central vein. Severe liver damage was detected in group IV, Cr treated, with congestion of the hepatic blood vessels and dilation of hepatic sinusoids was seen throughout the hepatic tissue (Figure 5D). The portal area showed moderate mononuclear cells infiltration (Figure 5E).

DISCUSSION

Melatonin has a number of beneficial effects in the treatment of various types of diseases. This study investigated the protective effect of melatonin against Cr-induced hepatotoxicity. The transaminases (AST and ALT) are considered the most sensitive biomarkers to evaluate the extent of the toxicity and the cellular damage. The hepatotoxicity of Cr resulted in increase the activity of these hepatic enzymes (AST and ALT). Because these enzymes are located in the hepatic cytoplasm and after cellular damage they are released in the blood stream [20]. Chromium (Cr) might be responsible for leakage of these enzymes from the liver cytosol into the circulation or it might be due to disturbance in their biosynthesis due to the liver dysfunction with alteration in the permeability of the hepatic membrane [21]. Our results were confirmed by the histopathological observations. Significant decrease in the serum AST and ALT levels was observed with melatonin pre-treatment. Protective effects of melatonin in decreasing the level of these hepatic markers correlated with the histological findings. Our results are in accordance with A'maal *et al.* [22] where similar decrease was reported in these hepatic marker in rats administered melatonin as protective agent against chlorpromazine-induced liver disease in rats.

Serum total protein, albumin and globulin reflect the damage in the hepatocytes. In the present study, serum total protein and albumin were significantly decreased in Cr-treated rats. These results demonstrate that Cr toxicity was associated with hepatotoxicity. This may be due to the impairment in their synthesis or poor liver function [21]. Similar results were observed [23, 24]. On the other hand, a significant increase in the level of serum total protein and albumin was observed in rats pretreated with melatonin. Our results were confirmed with the histopathological investigation. In the present study, the hepatic histoarchitecture of the chromium-treated rats resulted in congestion of the hepatic blood vessels, dilation of hepatic sinusoids and mononuclear cells infiltration throughout the hepatic

tissue. This may be attributed to that melatonin decreases the possess of lipid peroxidation and increases the activities of the plasma protein thiols [25].

The increase in the serum total bilirubin in Cr-treated rats, group IV, was due to the increase in the direct bilirubin level. It might be result from a decrease in the hepatic uptake or conjugation [26]. Similar results were observed [27]. They reported that chromium toxicity is responsible for jaundice as it increase the total and direct bilirubin. On the other hand, melatonin prevented the increase in the serum total and direct bilirubin. Our results were similar to Ogeturk *et al.* [28] who reported that melatonin administration to carbon tetrachloride (CCL4) treated rats, significantly reduced the increase in the total and conjugated bilirubin.

The present study showed that the K_2CrO_2 is genotoxic. DNA damage was observed in hepatic cells of Cr-treated rats. The DNA damage in the liver could be due to the elevated levels of reactive oxygen species (ROS) which attack the DNA and damage its integrity. Chromium might binds to the macromolecules and forms adducts with the thiol groups on proteins to creates DNA adducts and breakage the DNA strand [29]. Our results are corroborated with the earlier reports [2]. Melatonin pre-treatment protected the liver tissue from the damage-induced by chromium. The result could be due to that melatonin protects the cellular DNA from damage and stimulates the DNA polymerase enzymes, which are responsible for DNA repair [30]. Our results are similar to Conti *et al.* [31] who reported that melatonin was able to repair the hepatic DNA damage.

CONCLUSIONS

Our findings provide evidence of the protective effect of melatonin. Melatonin restored the hepatic toxicity that induced by chromium. The restoration of hepatic activity that was induced by melatonin was evaluated by the significant improvements in the liver function and the histological architecture. There are currently no data on the treatment of Cr-induced hepatic toxicity with melatonin that would indicate whether this substance has a significant clinical impact. We have elucidated a potential role for melatonin in the treatment of hepatic toxicity that needs to be intensively investigated in the future research. Our results provide evidence of the beneficial effects of melatonin in ameliorating chromium-induced hepatic damage. The lack of toxicity of melatonin makes it of interest for clinical applications and it may be of potential use as part of the treatment protocol for chromium toxicity in the future.

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