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Investigation of Cadmium Toxicity in Mice Spleen Cells

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Abstract

Background: *Cadmium (Cd) is a heavy metal which is widely spread in our surroundings and has a very strong ability to accumulate in the body organs such as spleen. In this study we aimed to determine the genotoxicity and cytotoxicity effects on mice spleen treated with cadmium.*

Methods: *Thirty male mice were enrolled in this study and kept in standard conditions. Mice were randomly divided into2 experimental groups (control and treatment). The treatment group was exposed to Cd intraperitoneally (i.p) (300µm/kg) at different time intervals (0, 6, 12, 24, 48 hrs). Twenty four hours after the last exposer mice were killed and the spleen was removed, then for studying cytotoxicity, oxidative stress markers namely malondialdehide (MDA), glutathione (GSH) and superoxide dismutase (SOD) were assayed on homogenized spleen, and comet assay was applied on isolated spleen cells for genotoxicity & DNA damage studies. Statistical analyses (T-test and ANOVA) were performed using SPSS 15 software.*

Results: *The concentration of MDA and GSH in control group spleen cells were 278.01±35.30 nmol/g.pr and 16.61±4.89 µmol/g.pr and for Cd- treated spleen cells were 612.24±32.87 nmol/g.pr and 32.52±4.22 µmol/g.pr, respectively which were statistically significant (P<0.003). In addition, SOD activity in control and Cd exposed spleen cells were 69.75±3.12 and 226.91±3.40 U/mg.pr (P<0.001). The comet assay include content comet length, tail length and head diameter showed DNA breakage in treated group which was not observed in the control group.*

Conclusions: *The results demonstrate that Cd has the ability to induce genotoxicity in spleen cells. Moreover, our results show that it is plausible to expect Cd induced -cytotoxicity in spleen cells.*

Keywords: Cadmium, Spleen, Comet assay, Oxidative stress, DNA damage.
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Introduction

One of the most toxic heavy metals in our surroundings is Cadmium (Cd). Cd and its corresponding salts, such as cadmium chloride, can accumulate in internal organs. Because they have been widely used by other metals, such as lead, copper, zinc smelter, alkaline accumulator and paint and plastic industries, they are one of the most prevalent air and water pollutants.[1]

In our previous studies, we reported Cd toxicity, and it has been greatly studied and reported by the other studies. According to its accumulation in organs, exposure to Cd causes damage to living organs, especially in humans, which leads to Itai-Itai disease.[2,3] Cd and all Cd compounds have been suggested as human carcinogenic elements.[4]

Owing to industrial production development, main sources of exposure occur mainly by inhalation,[5] cigarette smoking, and alcoholic beverages.[6] In addition, Cd compounds can enter the body by working with them, such as oral or dermal contact.

The spleen is an organ that has a very strong ability to accumulate Cd, and this can be dangerous for the spleen. Brzoska et al.[7] Shows that chronic exposure to Cd compounds can damage the renal proximal tubular epithelial cells as a result of dysfunctional proximal tubular manifested by low molecular weight proteinuria, glucosuria, aminoaciduria, and phosphaturia.[7-9]

According to the reported studies, Cd and its salts are unable to generate damage themselves, and an association is suggested between Cd and free radicals, but the mechanism and pathway for the toxic effects of Cd are not yet well understood.[10] The relationship between Cd and oxidative stress is shown by many studies, and compounds with this metal reduce the antioxidant system in animals. Mostly this is due to the decreased levels of glutathione and lipid peroxidation.[11,12]

Animal studies have shown that Cd can act as an inducer for reactive oxygen species (ROS) generation[13] along with hydrogen peroxide[14] and hydroxyl radicals[15] production. These free radicals amplify lipid peroxidation and DNA damage and alter calcium and sulfhydryl homeostasis.[16-18] These free radicals also affect cellular function by disturbing signal transduction such as protein kinase C (PKC), mutagen activated protein kinase (MAPK), and cyclic AMP pathway; however, the exact mechanism is not completely understood. [19,20]

Lipid peroxidation is the primary mechanism for Cd-induced toxicity.[21] As a result of the Fenton reaction, oxidative stress produces hydroxyl radical species that are believed to initiate lipid peroxidation.[10,22] Following this process, free radicals are produced, attacking any available molecule in intra- or extra cellular environment and leading to cellular damage.[10,22]

These damages are enhanced when the antioxidant defense systems, such as super oxide dismutase (SOD), Catalase (CAT), or reduced GSH, have been suppressed owing to an increase in ROS generation.[23,24]

Since the spleen is one of the main organs where Cd accumulate,[7,25] in this study, we aim to investigate the spleen

damage induced by Cd, which was related to the oxidative damage and DNA breakage in the spleen cells.

Materials and Methods

Animals: Twenty male mice with the age of 5-6 weeks old and initial body weight of 30±5 g were obtained from the laboratory animal house of Baqiyatallah University Medical of Sciences. The mice were kept under standard conditions (temperature 23±2°C, natural light-dark cycle). The mice were randomly divided into two groups (control & treatment)—housed in four plastic cages, five mice per cage—had access to drinking water and a standard diet for one week. Then, the mice were exposed by peritoneal injection five times to cadmium chloride at a dose of 300 µm/kg b.wt dissolved in 0.2-ml distilled water, at time intervals 0, 6, 12, 24, and 48 h. The control group received 0.2 ml of 0.9% normal saline solution as a placebo.

Tissue preparation: Twenty hours after the last injection, the animals were anesthetized by chloroform and their spleens were rapidly obtained. These tissues were transferred to 3-ml ice-cold PBS for biochemical and comet assays.

Biochemical assay: The tissues stored in the PBS were minced, and each part was homogenized according to the analytic assay protocol.

Total glutathione (GSH) concentration was estimated by CUSABIO BIOTECH CO, Rat Glutathione Peroxidase (GSH-PX) ELISA Kit Catalog No. CSB-E12146r (96T). The assay was performed according to the instructions provided by the manufacturer.

Malondialdehide (MDA) concentration was measured based on the method of OXItek TBARS Assay Kit ZMC Catalog: 0801192.

Superoxide dismutase (SOD) activity was determined following to the Kamiya Biomedical Company kit for the measurement of Superoxide Dismutase (SOD) Inhibition Activity (K-ASSAY, SOD Assay KT-219 (100 tests)).

Total protein was measured following the brad-ford method.

DNA damage assessment using the comet assay: Single cell gel electrophoresis/ comet assay was performed for rapid genotoxicity assessment according to the following method: 1.0% agarose (500 mg per 50-ml PBS), 0.5% LMPA (250 mg per 50 ml PBS), and 1.0% NMA (500 mg per 50 ml in Milli Q water) were prepared using a microwave or heater until near boiling and the agarose dissolved. For LMPA, aliquots of 5 mL samples were poured into scintillation vials and refrigerated until needed. MPA vials were placed in a 37°C water bath to warm and stabilize the temperature.

In the next step, a suspension of 500 - 1000 isolated spleen cells in 100 µL of 0.5% LMPA was prepared and mounted on the upper side of a 1.0% agarose dipped slide. Prepared slides were placed in a refrigerator for 20 minutes to dry, and then covered by lysing solution (tries-base 10 mM-pH=8, 1%SDS, 1%triton X100) for 2 h at 4°C.

After at least 2 h at ~4°C, slides was gently removed from the lysing solution and placed on the side on the horizontal gel box near one end, and sliding them as close together as possible. The buffer reservoirs were filled with a freshly made (pH>13) Electrophoresis Buffer until the liquid level completely covers the slides (avoids bubbles over the agarose). Slides were set in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the appearance of alkali–labile damage. The power was turned on and the voltage was adjusted to 24 volts (~0.74 V/cm), the current was set to 300 milliamperes by raising or lowering the buffer level, and the slides were electrophoresed for 30 mins. Then the slides were gently lifted from the buffer and placed on a drain tray. The slides were dropped wise coated with a neutralization buffer, and the coating was allowed to dry for at least five minutes. Then the slides were drained and the process was repeated two more times.

Prepared slides were stained with 80µL 1X Ethidium Bromide, left for 5 min, and then dipped in chilled distilled water to remove any excess staining solution. The stained slides were assessed by a fluorescent microscope (Nikon) to determine the DNA breakage. Captured image was analyzed by comet score software to determine quantifiable DNA breaking index factors (Tail length, %DNA in Tail, Head diameter, %DNA in head).

Statistical analysis: All data were expressed as mean±SD for 10 experiments in each group (n=10). Statistical analysis was performed with the T-test. Furthermore, experimental groups were compared using a one-way analysis of variance (ANOVA). We used SPSS version 15.0 for analysis, and $P<0.05$ was considered as significant.

In the groups that exposed to the Cd, our results showed an increase in the activity of SOD in compare with the control group ($P<0.001$). Moreover, the concentrations of MDA and GSH had shown a significant increase in compare with control group ($P<0.003$).

Results

In the groups that were exposed to the Cd, our results showed an increase in the activity of SOD in comparison with the control group ($P<0.001$). In addition, the concentrations of MDA and GSH showed a significant increase in comparison with the control group ($P<0.003$) (Table1).

Table1. SOD activity and GSH& MDA concentrations in homogenized spleen

Groups	GSH(µmol/g.pr)	MDA(nmol/g.pr)	SOD(U/mg.pr)
Control	16.61±4.89	278.01±35.30	69.75±3.12
Treatment	32.52±4.22*	612.24±32.87*	226.91±3.40**

* significant with control group ($P<0.003$)

** significant with control group ($P<0.001$)

By using this method, we found that DNA of spleen cells that were exposed to Cd were damaged, and moved to a tail from nucleus during the alkaline electrophoresis. (Figure 1).

Output results from the comet score software showed that Cd has a potential for DNA breakage (Figure 2). This diagram shows DNA amount in the nucleus (DNA in Head), DNA

movement during the Comet (Comet Length), and DNA amount in the tail. In this diagram, we show that the DNA tail in the Cd-exposed group has a significant increase in comparison with the control group (P<0.03).

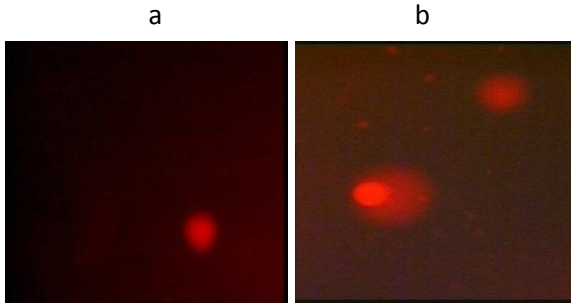

Figure 1. Image from comet assay on spleen cells. (a) A normal spleen cell: this image fails to show any DNA breakage and movement. (b) Spleen cells exposed to Cd: the DNA from these cells has significant breakage and movement that showed following the comet assay.

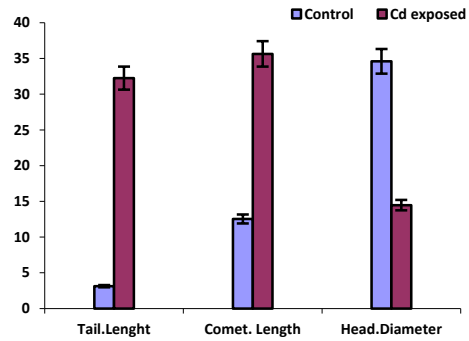

Figure 2. Analysis of comet picture with comet score software. This diagram showed DNA amount in nucleus (DNA in Head), DNA movement during the Comet (Comet Length) and DNA amount in tail. The result showed significant changes in treated group in compare with control group.

Discussion

Spleen has been recognized as a critical target organ of Cd accumulation. The present study was undertaken to assess the oxidative status, DNA damage, and cytotoxicity of a spleen after acute exposure to Cd. The mechanism of Cd-induced spleen damage has been related to increased oxidative status. Increased reactive oxygen species (ROS) production seems to be induced by the interaction of Cd with the mitochondrial structure.[26]

A comet assay was applied for detection of DNA damage in spleen cells.

A substantial alteration in GSH and MDA levels owing to Cd treatment has been reported previously.[26-28] Several studies have demonstrated that Cd exposure is associated with increased production of super oxide anions[29] and MDA[30] as well as decreased the tissue levels of GSH. (10). Thus, in the present study, we have endeavored to probe significant relationships between the spleen GSH, MDA, and SOD following acute peritoneal expose to Cd.

In our study, we have observed that MDA significantly increased in mice that were exposed to Cd in comparison with the control group. These results suggested that the spleen oxidative stress increased following an acute exposure to Cd, which has been reported previously by Babu et al.[30]

According to our results, the increase of spleen GSH could be explained by the spleen's stimulation to neutralize the increased oxygen free radicals in an acute exposing condition, which is in contrast with Murugavel.[10]

Accordingly, the that spleen content of SOD increased, which is not in agreement with Bin Xu et al.[31]

This different result in SOD activity can be explained in two different ways: first, condition of exposure. In this survey, we used an acute exposure; however, Bin Xu et al,[31] used a chronic exposure method. Second, in acute exposure, Cd is not able to interact between Zn and Cu at the active site of SOD; however, in chronic exposure, Cd has the ability to interact with Zn and Cu and inhibits the SOD activity.[7,25,32-36] Although, increased oxygen free radicals were associated with increased levels of antioxidant enzymes.

Cd-induced toxicity increasing MDA concentration in the spleen indicates that an elevation of lipid peroxidation in this organ due to the oxidative stress. Enhancing peroxidation of lipid intra- and extra-cellular, explains the damage to the cells, tissues, and organs that may be due to inability of antioxidant defense systems.

The result of comet assay on the spleen cells showed obvious DNA breakage in treated mice that was not seen in control group. This indicates that Cd can act as a carcinogen and mutagen.

Therefore, we conclude that Cd has ability to accumulate in spleen cells and could be harmful for nucleus and cell organelles. Therefore, we suggest appropriate screening of individuals who are exposed to Cd and regular follow up for any damage and improvement of the antioxidant defense system.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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