Ameliorative Role of Grape Seed Extract on Cadmium Induced Splenic Toxicity in Albino Rats

Adel Alkhedaide¹, ELkhateeb SA², Abass MA², Emam MA³, Amal S El-Shal⁴, Attia HF³

¹Medical Laboratory Dept., Faculty of Applied Medical Science, Taif University - Turabah, KSA.
²Forensic Medicine & Clinical Toxicology Dept., Faculty of Medicine, Zagazig University, Egypt.
³Histology and Cytology Dept., Faculty of Veterinary Medicine, Benha University, Egypt.
⁴Medical Biochemistry Dept., Faculty of Medicine, Zagazig University, Egypt.

Abstract: Cadmium (CdCl₂) is one of the most prevalent environmental and biologically hazardous toxicants among metals. This study was carried out to evaluate the ameliorative role of Grape seed extract (GSE) on CdCl₂ induced spleen toxicity in adult male albino rats. Forty adult male albino rats were equally divided into 4 groups. Group I (control); group II, administered GSE at dose 100 mg / kg bw/ day dissolved in distilled water; group III, administrated Cadmium chloride (CdCl₂) at a dose of 4.4 mg /kg bw /day dissolved in distilled water; and group IV, co-administrated CdCl₂ and GSE in the previous doses. Treatments were given by gavage for 3 months. Then rats were sacrificed and specimens were taken for biochemical, histological and immunohistochemical studies. CdCl₂ administration increased spleen level of MDA and decreased CAT, SOD GPx, GSH and plasma TAC. Also CdCl₂ up regulated mRNA expression of IL1, IL6, TNF, NFkB and down regulated IL10, COX-2. The treatment with GSE adjusted the alterations in all these parameters. Histological findings of the spleens from the CdCl₂ group showed thickening in the wall of the blood vessels, congestion, hemolysis, intracellular edema, depletion in the splenic follicles, and lymphocytic infiltration in the red pulp under the splenic capsule in comparison to control group. Also, the immunohistochemical finding showed higher expression of Bax and lower Ki76 in the spleens from CdCl₂ group in comparison to control group. However, appropriate improvement of histological and immunohistochemical features of the spleens from CdCl₂ + GSE group could be detected. In conclusion, this study suggested that concomitant use of GSE with CdCl₂ proved to be capable of ameliorating CdCl₂-induced splenic dysfunction.

Introduction

Cadmium (CdCl₂) is considered to be of major concern for public health by the World Health Organization as it is one of the most prevalent environmental and biologically hazardous toxicants among metals [1]. The increased industrial uses such as, electroplating, plastic production, pigments, battery-manufactures and pesticides that led to the entry of CdCl₂ into the soil and subsequently into ground, drinking water and storage in crops [2]. The presence of CdCl₂ over the permissible level has both acute and chronic health hazards. Chronic CdCl₂ administration affects lymphatic and hematopoietic organs [3]. Recent investigations reported the effects of CdCl₂ on spleen and its immunosuppressive effects [4, 5]. The mechanism of CdCl₂-induced cytotoxicity is mainly via induction of oxidative stress [6, 7]. Multiple studies indicated that CdCl₂ exposure induces oxidative stress at the cellular level by generation of (ROS) which lead to lipid peroxidation and malfunction of anti-oxidant system[8, 9].

Grape seed extract (GSE) is a good source of Gallic acid, which are potent antioxidant and anti-tumor agent[10, 11]. It contains numerous compounds called polyphenols that contain dimers, trimers, and other oligomers of catechin and epicatechin and their gallate derivatives that are called proanthocyanidins that protect against oxidative stress, protects the circulatory system, and has anti-inflammatory, and anticancer effects [12-15]. GSE’s antioxidative activities are found to be much stronger than those of vitamin C and vitamin E [16]. The aim of this study was to investigate CdCl₂ induced splenic dysfunction and explore the ameliorative role of GSE.
Material and methods:

Chemicals and Reagents:

Cadmium chloride \( \text{CdCl}_2 \) was purchased from Sigma (St. Louis, MO, USA). Grape seed extract (proanthocyanidins 95%) was obtained from GNC Holdings Inc. Pittsburgh, Pennsylvania, United States. Other chemicals and reagents were of analytical grade and obtained from Sigma-Aldrich (St Louis, MO, USA).

Animals

Forty healthy adult male Wister albino rats weighting 180–200 g were used. They were fed standard pellet diet and water, given ad libitum and housed in polypropylene cage in the departmental animal house of Faculty of Applied Medical Sciences, Turabah, Taif University, with 12 h light: dark cycle, and the temperature of 25°C ±2°C. All procedures for animal handling and treatments were approved by the Ethical Committee Office of the Scientific Deanship of Taif University (Taif, Saudi Arabia).

Experimental design

The animals were divided into four groups (10 male albino rats per group:

- **Group I** fed on balanced diet and was used as a control for 3 months
- **Group II** was administered GSE 100 mg/ kg body weight/day dissolved in distilled water for 3 months according to [17, 18].
- **Group III** was administered Cadmium chloride orally at a dose of 4.4 mg/ kg bw/ day (1/20 of LD50) that was dissolved in distilled water for 3 months.
- **Group IV** was given mixture 100 mg GSE diluted in distilled water in addition to 4.4 mg Cadmium chloride /day dissolved in distilled water for 3 months

Sample collection:

At the time of sacrifice, rats were anaesthetized with ether then blood samples were collected from the retro-orbital plexuses. The spleen of each rat was dissected out. Part of blood samples were collected in heparinized tubes and used for lymphocytes separation. Sera were separated by centrifugation at 3000 rpm and stored frozen at -20°C until the time of biochemical analyses.

Preparation of splenic homogenate

The spleen of each rat was excised and perfused with ice-cold saline till all the traces of blood were removed. A small portion of the tissue was used for histological, immune-histochemical examinations. The rest of spleen tissues were washed in ice cold phosphate buffer saline (PBS), dried, divided for the assessment of biochemical parameters and quantitative Real-time analyses and immediately frozen in liquid nitrogen at - 80°C.

Determination of antioxidant & oxidative stress markers

Measurement of Lipid peroxidation (LPO)

MDA, as a marker of lipid peroxidation, was measured calorimetrically in spleen homogenate (nmol / g.tissue) according to the method of Okhawa et al using commercial available kit (Biodiagnostic, Cairo, Egypt) [19]. Thiobarbituric acid reacts with MDA in acidic medium at 95 °C for 30 min to form thiobarbituric acid reactive product, and the absorbance of the resultant pink product can be measured at 534 nm.

Measurement of reduced glutathione:

Reduced glutathione (mmol/g) was determined according to the method of Beutler et al. using commercially available kit (Biodiagnostic, Cairo, Egypt) [20]. GSH determination is based on the reduction of 5, 5’-dithiobis (2-nitrobenzoic acid) with GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration, and its absorbance can be measured at 405 nm.

Determination of Antioxidant Enzymes

**Determination of catalase (CAT) activity**

CAT activity (U/g tissue) was assessed spleen homogenate by means of the method of Goth, L [21].

**Determination of superoxide dismutase (SOD) activity**

SOD activity was assessed in spleen homogenate (U/gm tissue) according to Nishikimi et al. using commercially available kit (Biodiagnostic, Cairo, Egypt)[22]. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. The activity was expressed as unit/mg protein.

**Estimation of glutathione peroxidase (GPx):**

GPx activity was assessed in spleen homogenate (U/gm tissue) according to Paglia and Valentine using commercially available kit (Biodiagnostic,
Cairo, Egypt)[23]. The assay is an indirect measure of the activity of GPx. Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase (GR). The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm (A340) providing a spectrophotometric means for monitoring GPx enzyme activity.

Assessment of the total antioxidant capacity (TAC):
Total antioxidant capacity (TAC) (Biodiagnostic, Cairo, Egypt) was measured in serum (µmol/L) according to Koracevic et al. to assess the effect of CdCl2 on systemic oxidative stress [24]. The method is based on determination of the ability to eliminate added hydrogen peroxide. The remaining H2O2 is determined calorimetrically by an enzymatic reaction converting 3,5-dichloro-2-hydroxylbenzenesulfonate to a colored product that is measured at 532 nm.

Quantitative Real-time analyses
RNA Extraction
Total RNA was extracted from spleen tissue homogenate using a Quiagen RNA isolation kit (RNasey, Qiagen Ltd, Crawley, West Sussex, UK) according to the manufacturer’s protocol. The total RNA was quantified by the measured absorbance at 260 nm in a spectrophotometer.

Reverse Transcriptase and Polymerase Chain Reaction (RT-PCR)
RNA is converted into CdCl2 by reverse transcriptase (QuantiTect Reverse Transcription Kit, QIAGEN, # 205310, Germany). This extracted RNA was used for determination of pro-inflammatory and anti-inflammatory cytokines and COX-2 gene expression. Beta actin (β-actin) gene was used as a housekeeping gene for rat genes. SYBR Green RT-PCR amplification was carried out in 25-µl reaction volume contained 12.5 µl of SYBR Green real time PCR Master Mix (Roche Diagnostics), 1 µl each of the forward and reverse primer, 2 µl of CdCl2 NA and 8.5 µl of distilled water.

Polymerase Chain Reaction (PCR):
All samples were run in duplicates, and PCR amplification consisted of 40 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 10 s, and extension at 72°C for 20 s. The primers were designed by the web based tool, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on the published rat sequence. Moreover, primers sequences were in accordance with the previous reports of Peinnequin et al. for IL-1β, IL-6, TNF-α, NFκB, β-actin, Lopes et al. for IL-10 [25, 26]. The sequence of the primers used is listed in Table 1.

The amount of cDNA in the reactions was normalized with an internal control, the constitutively expressed gene β-actin. Relative gene expression was calculated from the formula: 2ΔCT; (ΔCT=CT β-actin -CT target).

Table 1: Primer sequences used for cytokines and antioxidant real-time PCR assays

<table>
<thead>
<tr>
<th>Gene name</th>
<th>5'-3' primer sequence</th>
<th>Annealing Temp (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>FW: 5<code> CACCTCTCAAGGACGACCT 3</code></td>
<td>58 C</td>
<td>95 bp</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> GGGTTCATGGTGAGTCAAC 3</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>FW: 5<code> TCTTACCCCAACTCAAATGC 3</code></td>
<td>65 C</td>
<td>79 bp</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> TTGGATGCGTGGTCCTTACC 3</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>FW: 5<code> AATGCGTCTCCCTCATCAGTC 3</code></td>
<td>58 C</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> TCTCGTGGTGGTGTTGACAG 3</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>FW: 5<code> TGTATGTCACATGCTGTCG 3</code></td>
<td>60 C</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> GGGTGACGATGTGCTGACT 3</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFκB</td>
<td>FW: 5<code> TACTCCATTCTCTTTGTGGTT 3</code></td>
<td>85 C</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> TCAAGTACCAAAGTCTCTTC 3</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>FW: 5<code> AAATGGGCTCCCTCATCAAAAT 3</code></td>
<td>58 C</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> ACACCTCTGGTCCTGGAGGGT 3</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>FW: 5<code> AGTTCCCTCACCTCTCAAAAAG 3</code></td>
<td>58 C</td>
<td>97 bp</td>
</tr>
<tr>
<td></td>
<td>RW: 5<code> AAGCAATGCTGTCACCCTCCC 3</code></td>
<td></td>
<td></td>
</tr>
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</table>

Histological examination:
Cross sectioned specimens from the middle parts of the spleens of the different groups were fixed in 10% neutral buffered formalin solution. After fixation, the specimens were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax. Paraffin sections of 5µm thickness were stained with haematoxylin and eosin and Masson’s trichrome stains as outlined by Bancroft and Gamble [27].

Immunohistochemical staining
Paraffin sections (5µm thickness) of the spleen were collected on positive charged slides. After deparaffinization and rehydration, sections of the spleen were treated with 3% hydrogen peroxide for 10 minutes to reduce peroxidase activity. Then after, sections were incubated with the following primary antibodies; mouse monoclonal anti-Bax (Clone 2D2, Neomarkers, USA), rabbit polyclonal anti Bcl-2 (Sigma-Aldrich Co. LLC., Taufkirchen Germany) and rabbit polyclonal anti Ki-67 (DAKO Corp., Carpinteria, CA) for 30 minutes at room temperature. The sections were then stained with immune-peroxidase technique employing commercially available reagent ABC kit (Labvision, Fermont, CA, USA). For demonstration of binding sites, DAB chromogen was applied. Phosphate buffered saline was used for rinsing between each step and finally all sections were counterstained with Mayer’s hematoxylin [28].
Alkaline comet assay

The alkaline Comet assay (single cell gel electrophoresis): 3 ml of blood samples were collected in heparinized tubes and used for lymphocytes separation. Then lymphocytes subjected to comet assay which was carried out in Animal Venerology Research Institute (El-Haram, Giza, Egypt). It was performed according to (Singh et al.[29]). Slides were examined at 40x objective on an epifluorescence microscope equipped with a 515–560 nm excitation filter. Images of 100 randomly selected cells from each subject were analyzed with an automatic digital analysis system, Comet Image analysis software developed by Kinetic Imaging linked to a CCD camera (Ltd. Liverpool, UK) to determine the quantitative extent of DNA damage in the cells by measuring the length of DNA migration were calculated from this program.

Statistical Analysis

Data were represented as means ± SD. The differences were compared for statistical significance by ANOVA and post hoc Tukey's tests. Difference between groups was considered significant at P<0.05. The statistical analysis was performed using software (SPSS Inc., Chicago, Illinois, USA).

Results

MDA, antioxidant enzymes activities and reduced glutathion in spleen and TAC in serum of treated rats:

Administration of CdCl₂ induced oxidative stress that manifested by a significant increase in lipid peroxidation product MDA in spleen with respect to control group (P < 0.0001). Meanwhile, marked depletion in tissue levels of CAT, SOD, GPx activity and GSH contents as well as serum TAC was observed in CdCl₂ administered rats comparing to control ones as shown in table 2. In addition, table 2 demonstrated that GSE treatment significantly attenuated oxidative stress by reducing splenic MDA and improving GSH content as well as antioxidant enzymes activities in spleen tissues and increasing serum TAC as compared to CdCl₂ treated group (P < 0.0001).

Effect of Cd Cl₂ on mRNA expression of IL-1β-, IL-6, TNF-α, NFκB and IL-10:

Administration of CdCl₂ for 3 months showed significant increase in mRNA expression of proinflammatory cytokines IL-1β-, IL-6, TNF-α and NFκB. This increase was attenuated on co-administration of GSE with CdCl₂ (p<0.001) corresponding to control as shown in table3. Furthermore, table3 shown a marked decrease in mRNA expression of IL-10 in rats received CdCl₂. On the other hand, rats receiving GSE along with CdCl₂ showed significant increase in mRNA expression of the previously mentioned genes relative to CdCl₂ group (p<0.001) corresponding to control group as shown in table3.

### Table 2: Oxidative and anti-oxidative markers in all studied groups using ANOVA test and post hoc Tukey’s.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=10)</th>
<th>GSE (n=10)</th>
<th>CdCl₂ (n=10)</th>
<th>CdCl₂ + GSE (n=10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μmol/g spleen)</td>
<td>3.5 ± 0.02</td>
<td>3.3 ± 0.05</td>
<td>6.7 ± 1.7*</td>
<td>4.7 ± 1.2*</td>
<td>0.000*</td>
</tr>
<tr>
<td>CAT (μmol/min/g protein)</td>
<td>40.2 ± 10.4</td>
<td>39.1 ± 3.7</td>
<td>20.9 ± 6.5*</td>
<td>37.4 ± 3.7*</td>
<td>0.000*</td>
</tr>
<tr>
<td>SOD (U/g protein)</td>
<td>32.6 ± 9.1</td>
<td>31.1 ± 9.8</td>
<td>20.9 ± 6.5*</td>
<td>29.6 ± 7.5*</td>
<td>0.000*</td>
</tr>
<tr>
<td>GPx (U/g tissue)</td>
<td>119.6 ± 17.8</td>
<td>118.4 ± 13.4</td>
<td>75.8 ± 10.9*</td>
<td>103.7 ± 16.3*</td>
<td>0.000*</td>
</tr>
<tr>
<td>GSH (μmol/g spleen)</td>
<td>8.6 ± 2.9</td>
<td>8.2 ± 2.8</td>
<td>5.1 ± 2.7*</td>
<td>6.4 ± 2.7*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TAC (μmol/L)</td>
<td>0.76 ± 0.05</td>
<td>0.76 ± 0.05</td>
<td>0.04 ± 0.01*</td>
<td>0.05 ± 0.09*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Values are expressed as mean± standard deviation (SD) of n=10 animals; a significant as compared with control group; b significant as compared with Cd Cl₂ -treated group.

MDA: malondialdehyde; GSH: reduced glutathione; CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; TAC: total antioxidant capacity.

### Table 3: Quantitative gene expression of cytokines and NF-κB by real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control (n=10)</th>
<th>GSE (n=10)</th>
<th>CdCl₂ (n=10)</th>
<th>CdCl₂ + GSE (n=10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1.50 ± 0.08</td>
<td>1.7 ± 0.8</td>
<td>2.8 ± 1.4*</td>
<td>2.00±0.54*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.23 ± 0.08</td>
<td>1.13 ± 0.49</td>
<td>2.91 ± 1.1*</td>
<td>1.71±0.71*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.62 ± 0.4</td>
<td>1.42 ± 0.6</td>
<td>2.92 ± 0.9*</td>
<td>2.14±0.72*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>NFκB</td>
<td>1.31 ± 0.75</td>
<td>1.22 ± 0.61</td>
<td>2.91 ± 1.2</td>
<td>1.83±0.88</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.6 ± 0.9</td>
<td>2.7 ± 0.8</td>
<td>1.21 ± 0.5*</td>
<td>2.04±0.63*</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Values are expressed as mean± standard deviation (SD) of n=10 animals; a significant corresponding to control group; b significant corresponding to CdCl₂- treated group.

Histological and immunohistochemical observations

The animals of control group showed normal histological features of the spleen. The spleens were covered with dense fibrous tissue capsule, from which short trabeculae extended into the architecture of the spleen. The parenchyma of the spleen consists of white and red pulps. The red pulp consisted of...
venous sinuses and splenic cords of diffuse lymphocytes in addition to RBCs (Fig.1). The white pulp consisted of lymphocytic aggregation (splenic follicle), and marginal zone that located at periphery of the follicles (Fi.g1). Periarterial lymphatic sheath (PALS) cells were located around the central artery of the white pulp (Fig.2). Immunohistochemically, the splenic cells showed positive BAX immunostaining (Fig.3), and sporadic cells showed faint Bcl-2 immunostaining (Fig.4). Moreover, most of the splenic cells showed strong positive Ki67 immunostaining (Fig.5).

The spleen of GSE group showed an increase in the red pulp areas in comparison to the spleen of control group (Fig.6) while the immunostainings of BAX, Bcl-2, and ki67 were still as that of the control group. The spleen of the CdCl₂ group showed thickening in the wall of the splenic vessels, congestion, hemolysis and intercellular edema (Fig.7). In addition, depletion of the splenic follicles, and lymphocytic infiltration in the red pulp in the form of small aggregations under the capsule were detected (Fig.8). Immunohistochemically, there was an increase in the number of BAX positive cells in comparison to the control group however, the rest of the cells showed necrosis (Fig.9). Bcl-2 positive sporadic cells were still showed as that of the control group. The splenic cells showed less prominent Ki67 immunostaining in comparison to the control group (Fig.10).

In group co-administrated GSE with CdCl₂, the spleen showed appropriate improvement of its histological architecture. Disappearance of the splenic follicles depletion was a common feature (Fig.11). However, the sub capsular lymphocytic aggregation and intercellular edema in the red pulp were still persisted (Fig.12).
Fig. 7: Cross section of the spleen of CdCl₂ group showing thick wall of the splenic vessels (t), congestion (c), hemolysis (h) and intercellular edema (e). Masson’s trichrome stain. Objective lens x10.

Fig. 8: Cross section of the spleen of CdCl₂ group showing depletion of the splenic follicles (d), and lymphocytic infiltration in the red pulp in the form of small aggregations (g) under the capsule were detected in addition to, edema (e). H&E stain. Objective lens x10.

Fig. 9: Immunohistochemical staining of BAX in the spleen of CdCl₂ group showing an increase in the number of positive cells (arrows) and necrosis (N) in comparison to the control group. Objective lens x40.

Fig. 10: Immunohistochemical staining of Ki67 in the spleen of CdCl₂ group showing less Ki67 immunostaining (arrows) in comparison to the control group. Objective lens x40.

Fig. 11: Cross section of the spleen of group co-administrated GSE with CdCl₂ showing disappearance of the splenic follicles depletion in addition to, sub capsular lymphocytic aggregation (g) in the red pulp. H&E stain. Objective lens x4.

Fig. 12: Cross section of the spleen of group co-administrated GSE with CdCl₂ showing persistence of intercellular edema (e). Masson’s trichrome stain. Objective lens x4.

Fig. 13: Immunohistochemical staining of BAX in the spleen of group co-administrated GSE with CdCl₂ showing obvious decrease in number of positive splenic cells (arrows) in comparison to the CdCl₂ group. Notice no necrosis can be detected. Objective lens x40.

Fig. 14: Immunohistochemical staining of Ki67 in the spleen of group co-administrated GSE with CdCl₂ showing obvious an increase in the number of positive splenic cells (arrows) in comparison to the CdCl₂ group. Objective lens x40.

Immunohistochemically, there was obvious decrease in the number of BAX positive splenic cells (Fig.13) while Ki67 positive cells were increased (Fig.14) in comparison to the CdCl₂ group. No necrosis can be detected in the spleens of this group.

**Comet tail length results**

Regarding comet assay, there was major DNA damage in Cadmium chloride treated rats indicated by a high significant increase (P< 0.001) in mean values of the comet tail length corresponding to mean values of tail length of CdCl₂ treated rats (fig. 15-17)

1. Discussion

Cadmium Chloride (CdCl₂) shows high toxicity to different biological systems. Occupational exposure to CdCl₂, such as working with CdCl₂-containing pigments, plastic and electrode material in nickel-cadmium batteries, and environmental exposure, such as food, water and cigarette smoke [30]. CdCl₂ accumulates mostly in the liver, kidney, and spleen [31, 32]. Therefore, a great deal of scientific research is focused on ameliorating the toxic effects of CdCl₂ around the world including Egypt. Our main purpose was to examine how CdCl₂ induced spleen toxicity and explore the ameliorative role of GSE.

In this study, we found that chronic CdCl₂ exposure induced profound negative effects on splenic tissues. This was evident from increased levels of spleen lipid peroxidation, proinflammatory cytokines, DNA breakage, associated with concomitant decrease in antioxidant defense system, in addition to histological and immunohistochemical changes.

Previous studies had illustrated the harmful pro-oxidative, pro-inflammatory activities of CdCl₂ and reported that CdCl₂ is capable of eliciting a variety of ROS (O₂-, H₂O₂, and •OH), which could be the main mechanism of cellular toxicity induced by this heavy metal [33-37]. It can replace iron and copper from a number of cytoplasmic and membrane proteins like ferritin, thereby causing rise in the concentration of these free redox-active metals which directly enhance the production of hydroxyl radicals through the Fenton reaction.

The oxidative stress could induce many hazards, including DNA damage/mutations, oxidation of proteins and lipid peroxidation (LPO), which may cause alterations in lipid composition of cellular membranes and functions [38]. In this context, López, et al. and Toppo et al. have illustrated that CdCl₂ induced LPO in cortical neurons and liver due to increased concentration of ROS [39, 40].
Our results revealed reduction in the activities of CAT, GPx and SOD that might be secondary to usage in neutralizing the CdCl₂-induced ROS generation and scavenging free radicals. The interaction of cadmium with zinc, iron and selenium led to displacement and replacement of these beneficial metals from the active site of CAT, SOD and GPx enzymes with subsequent decrease in their activity. Moreover, CdCl₂ combines with thiol groups of SOD, GPx and CAT and inhibits their activities [41]. Reduced glutathione (GSH) is an endogenous antioxidant, highly abundant in cells, has many functions such as storage and transport of cysteine, maintaining the reduced state of proteins and thiols, participating in detoxification of many oxidative toxicants such as drugs, and heavy metal ions. Moreover, GSH functions as a substrate for detoxification enzymes; glutathione peroxidase and glutathione-S-transferase [42]. GSH is a primary target for free CdCl₂ ions where it acts by scavenging CdCl₂ to prevent its interaction with critical cellular targets. Therefore prolonged CdCl₂ exposure induced depletion of the reduced GSH pool with subsequent disturbance of the redox balance leading to an oxidative environment [43]. In our study, the increase in spleen level of MDA was associated with concomitant decrease in antioxidant defense system (GSH content, TAC and CAT, GPx and SOD activities).

The alkaline comet assay detects DNA single strand breaks and alkali-labile sites. It is used to investigate the genotoxic effects in human bio-monitoring studies [44]. In the current study, microscopic examination of peripheral blood lymphocytes, in Cadmium treated group showed cells with major DNA damage and long comet tails as compared to normal lymphocytes with undamaged DNA (without comet tail) in control group rats. This result confirms earlier results obtained regarding genotoxicity of Cadmium compounds [3, 45]. In our study the DNA damage was strongly correlated with oxidative stress induced by CdCl₂. Nemmiche et al. stated that CdCl₂-induced ROS and free radicals have the capacity to induce DNA strand breaks and lead to an increase in the total number of DNA lesions [9].

Apoptosis was an important process mediating CdCl₂-toxicity in different organs evidenced by increased lipid peroxidation after CdCl₂-exposure [46, 47]. Current study showed that CdCl₂ induced histological and immunohistochemical variations in the spleen comparison to the control group including thick splenic vessels, congestion, hemolysis and edema, small aggregations of lymphocytic infiltration in the red pulp that was in accordance with Randa AH et al. in CdCl₂ treated guinea pigs. In addition, depletion of the splenic follicles was common that was in consistence with Tarasub et al. suggesting CdCl₂ as lymphocyte toxic agent that can cause apoptosis or necrosis of lymphocytes of the splenic white pulp [48, 49]. Overall, our present finding about depletion of the white pulp with concomitant increase in the red pulp infiltrated by lymphocytes was in agreement with other findings[4, 50]. In addition, the subcapsular lymphocyte infiltration may be a marker of repeated tear and wear of tissues in response to the injury caused by CdCl₂. Immunohistochemically, CdCl₂ treated specimens showed increased number of BAX positive cells and necrosis but less Ki67 positive cells in comparison to the control group. The increase of BAX positive cells indicates apoptosis. Both apoptosis and necrosis can be induced by increased accumulation of ROS and increased lipid peroxidation after CdCl₂ exposure [46, 51]. Furthermore, our histological and immunohistochemical findings were strongly correlated with our biochemical results.

Another mechanism of CdCl₂-mediated pro-oxidative activity includes oxidative activation of redox-sensitive transcription factors such as nuclear factor kappa B (NFκB) [52, 53]. NF-κB is important in regulating cellular responses because it belongs to the category of “rapid-acting” primary transcription factors. It is present in cells, sequestered in the cytoplasm in an inactive state through its association with one of several inhibitory molecules, including IκBα, IκBβ IκBε, p105, and p100. The most classical form of NF-κB is a heterodimer of p50 and p65 [54, 55]. Diverse stimuli like ROS, TNFα and IL-1β induce the degradation of IκB or partial degradation of the C-termini of p105 and p100 precursors, allowing the translocation of NF-κB into the nucleus and its transformation into an active form where it induces transcription and expression of many genes including inflammatory cytokines leading eventually to cell death [56-58]. CdCl₂ induced proinflammatory mechanism was hypothesized by Monaco et al. and Sethi et al. and emphasized by Lee J. and Lim KT. who reported increased expression of NF-κB in mice treated with CdCl₂ [59-61]. Interestingly, increased expression of inflammatory cytokines (TNF-α, IL-1β) and IL6 in spleen tissues of animals treated with CdCl₂ mentioned in our study is a typical factor of NF-κB upstream activation.

Pro-inflammatory cytokines are known as traditional markers of inflammation that not only promotes the induction of acute phase proteins, but over-production of these agents lead to chronic inflammation and/or autoimmunity [62, 63]. The present study indicated upregulation in the expression of IL-1β, IL-6, TNF-α, and downregulation of IL-10 in CdCl₂ treated rats. The modulating effect of CdCl₂ on the expression of cytokines have been investigated in several in vivo and in vitro models. Previous studies have demonstrated that chronic exposure of Wistar rats...
CdCl₂ resulted in significant increase in IL-1β, IL-6 and TNF-α levels in heart tissue supernatants and plasma levels as compared to controls [64-66]. In contrast, IL-10 is a pleiotropic, immunoregulatory cytokine produced in large amounts from activated B-lymphocytes that mature in the marginal zone of the spleen. It plays a regulatory role in protecting the host from infection-associated immunopathology, autoimmunity, and allergy, enhances B cell survival, proliferation, and antibody production [67, 68]. It was suggested that IL-10 may exert a significant part of its anti-inflammatory properties by inhibiting transcription factor NF-KB where it blocks NF-xB nuclear translocation by inhibiting IKK activity; and blocks DNA-binding of NF-xB already present in the nucleus [69]. Our results revealed down regulation in IL-10 gene expression in CdCl₂ treated rats that could be contributed to the hyperoxidative state leading to organ damage by ROS as mentioned above [70, 71]. In support of this notion, the current data indicated the changes in IL- 10 levels were associated with loss of splenic anti-oxidants. This study was the first to show that co-treatment with GSE attenuated CdCl₂ induced spleen toxicity by modulation of oxidative damage, CdCl₂ -induced over expression of proinflammatory cytokines, DNA breakage. GSE contains polyphenols including proanthocyanidins and procyanidins that showed antioxidant and free radical scavengers. GSE inhibit enzyme systems that are responsible for the production of free radicals [72]. They are also known as sustained release antioxidants, and can remain in the plasma and tissues for up to 7 – 10 days and exert antioxidant properties, which is mechanistically different from other water soluble antioxidants [73]. Commensurate with this, co administration of GSE with CdCl₂ elicited significant reduction in MDA levels in the spleen tissues and increased TAC,CAT, GPx, SOD activities and GSH content. Moreover, the spleen specimens showed appropriate improvement of its histological architecture. Immunohistochemically, there was obvious decrease in the number of BAX positive splenic cells while Ki67 positive cells were increased in comparison to the CdCl₂ treated group. Active compound in GSE may be act as immune stimulant and increased splenocyte proliferation and hyperplasia of lymphoid tissue of white pulp of spleen. In accordance with these findings, experimental studies have shown that oral administration of GSE increased antioxidant activities, and inhibited lipid peroxidation [74, 75]. Similarly, Chena, et al. have reported that GSE has protective effect against renal oxidative damage induced by CdCl₂, where it attenuated CdCl₂ -induced lipidperoxidation, and antagonized renal apoptosis [76]. Also, our findings revealed that GSE administration significantly downregulated the activation and expression profiles of NF-KB. Additionally, it markedly reduced TNF-α and IL-1β productions. Alkedaide has reported significant reduction in serum TNF-α level in animals that were co-treated with both CdCl₂ and GSE. GSE also showed marked ameliorative effects on CdCl₂ induced DNA damage in lymphocytes [37]. Furthermore, Sehirli et al. have reported that GSE could minimize organ injury through its ability to balance the oxidant-antioxidant status, and to regulate the release of inflammatory mediators [77].

2. Conclusion:

Finally, it can be concluded from all these findings that GSE (a potent anti-oxidant) had beneficial impacts on the spleen of CdCl₂ -treated rats through alleviating the oxidative stress, suppressing inflammation and DNA damage.

References


