Materials & Methods
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Design of Study

Four to six week-old juvenile male Balb/c mice weighing 20-25 grams were procured from the Central Animal House, Panjab University, Chandigarh. All animals were kept in cages, given food and water *ad libitum* and maintained on a 12-hour light/dark regime. They were acclimatized for 7 days prior to experimental use. This study was approved by the Animal Ethical Committee, Panjab University, Chandigarh.

The study was divided into two parts *i.e.*, (i) acute and chronic Cd exposures (Table 3.2: A and B) and (ii) modulating role of curcumin pre-treatment on acute Cd exposure (Tables 3.3). The animals were divided into different experimental groups of 7 mice each and doses were administered intraperitoneally. The acute exposure doses were based on experimentally determined LD50 values (1.12 mg CdCl2/kg bw) in mice (calculated using Sun’s formula, 1963) (Table 3.1). The chronic exposure dose was similar to the WHO approved Cd intake for humans (7 μg CdCl2/kg bw per week).

Experimental Model

*Selection of Animal Model - Mice*

The selection of juvenile mice was based upon their sensitivity to toxicity, and ability to absorb and retain the administered dose (Matsusaka *et al.*, 1972). Mice are genetically very similar to humans and over 90% of the mouse and human genomes can be partitioned into corresponding regions of conserved synteny (all loci on one chromosome are said to be syntenic). The proportion of mouse genes with a single identifiable orthologue in the human genome seems to be approximately 80%, while the proportion of mouse genes without any homologue currently detectable in human genome (and *vice versa*) seems to be less than 1% (Mouse Genome Sequencing Consortium, 2002). Due to this genetic homology, the data obtained from studies on mice can be successfully extrapolated to humans and extent of toxicity may be determined.

*Selection of Cd Compound - Cadmium chloride (CdCl2)*

Organic compounds of Cd are generally insoluble in aqueous media and may interfere with metabolic processes, therefore, the inorganic compounds of Cd are a better alternative to study toxicity of Cd. CdCl2 has the maximum solubility in water out of all the inorganic Cd compounds and is a major source of Cd exposure by ingestion of contaminated food and water in the non-industrially exposed human populations.
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(ASTDR, 1999). Also, it does not require bio-activation and can better define the biological events responsible for injury of liver and kidney following exposure (Prozialeck, 2000).

Determination of LD\textsubscript{50}

A pre-test was conducted to establish the range of toxicity so that proper dose levels could be established for LD\textsubscript{50} determinations (Jing-Hui and Marsh, 1988). The pretest used groups of 5 male Balb/c mice each. Three dose levels (0.2 mg, 0.8 and 2.0 CdCl\textsubscript{2}/kg bw) were used in the pre-testing for acute exposure studies. Based on pretest results, final doses were established with each group comprising of 10 animals. The LD\textsubscript{50} determinations were based on dose levels that increased by a geometrical progression (Table 3.1) and five dose levels were used for establishing the LD\textsubscript{50}. Calculations were based on Sun’s formula (1963) (Appendix I).

<table>
<thead>
<tr>
<th>Dose (mg CdCl\textsubscript{2}/kg bw)</th>
<th>Mortalities (Dead/tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>0/10</td>
</tr>
<tr>
<td>0.6</td>
<td>0/10</td>
</tr>
<tr>
<td>0.8</td>
<td>3/10</td>
</tr>
<tr>
<td>1.2</td>
<td>6/10</td>
</tr>
<tr>
<td>1.75</td>
<td>10/10</td>
</tr>
<tr>
<td>Total</td>
<td>19/50</td>
</tr>
</tbody>
</table>

LD\textsubscript{50} ± SE (mg CdCl\textsubscript{2}/kg bw) 1.02 ± 0.05

Chemicals Used in the Study

All chemicals of analytical grade specifications were obtained from HIMEDIA Ltd, India. Taq DNA polymerase and dNTPs were procured from MBI Fermentas, USA, while restriction enzymes (EcoRI and Hinfl) and DNA ladder from Bangalore Genei, Bangalore. Cadmium chloride and curcumin were obtained from Sigma Chem. Co., St. Louis, MO, and primer sequence for β-catenin gene from Sigma Aldrich, USA.

Experimental Layout

1. Acute Toxicity

Table 3.2 (A) shows the design for acute exposure studies to investigate the effect of Cd on mice liver and kidney. The control group (AC) received the vehicle
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(normal saline), while single i.p. injections of Cd doses- 0.2, 0.4 and 0.8 mg CdCl₂/kg bw were administered to the groups AT-I, AT-II and AT-III, respectively. Cd reached a steady-state concentration in body tissues by 6 hours, which was maintained for upto 24 hours (Zheng et al., 1996). Therefore, the animals were sacrificed after 18 hours of treatment.

Table 3.2 (A) - Dose and Durations of Treatments of Cadmium (as CdCl₂) for Acute Exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>Duration (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (AC)</td>
<td>Normal saline</td>
<td>18</td>
</tr>
<tr>
<td>Cd (AT-I)</td>
<td>0.2 mg CdCl₂/kg bw</td>
<td>18</td>
</tr>
<tr>
<td>Cd (AT-II)</td>
<td>0.4 mg CdCl₂/kg bw</td>
<td>18</td>
</tr>
<tr>
<td>Cd (AT-III)</td>
<td>0.8 mg CdCl₂/kg bw</td>
<td>18</td>
</tr>
</tbody>
</table>

2. Chronic Toxicity

To study the effect of chronic Cd exposure on liver and kidneys of mice, a dose of 7 µg CdCl₂/kg bw was administered once a week for 4 weeks regularly. This dose was similar to the WHO approved Provisional Tolerable weekly intake for humans (WHO, 2000). Parallel controls were also maintained (Table 3.2: B).

Table 3.2 (B) - Dose and Durations of Treatments of Cadmium (as CdCl₂) for Chronic Exposures

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>Frequency of Exposure</th>
<th>Duration (Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CC)</td>
<td>Normal saline</td>
<td>Once a week</td>
<td>4</td>
</tr>
<tr>
<td>Cd (CT)</td>
<td>7 µg CdCl₂/kg bw</td>
<td>Once a week</td>
<td>4</td>
</tr>
</tbody>
</table>

3. Modulating Role of Curcumin Pre-treatment

For studying the modulating role of curcumin pre-treatment, mice were divided into 6 groups. Curcumin was dissolved in DMSO, an amphiphilic compound that is known to increase the permeability of the membranes (Perkins et al., 2002). Therefore, a DMSO-treated control was also studied along with a normal saline injected control. The dose of Cd used in this study was 0.8 mg CdCl₂/kg bw. A single i.p. injection of 100 mg curcumin/kg bw dissolved in 100 µl DMSO was administered to the group DMSO+Cur and animals were sacrificed after 24 hours of exposure.
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The groups DMSO+Cd and DMSO+Cd+Cur were injected 100 μl DMSO or 100 mg curcumin/kg bw dissolved in 100 μl DMSO, respectively. Another treatment of 0.8 mg CdCl₂/kg bw was given to these groups 24 hours after the first treatment and finally the animals were sacrificed after 18 hours of the second treatment (Table 3.3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>Duration (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Normal Saline</td>
<td>18</td>
</tr>
<tr>
<td>Cd</td>
<td>0.8 mg CdCl₂/kg bw</td>
<td>18</td>
</tr>
<tr>
<td>DMSO (Control)</td>
<td>100 μl DMSO</td>
<td>24</td>
</tr>
<tr>
<td>DMSO + Cur</td>
<td>100 mg Curcumin/kg bw in 100 μl DMSO</td>
<td>24</td>
</tr>
<tr>
<td>DMSO + Cd</td>
<td>Treatment 1 – 100 μl DMSO</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Treatment 2 - 0.8 mg CdCl₂/kg bw</td>
<td>18</td>
</tr>
<tr>
<td>DMSO + Cur + Cd</td>
<td>Treatment 1 – 100 mg Curcumin/kg bw in 100 μl DMSO</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Treatment 2 - 0.8 mg CdCl₂/kg bw</td>
<td>18</td>
</tr>
</tbody>
</table>

The mice were sacrificed by cervical dislocation and the liver and kidney tissues perfused with normal saline. These were then washed with ice-cold normal saline and further processed separately as described below for morphological studies (light and scanning electron microscopic), biochemical measurements (lipid peroxidative indices, cellular non-enzymatic anti-oxidants, anti-oxidant and detoxifying enzyme activities) and molecular studies (DNA apoptosis and state of the cancer critical gene Ctnnb1).

Morphological Studies

Morphological alterations are generally indicative of irreversible damage (Hinton et al., 1992). Histopathological alterations were observed using light microscopy (H and E staining) and further substantiated at finer scale under scanning electron microscope.

Histopathological Studies

Liver and kidney tissues of mice were fixed in 10% buffered formalin and processed routinely. The blocks were embedded in paraffin wax. Sections of 5-6 μm thickness
were cut by rotary microtome, stained with Haematoxylin-eosin (H and E) stain and examined under light microscope (Leica DC 100, PC I Interface Digital Camera).

**Scanning Electron Microscopy**

The liver and kidney tissue slices were washed with phosphate buffer and fixed in 4% gluteraldehyde prepared in 0.2 M phosphate buffer (pH-7.2 - 7.4 at 4°C), followed by post-fixation with 1% osmium tetroxide prepared in the same buffer. The tissue samples were rinsed in phosphate buffer with frequent changes. These were then dehydrated in ascending acetone grades and critical point dried (C.P.D.) through transitional fluid amyl acetate. The dried samples were mounted on metal stubs with double adhesive tape for gold sputtering. The stubs so prepared were examined using JEOL JSM 6100 scanning electron microscope and exposures were taken at different magnifications.

**Biochemical Measurements**

Homogenate and post-mitochondrial supernatants of liver and kidney tissues were prepared and various biochemical tests were performed.

**Preparation of Homogenate**

10% homogenates of liver and kidney tissues were prepared in 50 mM Tris-HCl buffer (pH-7.4) using a Potter-Elvejhem homogenizer at 0-4°C. The homogenate was used for the spectrophotometric determination (using Jenway 6305 uv/vis spectrometer) of lipid peroxidation (LPO), superoxide anion (SA), hydroperoxides (HP), total cellular thiols (TT), reduced glutathione (GSH) and protein content.

**Preparation of Post Mitochondrial Supernatant (PMS)**

Liver and kidney homogenates were centrifuged at 9,200 rpm for 10 minutes at 4°C. The supernatant was stored at -20°C and used for the estimation of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GR) and Glutathione-S-transferase (GST) activities, by continuous spectrophotometric rate determination (using Perkin Elmer Lamda 35 uv/vis spectrometer) and protein content (using Jenway 6305 uv/vis spectrometer).

**Oxidative Stress Parameters**

**Lipid Peroxidative Indices**

The reactive oxygen species (ROS) or the oxygen free radicals such as superoxide
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Anion and hydroperoxides react with critical cellular macromolecules such as lipids resulting in LPO and subsequent oxidative stress (Oost et al., 2003). To determine the extent of oxidative stress following various treatments, LPO, SA and HP generation were calculated using the measured spectrophotometric data (optical density).

Lipid Peroxidation (LPO)

Lipid peroxidation as evidenced by the formation of MDA-TBA (Malondialdehyde-thiobarbituric acid) chromophore was measured by the method of Beuge and Augst (1978). 0.1 ml homogenate was incubated in 0.1 ml each of 150 mM Tris-HCl (pH-7.1), 1.5 mM ascorbic acid and 1 mM ferrous sulphate in a final volume of 1 ml at 37°C for 15 minutes. 1 ml of 10% trichloroacetic acid (TCA) and 2 ml of 0.375% trichlorobutric acid (TBA) were added and kept in boiling water bath for 15 minutes. The contents were then centrifuged at 3,000 rpm for 10 minutes. The absorbance of the clear supernatant was measured against reference blank at 532 nm. Data were expressed as nmoles MDA formed/mg protein using 1.56 x 10^5 pM^-1 cm^-1 as the molar extinction coefficient of MDA.

Superoxide Anion Generation (SA)

Superoxide anion was assayed in the liver and kidney tissues by the method of Elferink (1984). McEvan tissue slicer was used to prepare 70 mg of liver/kidney slices. These were incubated in 1.2 ml of HEPES buffer [consisting of 20 mM HEPES (N-2 hydroxyethylpiperazine-N-2-ethane sulphonic acid), 1 mM calcium chloride, 10 mM glucose, 5 mM potassium chloride and 140 mM sodium chloride, pH-7.3] and 0.8 ml of 0.04% NBT (Nitroblue tetrazolium) in buffer at 37°C with a speed of 200 oscillations/minute in a metabolic shaker (Scintronic, New Delhi) for 30 minutes. The contents were centrifuged at 3,000 rpm for 10 minutes and the pellet was suspended in 2.5 ml of 0.5 N HCl (hydrochloric acid). Following re-centrifugation, supernatant was discarded and pellet was suspended in 2 ml pyridine. Tubes were kept in boiling water for 10 minutes and absorbance of the pyridine extract was read at 510 nm. Superoxide anion generation was expressed as nmoles/min/gm tissue using 30,000 as the molar absorptivity of NBT-formazon cm^-1.

Hydroperoxide Generation (HP)

Hydroperoxides were measured by the method of Jiang et al. (1992). 0.1 ml of homogenate was reacted with 0.9 ml of Fox reagent [88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium sulphate were added to 90 ml of
methanol and 10 ml of 250 mM sulphuric acid] and incubated at 37°C for 30 minutes. The colour developed was read at 560 nm and hydroperoxides were expressed as mmol/mg protein.

Non-Enzymatic Anti-oxidants

The most obvious direct effect of certain pollutants is a decrease in thiol status, due to either direct scavenging or increased peroxidase activity (Oost et al., 2003). The total cellular thiols and GSH content of liver and kidney homogenates were measured spectrophotometrically.

Reduced Glutathione (GSH)

Reduced glutathione was determined by the method of Beutler et al. (1963). 0.1 ml of homogenate and 2 ml of 0.2 M di-sodium hydrogen phosphate buffer were mixed thoroughly followed by addition of 1 ml of 1 mM dithio-bisnitro benzoic acid (DTNB) prepared in 1% (w/v) potassium citrate. The contents were centrifuged at 3,000 rpm for 15 minutes and absorbance of the clear supernatant was measured against reference blank at 412 nm. Glutathione content was expressed as nmoles/mg protein.

Total Cellular Thiols (TT)

Total cellular thiols were estimated by the method of Sedlak and Lindsay (1968). 0.5 ml homogenate was incubated with 1.5 ml of 0.2 M Tris buffer (pH-8.2), 0.1 ml of 0.01 mM dithio-bisnitro benzoic acid (DTNB) prepared in methanol and 7.9 ml absolute methanol. The colour was allowed to develop for 15 minutes and then the contents were centrifuged at 3,000 rpm for 15 minutes. The absorbance of the clear supernatant was measured against reference blank at 412 nm and total cellular thiol content was expressed as nmoles/mg protein.

Enzymatic Activities (Detoxifying and Anti-oxidant Enzymes)

Defense systems that tend to inhibit oxyradical formation include detoxifying and anti-oxidant enzymes such as SOD, CAT, GPx, GR and GST (Oost et al., 2003).

Superoxide Dismutase [(SOD), Superoxide:Superoxide Oxidoreductase, E. C. 1. 15. 1.1]

This was assayed according to the method of Kono et al. (1978). The reaction mixture containing 1.2 ml of solution A (containing 50 mM sodium carbonate in 0.1 mM
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EDTA buffer, pH-10.8), 0.5 ml of 96 mM nitroblue tetrazolium ([NBT] in solution A) and 0.1 ml of 0.6% Triton X-100 (w/v) in solution A, were incubated at 37°C for 10 minutes. Reaction was initiated by adding 0.1 ml of 20 mM hydroxylamine hydrochloride (pH-6). The rate of NBT dye reduction by O$_2^*$ anion generated due to photoactivation of hydroxylamine hydrochloride was recorded at 560 nm for 2 minutes, in the absence of PMS for blank. Then 0.1 ml PMS was immediately added after addition of hydroxylamine hydrochloride to the reaction mixture. After mixing thoroughly, 50% inhibition in the rate of NBT reduction by SOD present in the enzyme source was recorded. Enzyme activity was expressed as Units/mg protein. 1 unit enzyme activity is defined as the amount of enzyme inhibiting 50% nitroblue tetrazolium reduction.

Catalase [CAT], Hydrogen Peroxide:Hydrogen Peroxide Oxidoreductase, E. C. 1.11.1.6

This was assayed according to the method of Luck (1971) in the post mitochondrial (PMS) fraction. Three ml of assay mixture consisted of 2.9 ml of H$_2$O$_2$-phosphate buffer (0.16 ml H$_2$O$_2$ diluted to 100 ml with 0.067 M phosphate buffer, pH-7) and 0.01 ml PMS (DDW for blank). The decrease in O.D./30 seconds at 240 nm was read for 3 minutes. The enzyme activity was expressed as μmoles of H$_2$O$_2$ decomposed/min/mg protein using 0.071 mM$^{-1}$ cm$^{-1}$ as the molar extinction coefficient of H$_2$O$_2$.

Glutathione Peroxidase [GPx], NAD(P)H:Oxidised Glutathione Oxidoreductase, E. C. 1.11.1.9

Glutathione peroxidase was measured by the method described by Paglia and Valentine (1967). Three ml of reaction mixture contained 2.4 ml of 50 mM phosphate buffer (containing 1 mM EDTA, pH-7.0), 0.1 ml of 1 mM sodium azide, 0.1 ml of 0.2 mM NADPH, 0.1 ml of 1 U glutathione peroxidase and 0.1 ml of 1 mM reduced glutathione. After adding 0.1 ml of PMS (homogenized in 50 mM phosphate buffer pH-7.0) [0.1 ml buffer for blank], it was allowed to equilibrate at 25°C for 5 minutes. The reaction was initiated by the addition of 0.1 ml of 2.5 mM hydrogen peroxide. Absorbance at 340 nm was recorded for 5 minutes and values were expressed as nmoles of NADPH oxidized to NADP by using extinction coefficient of 6.2 × 10$^3$ M$^{-1}$ cm$^{-1}$ at 340 nm. The levels of GPx were expressed in terms of nmoles NADPH consumed/min/mg protein.
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Glutathione Reductase [(GSSG-R), NADPH-Oxidized Glutathione Oxidoreductase, E. C. 1. 6. 4. 2]

It was assayed according to the method of Horn (1971) in the post mitochondrial (PMS) fraction. Three ml of assay mixture consisted of 2.5 ml of 0.067 M phosphate buffer (pH-6.6), 0.2 ml of 6 mM NADPH (in 1% (w/v) sodium bicarbonate), 0.1 ml PMS (0.1 ml DDW for blank) and 0.2 ml of 7.5 mM oxidized glutathione (in buffer). Enzyme activity was monitored for 3 minutes by recording the change in O.D./30 seconds at 340 nm. GSSG-R activity was expressed as nmoles NADPH oxidized/min/mg protein using $6.22 \times 10^3 \mu M^{-1} cm^{-1}$ as the molar extinction coefficient of NADPH.

Glutathione-S-Transferase [(GST), Transferase, E. C. 2. 5. 1. 18]

It was measured by the method of Habig et al. (1974). 0.1 ml PMS was incubated in 1 ml of 0.2 M phosphate buffer (pH-6.5), 0.1 ml of 20 mM 1-chloro-2, 4-dinitrobenzene (CDNB) prepared in 95% ethanol and 0.8 ml of DDW. After mixing thoroughly, incubation was carried out at 37°C for 5 minutes. 0.1 ml of 20 mM GSH (dissolved in DDW) was added just before reading the O.D./30 seconds at 340 nm for 5 minutes. In case of blank, 2.9 ml phosphate buffer, 0.1 ml CDNB were taken and PMS along with GSH were omitted. Enzyme activity was expressed as µmoles GSH adduct formed/min/mg protein using $9.6 \times 10^3 \mu M^{-1} cm^{-1}$ as the molar extinction coefficient of GSH adduct formed with CDNB.

Estimation of Protein

Protein content was assayed in the homogenate and PMS of liver and kidney by the method of Lowry et al. (1951). To 10 µl of each sample, 990 µl of DDW and 5 ml of Lowry’s reagent (1% copper sulphate, 2% sodium potassium tartarate and 2% sodium carbonate in 0.1 N NaOH in ratio 1:1:98 v/v/v) were added. The contents were shaken well and allowed to stand for 10 minutes at 37°C. It was followed by addition of 0.5 ml of Folin-Ciocalteau’s reagent (1:1 diluted with water before use). The contents were thoroughly mixed and allowed to stand at room temperature for 30 minutes for maximum colour development. Blank and BSA standard (Bovine serum albumin) [(2 mg%): 200 mg of BSA in 10 ml DDW stored at 4°C] were run simultaneously and the absorbance was read at 670 nm.
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Statistical analysis

The data were analyzed using one-way ANOVA (analysis of variance). Significance between pair of means for control and treated groups was determined by Student’s t-test. The data were expressed as mean ± standard error (Mean ± SE) of seven mice and p < 0.05, p < 0.01, p < 0.001, were considered to be levels of significance.

Genotoxicity

Exposure to toxic chemicals may induce a cascade of events that cause DNA damage with subsequent expression in mutant gene products or progression of DNA damage leading to its deregulation (Shugart et al., 1992).

DNA Apoptosis (Isolation of Genomic DNA)

Apoptosis is characterised by DNA fragmentation and the appearance of nucleosomal-sized fragments on agarose gel electrophoresis has been used as a hallmark of apoptosis (Winter et al., 1998).

To detect apoptosis, DNA was isolated from liver and kidney tissues by the method described by Chen et al. (2004). Liver and kidney tissues were homogenized in Tris-EDTA (TE) buffer, containing 100 mM Tris-HCl and 0.5 M EDTA (pH-7.5). The homogenate was centrifuged at 10,000 rpm for 5 minutes at 4°C, following which the pellet was washed twice with PBS (phosphate buffered saline, pH-7.4). The pellet was incubated overnight at 55°C in lysis buffer [containing TE, 10% SDS and proteinase K (25 mg/ml)] and then centrifuged at 10,000 rpm for 5 minutes at 4°C. DNA was precipitated by adding 3.3 M ammonium acetate and chilled ethanol (99.5%) to the supernatant. The pellet obtained after centrifugation at 10,000 rpm for 5 minutes at 4°C was washed twice with 70% ethanol. Finally the dried pellet was dissolved in 100 µl of TE buffer and stored at -20°C.

DNA fragmentation (laddering of DNA) and the isolated genomic DNA were detected by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide (0.5 µg/ml) under Gel Doc (Bio Rad).

Restriction Fragment Length Polymorphism (RFLP) Assay for β-catenin Gene.

The DNA extracted by the method of Chen et al. (2004) was used to detect mutations in the β-catenin gene. The concentration of extracted DNA was determined first, followed by PCR (polymerase chain reaction) and restriction enzyme cutting as