Effect of Short-term Curcumin exposure and its Modulatory Role on Acute Cadmium Hepatotoxicity.

Tammanna R. Sahrawat1, Ranbir C. Sobti2, Sukesh C. Sharma3, Uma N. Saikia4 and Madan L. Sharma5.

Abstract: Curcumin, presents chemo-preventive, antioxidant and anti-inflammatory properties, whereas cadmium is a serious contaminant due to industrial and agricultural practices. Most of the work on curcumin deals with repeated exposures for longer durations. The present study was designed to investigate the effect of a single short-term (24 hour) curcumin treatment with FDA approved dose of 100 mg/kg body weight for humans, on Male Balb/c mice liver. Further, the modulatory role of its pre-treatment on acute cadmium hepatotoxicity was also studied. Animals treated with curcumin for 24 hours were divided into two groups, and one group was sacrificed. The other group was sacrificed after additional exposure to cadmium for 18 hours with corresponding positive and negative control groups. Oxidative stress was measured using a multi-parametric biochemical approach, and histopathological changes were studied using light and electron microscopes. Administration of curcumin for 24 hours resulted in an increase in oxidative stress in liver suggesting a pro-oxidant role, which might be due to the generation of reactive oxygen species, while post-treatment with Cd resulted in a synergistic effect on oxidative stress. Concurrent marked histological alterations were observed under the light microscope in the form of basophilic depositions, Kupffer cell hyperplasia, and lobular inflammation. Electron micrographs also revealed similar features along with pronounced damage to the endothelial cell fenestrations and bile canaliculi with crystalline deposits on hepatocytic surfaces. Therefore, it was concluded that after 24-hour exposure to curcumin, it acted as a pro-oxidant in mice liver and was not found to have an ameliorative effect on acute Cd-induced hepatotoxicity.

KeyWords: Curcumin, Cadmium, acute exposure, liver, oxidative stress, pro-oxidant scanning electron microscopy, light microscopy.

Introduction

Antioxidants play an important role to nullify the deleterious effects of free radicals such as reactive oxygen species, thereby not only protecting the cells and tissues but also regulating various pathological and physiological processes. Curcumin, derived from the rhizome of the herb Curcuma longa (turmeric spice), is one such antioxidant which is a bioactive hydrophobic polyphenol compound. It is a safe nutritional dietary supplement that has been widely used in traditional medicine and as a spice/coloring agent since time immemorial.

In India and Southeast Asia, the diverse biological functions of turmeric such as antioxidant activities, anti-inflammatory, and anti-mutagenic were realized since long, and it has been used in the treatment of inflammation, skin wounds, and tumors. In recent years, there is growing evidence that curcumin is a potentially important chemopreventive agent against cancer and may play a crucial role in both the prevention and treatment of various neurodegenerative diseases such as Parkinson’s and Alzheimer’s disease.

Garcia-Niño and Pedraza-Chaverri (2014) in a review on the protective effects of curcumin against heavy metals-induced liver damage, gave an account of the various mechanisms by which curcumin confers protection. These mechanisms included suppression of oxidative stress, inflammation, and activation of stellate cells in the liver, with concomitant up-regulation of enzymes involved in detoxification and Keap1/Nrf2/ARE pathway expression. Various studies on guinea pigs, rats, monkeys, and pigs have reported that on the feeding of curcumin or turmeric there were no toxic effects. On the other hand, Deshpande et al., (1998) reported that on feeding turmeric or ethanolic turmeric extract, in high doses for long-term exposure, to mice and rats, hepatotoxicity was observed in the form of profuse focal necrosis along with decreased body weights. Similarly, Kandarkar et al., (1998), while analyzing the effects of doses of whole spice turmeric or ethanolic turmeric extract reported being cancer protective, observed coagulative necrosis with areas of parenchymal regeneration.

Cadmium (Cd) is a toxic heavy metal that has no physiological function in the body and causes deleterious effects on health following both acute and chronic exposures. Moreover, Cd accumulates in the body over time as in humans its biological half-life is 17-30 years. Non-occupational exposure is generally via consumption of food and drinking water contaminated with Cd along with cigarette smoke. Occupational exposure mainly results from numerous modes of exposure, such as CdO, utilized in paint manufacture and Cd fume inhalation in battery industry. Exposure to Cd has been reported to affect a myriad of organs in humans, but most commonly it affects kidney, lung, bone and skeletal, cardiovascular, and nervous systems.

The liver is a major organ that is involved in metabolism and detoxification reactions, through which all substances that are absorbed by the intestine pass. The liver is known to accumulate toxins, including heavy metals such as Cd following acute or chronic exposures that result in hepatotoxicity.

Tarasub et al. in 2008 reported that following co-treatment

1 Assistant Professor, Centre for Systems Biology and Bioinformatics, Panjab University, Chandigarh, India.
2 Professor Emeritus, Department of Biotechnology, Panjab University, Chandigarh, India.
3 Professor, Department of Biochemistry, Panjab University, Chandigarh, India.
4 Professor, Department of Histopathology, P.G.I.M.E.R., Chandigarh, India.
5 Professor Emeritus, Department of Biotechnology, Panjab University, Chandigarh, India.

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with cadmium acetate and curcumin, curcumin was unable to ameliorate oxidative damage induced due to cadmium. In another study, they reported that combined treatment of curcumin and vitamin C was more effective in protection against Cd-induced hepatic injury by scavenging free radicals.

Hepatotoxic effects of Cadmium and beneficial effects of antioxidant compounds such as curcumin following long-term exposures have been studied in detail in previous studies. However, there is a lacuna in knowledge related to the effect of short-term treatment of curcumin on the liver and its modulatory role on subsequent treatment with cadmium chloride (CdCl₂) in mice. Therefore, the present study was undertaken to investigate the effect of short-term exposure to curcumin in the liver of mice. Further, the ameliorative effects of short-term pre-treatment with curcumin in acute cadmium chloride induced hepatotoxicity would be evaluated by assessment of biochemical parameters of oxidative stress and ultrastructural alterations.

**Materials and methods**

**Chemicals**

All chemicals were of analytical grade specifications and obtained from HIMEDIA Ltd, India. Cadmium chloride (CdCl₂) and curcumin were obtained from Sigma Chem. Co., St. Louis, MO, USA.

**Animals and Treatments**

Four to six-week-old Balb/c male mice weighing 20-25 grams were procured from the Central Animal House, Panjab University, Chandigarh. Mice were kept in cages, given food and water ad libitum and allowed to acclimatize for seven days, maintained at 12 hours light/dark regime before experimental use.

The animals were divided into five experimental groups, each containing seven mice and the doses was administered intraperitoneally. Curcumin dose of 100 mg/kg body weight (bw) was used, as it is the FDA approved dose for humans. Curcumin was dissolved in DMSO (dimethyl sulfoxide), an amphiphilic compound that increases the permeability of the membranes, for its effective uptake by the cells. A single i.p. injection of 100 mg curcumin/kg bw dissolved in 100 ml DMSO was administered to the group T-II and animals were sacrificed after 24 hours of exposure, and therefore, a DMSO-treated group was also studied (group T-I). Curcumin chloride was dissolved in water and dose used in this study (0.8 mg CdCl₂/kg bw) was two-thirds the experimentally determined LD₅₀ value for intraperitoneal exposure in mice (calculated using Sun’s formula, 1963) to which group T-III were exposed for 18 hours.

The groups T-IV and T-V were injected 100 ml DMSO or 100 mg curcumin/kg bw dissolved in 100 ml DMSO, respectively and after 24 hours were given another treatment of 0.8 mg CdCl₂/kg bw and finally sacrificed after 18 hours of the second treatment following various protocols and ethical procedures.

**Hepatic Biochemical Estimations**

**Preparation of Homogenate**

10% homogenates of liver were prepared in 50mM 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 by measuring the tissue malondialdehyde (MDA) level, superoxide anion-SA, hydroperoxides-HP, reduced glutathione-GSH, and protein content.

**Preparation of Post Mitochondrial Supernatant -PMS**

Liver 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 activities of Superoxide dismutase-SOD, Catalase-CAT, Glutathione peroxidase-GPx, Glutathione reductase-GR, and Glutathione-S-transferase-GST, by continuous spectrophotometric rate determination (using Perkin Elmer Lambda 35 UV/vis spectrometer) and protein content.

**Scanning electron microscopy**

The liver slices were washed with phosphate buffer and fixed in 4% glutaraldehyde in phosphate buffer. They were then dehydrated in ascending acetone grades and critical point dried through transitional fluid amyl acetate. The dried samples were fixed on metal stubs with double adhesive tape for gold sputtering. The stubs so prepared were examined using JEOL JSM 6100 Scanning Electron Microscope and captured at different magnifications to study the ultrastructural features.

**Statistical analysis**

Student’s t-test determined significance between pair of means for control and treated groups. The data were expressed as mean ± standard error of seven mice and the level of significance considered was P < 0.05.

**Results and Discussion**

Effects of short-term curcumin treatment and pre-treatment for 24 hours, followed by acute Cd exposure for 18 hours were studied in male Balb/c mice. To investigate whether the exposure to curcumin alone or followed by Cd exposure causes hepatotoxicity resulting from oxidative stress, the levels of hepatic superoxide anion, hydroperoxides, GSH, total thiols, and MDA were measured in the liver homogenates of treated mice while activities of enzymes of the antioxidant system were measured in the PMS.

DMSO treated control group T-I was compared with the treated groups T-II to T-V. The GSH and total thiol levels in the liver of group T-I were 0.80 ± 0.03 and 31.1 ± 2.1 nmolies/mg protein, respectively. Both GSH and total thiol levels decreased in all the treated groups T-II to T-V as compared to the controls (T-I). Exposure to curcumin for 24 hours (group T-II) or pre-treatment with curcumin followed by acute Cd exposure (group T-V) resulted in increased oxidative stress as seen by an increase in LPO, SA, HP and activities of antioxidant and detoxifying enzymes SOD, CAT, GPx, GR and GST, with a concomitant decrease in cellular antioxidant GSH for the groups T-II and T-V (Figs. 1-3). These
observations indicate pro-oxidant effects following short-term exposure to curcumin in mice. The increase in activities of antioxidant/detoxifying enzymes SOD, CAT, GPx, GR and GST following treatments with curcumin, Cd or curcumin and Cd combinations is suggestive of the activation of the cellular protective mechanisms involving antioxidant and detoxifying enzymes. However, the protective mechanisms of the cells i.e. antioxidant and detoxifying enzymes were insufficient to neutralize the overwhelming effect of ROS generation, thereby resulting in the observed oxidative stress for the doses and duration of exposure in the present study.

The basal MDA level, indicative of lipid peroxidation, in control liver was 0.36 ± 0.07 nmoles/mg protein. MDA was significantly increased by 1.5, 1.6 and 1.8 fold, following Cd exposure in groups T-III to T-V respectively (Fig. 1). On the other hand, total thiols showed a significant decrease of 4.7, 5.4 and 8.1 fold for Cd-exposed groups, i.e. groups T-III to T-V, respectively, irrespective of the pre-treatment with curcumin in group T-V (Fig. 2). A corresponding decrease of 2.3, 2.5, and 5.7 fold in GSH levels in groups T-III to T-V, respectively, was observed (Fig. 2). Similar increase ranging between 1.1 to 2 fold was observed in the activities of the liver antioxidant enzymes SOD, GPx, CAT and GSH pathway enzymes GPx and GR for groups T-III to T-V (Figs. 2 and 3).

Acute Cd exposure has been reported to be associated with an increase in LPO, SA and HP and disruption of the cellular antioxidant system with a subsequent decrease in GSH31, 32. Waisberg et al. (2003) had reported that Cd cannot directly generate free radicals and rather is involved in the indirect formation of ROS and RNS33. An imbalance between lipid peroxidation and antioxidant system results in oxidative stress. One of the main manifestations of oxidative damage is lipid peroxidation34. In the present study, the increase in MDA levels with a concurrent decrease in GSH and total thiols, indicates the depletion of reduced glutathione and total thiols, due to increased lipid peroxidation. The increase in the levels of hydroxyl radicals, superoxide anions, hydrogen peroxide along with the enzymes of the antioxidant system indicated that short-term exposure to curcumin and/or Cd results in oxidative stress.

Pro-oxidant effects of curcumin have also been previously reported, though mainly in terms of reactive oxygen species induced DNA damage35-37. Rukkumani et al. (2004) investigated the effect of curcumin on alcohol and poly-unsaturated fatty acid-induced oxidative stress and had also reported an increase in LPO and HP with an associated decrease in GSH following curcumin administration38. In a study following co-treatment with cadmium acetate and curcumin, it was reported that curcumin did not have a protective effect against Cd-induced oxidative stress39. Following long-term pre-exposure, to curcumin, there are reports that tissues are protected against LPO as curcumin increases intracellular glutathione concentration and acts as a powerful oxygen free radical scavenger39.

Masuda et al. (1999) explained the anti-oxidative role of curcumin, suggesting a two-stage mechanism involving a radical trapping stage and radical termination stage40. In a subsequent study, they elaborated that radical terminations were of two types; (i) dimer formation between two curcumin radicals and (ii) a coupling product between curcumin and lipid hydroperoxide with the latter being fundamental in the antioxidant mechanism of curcumin40. Thus, the increase in ROS generation observed in the groups exposed to curcumin viz. groups T-II and T-V may be explained based on the formation of free radicals, before the radical tapping stage during the 24 hours of exposure in the present study resulting in pro-oxidant effects.

Kawanishi et al. (2005) reported that like many antioxidants, curcumin could be a “double-edged sword,” having carcinogenic and pro-oxidant effects on one hand and anticancer and antioxidant effects on the other41. The antioxidant, protective and ameliorative effects of curcumin have been reported after long term, sustained exposures of curcumin by exerting effects of a potent scavenger of different ROS including superoxide anion radicals (O2-) and hydroxyl radicals (·OH)43.

In our previous study on dose-response effects following acute Cd exposures in mice, we reported that with an increase in CdCl2 dose there were significant changes in the biochemical parameters indicative of oxidative stress, while a certain amount of Cd load was required for histopathological changes to take place in hepatic tissue44. The hepatic tissues of the mice administered different treatments were further investigated to identify the correlation between oxidative stress and histopathological alterations.

Liver from the control group T-I and curcumin-treated
group T-II did not exhibit any macroscopic alterations in the morphology of hepatic tissue of mice. On the other hand, exposure to Cd (groups T-III and T-IV) or curcumin pre-treatment followed by Cd exposure (group T-V) resulted in marked alterations in the histology of the liver as observed under the light and scanning electron microscopes.

Light micrograph examination of sections of the groups T-II, T-III/T-IV, and T-V revealed signs of cell injury as compared to control mice (group T-I) that had normal morphology and occasional foci of lobular inflammation (Fig. 4A). Multiple foci of lobular inflammation by mononuclear cells and Kupffer cell hyperplasia were observed in all the other groups T-II to T-V (Figs. 4 B-D), though cell injury was more pronounced in group T-V with prominent sinusoidal dilation and giant cell transformation along with depositions of basophilic material (Fig. 4D). The morphological changes were diffused and not localized to any specific area, suggesting that Cd and curcumin acted as general hepatotoxins following short-term exposures.

Investigation of the morphological features of the livers under the scanning electron microscope revealed that the mesothelial layer of the liver was apparently normal in the control group T-I, groups T-II and T-V (Figs. 5 A, B and D), whereas hyperplasia with necrotic damage was observed in the liver of mice in the groups T-III/T-IV (Fig. 5C). Kupffer cells were seen lying over the endothelial cells lining the sinusoids in groups T-I and T-II (Figs. 6 A & B). Similar to the observations of KC hyperplasia seen in the light micrographs of groups T-II and T-V (Figs. 4 C & D), KC lying over necrotic hepatocytes were also observed on the electron micrographs (Fig. 6 C) with crystalline deposits on the hepatocytic surface in the group T-V (Fig. 6 D).

Endothelial cells lining the sinusoidal wall of the liver have characteristic fenestrations with well-defined porosity of bile canaliculi, as observed in the liver of groups T-I and T-II (Figs. 7 and B: A and B). However, loss of endothelial cell fenestrations and blockage of bile canaliculi due to blebbing of the surface epithelia due to necrotic damage were seen in the liver of mice in the groups exposed to Cd, i.e. T-III/T-IV and T-V (Figs. 7 and B: C and D).
Figure 4. Micrographs of liver of treated mice. (A) Normal morphology with occasional foci of lobular inflammation in control group T-I. H & E × 550. (B) Portal tract inflammation and Mononuclear cell (MNC) infiltration in group T-III/T-IV. H & E × 550. (C) MNC infiltration around the central vein, KC (Kupffer cell) hyperplasia and lobular inflammation in group T-II. H & E × 280. (D) Hepatocytic damage with giant cell transformation, sinusoidal dilation and KC hyperplasia with basophilic deposits within heptocytes and sinusoids in group T-V. H & E × 550.

Figure 5. Scanning electron micrographs of mesothelial layer of liver of mice. (A) Group T-I (SEM 10 µm), (B) Group T-II (SEM 100 µm) and (D) Group T-V (SEM 10 µm) - Normal mesothelial layer. (C) Group T-III/IV show hyperplasia and necrotic damage of mesothelial layer (SEM 10 µm).

Figure 6. Micrographs of liver showing. (A) Group T-I (SEM 1µm) and (B) Group T-II (SEM 1µm) Kupffer cell lying on the surface of normal endothelial cells lining the sinusoids. (C) Group T-III/IV has Kupffer cells lying over necrotic hepatocytes (SEM 1µm). (D) Crystalline deposits observed on the hepatocyte surface group T-V (SEM 1µm).
A plausible mechanism of curcumin and Cd-induced hepatotoxicity may be that the damage results cumulatively due to release of endogenous inflammatory mediators (ROS) and concurrent Kupffer cell and mononuclear cell activation as seen on light and electron micrographs. Blockage of the bile canaliculi and loss of endothelial cell fenestrations may further result in ischemia\(^\text{44}\) with subsequent loss of function of hepatocytes.

**Conclusions**

In summary, the present results suggested that curcumin exposure for 24 hours or curcumin pre-treatment followed by Cd exposure for 18 hours, in mice resulted in increased oxidative stress that was measured in terms of increased levels of LPO, SA, HP and activities of enzymes of antioxidant and detoxifying systems with concomitant decrease in the GSH and total thiols. Corresponding histopathological alterations were observed in the liver sections in the light and electron micrographs of the treated mice, suggesting that on short-term exposure to curcumin, it acts as a pro-oxidant and has a synergistic effect on acute Cd hepatotoxicity.

**Conflict of Interests**

The authors declare that there is no conflict of interests.

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