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Protective Effects of Curcumin, Vitamin C, or their Combination on Cadmium-Induced Hepatotoxicity

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ABSTRACT

Curcumin, a biologically active compound from turmeric, and vitamin C act as a natural antioxidant and potent chemopreventive agent. The objective of the study was to investigate whether the combined pretreatment with curcumin and vitamin C offers more beneficial effects than that provided by either of them alone in reversing cadmium (Cd)-induced hepatotoxicity. For this purpose, 64 adult male Wistar rats, equally divided into control and seven treated groups, received either Cd (as $CdCl_2$ 5 mg/kg), curcumin 400 mg/kg, curcumin 200 or 400 mg/kg + $CdCl_2$, vitamin C 100 mg/kg + $CdCl_2$, curcumin 200 or 400 mg/kg + vitamin C + $CdCl_2$. All groups were treated by gavage for 27 days. The results showed that Cd treatment increased significantly lipid peroxidation levels,decreased significantly the glutathione levels, increased significantly on metallothionein (MT) expressions including the degenerative changes of liver histological tissues were observed. The treatment of Cd-exposed rats with curcumin or vitamin C alone could not reverse Cd-induced the above changes. The combined treatment with curcumin along with vitamin C before Cd intoxication was more effective than that with either of them alone in reducing such changes and reverse the changes almost similar to that of control. In conclusion, the results demonstrated that the combined pretreatment with curcumin along with vitamin C could recover the alterations and offer more protection than curcumin or vitamin C alone against Cd hepatotoxicity.

KEY WORDS

curcumin, vitamin C, cadmium toxicity, liver, metallothionein

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INTRODUCTION

Cadmium (Cd) is a widespread environmental pollutant, having diverse toxicity in various organs of man and animals and is classified as a human carcinogen [1]. Cd contamination of environment is a subject of serious international concern since the metal is known to enter the food chain and can undergo bioaccumulation, end angering human health. The liver makes up the bulk of total body burden to Cd because of its ability to produce large amount of metallothionein, a metal-binding protein with high affinity for Cd [2]. The mechanism responsible for Cd toxicity may be multifactorial. Cd can cause oxidative damage within tissues, which is considered an early sign of toxicity and has been linked with carcinogenesis [3].

Cd exerts its toxic effects via oxidative damage to cellular organelles by inducing the generation of reactive oxygen species (ROS) [4] which consist mainly of $^{\bullet}O_2$, H_2O_2 and $^{\bullet}OH$. The molecular mechanism by which the generation of free radicals are far from being understood but reports have indicated that Cd does this via an indirect phenomenon [5]. In addition to that depletion of glutathione and other endogenous antioxidants may also contribute significantly to the development of Cd-induced toxic oxidative stress [6]. If these ROS-mediated stress events are not balanced by repair processes, affected cells undergo apoptosis or necrosis [7].

Antioxidants are the natural defense mechanism existing in our system and these are capable of scavenging the deleterious free radicals. A number of dietary antioxidant compounds have been shown to influence the membrane characteristics such as fluidity, stability and susceptibility to membrane oxidative damage [8]. Recently, a great deal of attention has focused on the protective biochemical functions of naturally occurring antioxidants in biological systems against toxic heavy metals. Thus it is believed that antioxidant should be one of the important components of an effective treatment of Cd poisoning. Curcumin (diferuloylmethane), a yellow coloring ingredient of the spice turmeric obtained from the rhizomes of *Curcuma longa* Linn (Zingiberacea), a perennial herb distributed mainly throughout tropical and subtropical regions of the world. Curcumin represents a class of anti- inflammatory and antioxidants reported to be a potent inhibitor of ROS formation [9, 10]. Reddy and Lokesh [11] indicated that curcumin is a potent scavenger of a variety of ROS including superoxide anion radicals and hydroxyl radicals. Curcumin administration has been reported to prevent the arsenic, gentamicin and acetaminophen-induced oxidative stress in rats [12, 13, 14]. The protective effects of curcumin against chemically-induced hepatotoxicity are well documented, and have been attributed to its intrinsic antioxidant properties [15, 16]. In addition, vitamin C or ascorbic acid exhibit a protective effect against free radical-induced oxidative damage [17]. Its scavenging effect of oxygen radicals has been clarified. It can participate in the redox mechanism of the cell, and thereby neutralize ROS.

There are multitudes of reports available on the protective effects of curcumin, vitamin C individually against various xenobiotics induced

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oxidative stress in experimental animals. Still to date the reports are scanty regarding the combined alleviated efficacy of curcumin in combination with vitamin C on Cd induced hepatotoxicity in rats. As well as there are some controversies over the combined administration of curcumin in combination with vitamin C. In view of the above considerations, the present study was designed to evaluate the protective efficacy of curcumin in combination with vitamin C on Cd induced oxidative damages in the liver of rats.

MATERIALS AND METHODS

Chemicals

Curcumin was purchased from Cayman Chemical Company. $CdCl_2$, Vitamin C, Bovine serum albumin and Bradford reagent was purchased from Sigma-Aldrich Chemical Company, St. Louis, USA. The other chemicals used, eg. absolute ethanol was purchased from Merck (Darmstadt, Germany) and all the reagents were of analytical grade.

Animals

Adult male Wistar rats were used in the present study. The experimental animals were supplied by the National Laboratory Animal Center of Mahidol University and used for experiments after 1 week of acclimatization. The animals were maintained as national guidelines and protocols, approved by the Institutional Animal Ethics Committee and in an air-conditioned animal house with constant 12 h light and 12 h dark schedule. Animals were fed on standardized diet for rodents and water *adlibitum*.

Experimental design

Curcumin, vitamin C and $CdCl_2$was dissolved in sterile distilled water. The dose of $CdCl_2$, curcumin and vitamin C used in this study was selected on the basis of the previous study [18, 19, 20]. Curcumin or vitamin C was given daily by oral gavage for 1 h before $CdCl_2$ administration. The experiment was conducted over a period of 27 days. The animals, at 200-220 g initial body weight, were randomly divided into eight groups of 8 animals each.

Group 1: Control rats received only distilled water for a period of 27 days.
Group 2: Normal rats orally received curcumin (400 mg/kg BW) alone for a period of 27 days.
Group 3: Normal rats orally received cadmium chloride (5 mg/kg BW) for 27 days.
Group 4: Normal rats orally received curcumin (200 mg/kg BW) for 1 h prior to Cd (5 mg/kg BW) for 27 days.
Group 5: Normal rats orally received curcumin (400 mg/kg BW) for 1 h prior to Cd (5 mg/kg BW) for 27 days.
Group 6: Normal rats orally received vitamin C (100 mg/kg BW) for 1 h prior to Cd (5 mg/kg BW) for 27 days.
Group 7: Normal rats orally received curcumin (200 mg/kg BW) in combination with vitamin C (100 mg/kg BW) for 1 h prior to Cd (5 mg/kg BW) for 27 days.
Group 8: Normal rats orally received curcumin (400 mg/kg BW) in combination with vitamin C (100 mg/kg BW) for 1 h prior to Cd (5 mg/kg BW) for 27 days.

All groups of rats were treated by oral gavage once daily for 27 days. All the animals were sacrificed 24 h after the last treatment following protocols and ethical procedures.

The liver was immediately dissected out, weighed and washed using chilled saline solution. Tissues were minced and homogenized (10%w/v), separately, in ice-cold 0.1 M phosphate buffer (pH 7.4) using glass homogenizer. The homogenate was used for the determination of MDA and reduced GSH. The remaining liver was fixed in 10% neutral phosphate buffer formalin solution for histological analysis and immunohistochemistry of metallothionein.

Malondialdehyde (MDA) and reduced glutathione (GSH) assays

Lipid peroxidation (LPO) level was measured by the method of Buege and Aust [21] and evaluated by measuring the malondialdehyde (MDA) concentration which was the end product of LPO. The tissues homogenates were precipitated with trichloroacetic acid. After centrifugation (1500×g, 15 min), the supernatant was mixed with thiobarbituric acid (TBA) reagent and the mixture was kept at 100 °C for 15 min. The level of LPO was measured based on the formation of TBA reactive substance (TBARS) to produce a red colored complex with a peak absorbance at 535 nm. An extinction coefficient of 1.56 X 10^5 M^{-1} cm^{-1} was applied for calculation and results were expressed as nM/mg protein.

Reduced GSH was determined according to the method by Beutler [22] using Ellman's reagent. The procedure is based on the reduction of Ellman's reagent by SH groups to produce 5,5'-dithiobis (2-nitrobenzoic acid) which has an intense yellow color that is measured spectrophotometrically at 412 nm using Thermo Scientific Genesis 10S spectrophotometer. GSH levels were calculated using an extinction coefficient of 1.36×10^5 M^{-1} cm^{-1}. Results were expressed as μM/mg protein.

The protein content in supernatant was estimated by the method of Bradford et al. [23] using bovine serum albumin (BSA) as the standard.

Histopathological studies

Immediately after sacrifice, the liver was removed surgically and rinsed with ice cold physiological saline. For microscopic evaluation liver was fixed in 10% neutral phosphate buffer formalin solution for 48 h. Following dehydration in ascending series of ethanol (70, 80, 95, 100%), tissue samples were cleared in xylene and embedded in paraffin. Tissue sections of 5.0 μm were stained with hematoxylin and eosin (H&E). These sections were examined under light microscopy (Axioskop 40, Zeiss) and documented by digital photocamera (Axiocam, Zeiss).

Immunohistochemistry of Metallothionein

For immunohistochemical detection of MT protein, the sections of liver tissues on poly-L-lysine coated slides were deparaffinized in xylene and hydrated in ethanol series. Antigen retrieval was performed with 10 mM citrate buffer, pH 6 for 15 min. After incubation with 10% normal goat serum for 5 min at room temperature, sections were incubated overnight at 4 °C with the primary antibody mouse anti-metallothionein (Invitrogen, clone E9) in 1% BSA using 1:50 dilution. To detect the specific binding of the primary antibody, an immunohistochemical staining kit (Histostatin Plus, Zymed laboratories, USA) was used, with which tissues were incubated sequentially with a biotinylated secondary antibody for 2 h at room temperature. This was followed by streptavidin peroxidase complex. Finally, diaminobenzidine was used as chromogen. Sections were counter stained with hematoxylin. Preparations were evaluated by a bright field microscope and were photographed (Axioskop 40, Zeiss). Negative control sections were prepared by substituting the primary antibodies with phosphate-buffer saline.

MT immunostaining was considered positive when the nuclei and cytoplasm of the hepatocytes were stained prominently (purplish brown or reddish brown). The percentage of cells with immunostained MT was determined by an automated image analyzer (Axiocam, Zeiss) after total and MT-stain-positive cells were counted at 100× magnification in three random fields (90 nm^2) of three different animals. Results were expressed as percentage of positive cells.

STATISTICAL ANALYSIS

All the data were expressed as mean±SEM (standard error of the mean). One-way analysis of variance (ANOVA) followed by a post hoc test

Figure 1: Effects of curcumin and vitamin C on lipid peroxidation, expressed as MDA levels, in rat liver homogenate induced by Cd. Results were expressed as mean ± SEM from 8 animals in each group. [a]denote significantly different from control group at $p < 0.01$, [b]denote significantly different from Cd treated group at $p < 0.05$, [c]denote significantly different from Cd treated group at $p < 0.01$.

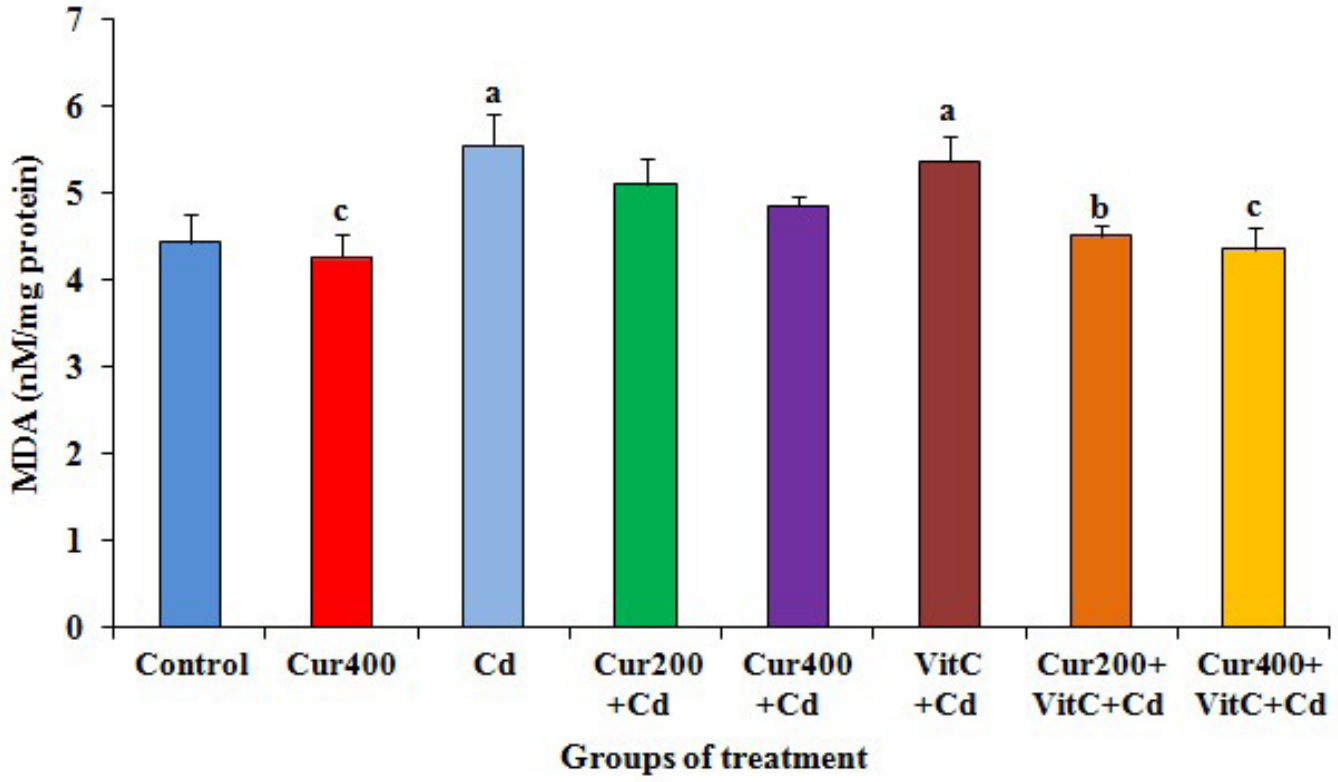


Figure 2: Effects of curcumin and vitamin C on reduced GSH levels in rat liver homogenate induced by Cd. Results were expressed as mean ± SEM from 8 animals in each group. [a]denote significantly different from control group at $p < 0.01$, [b]denote significantly different from Cd treated group at $p < 0.05$, [c]denote significantly different from Cd treated group at $p < 0.01$.

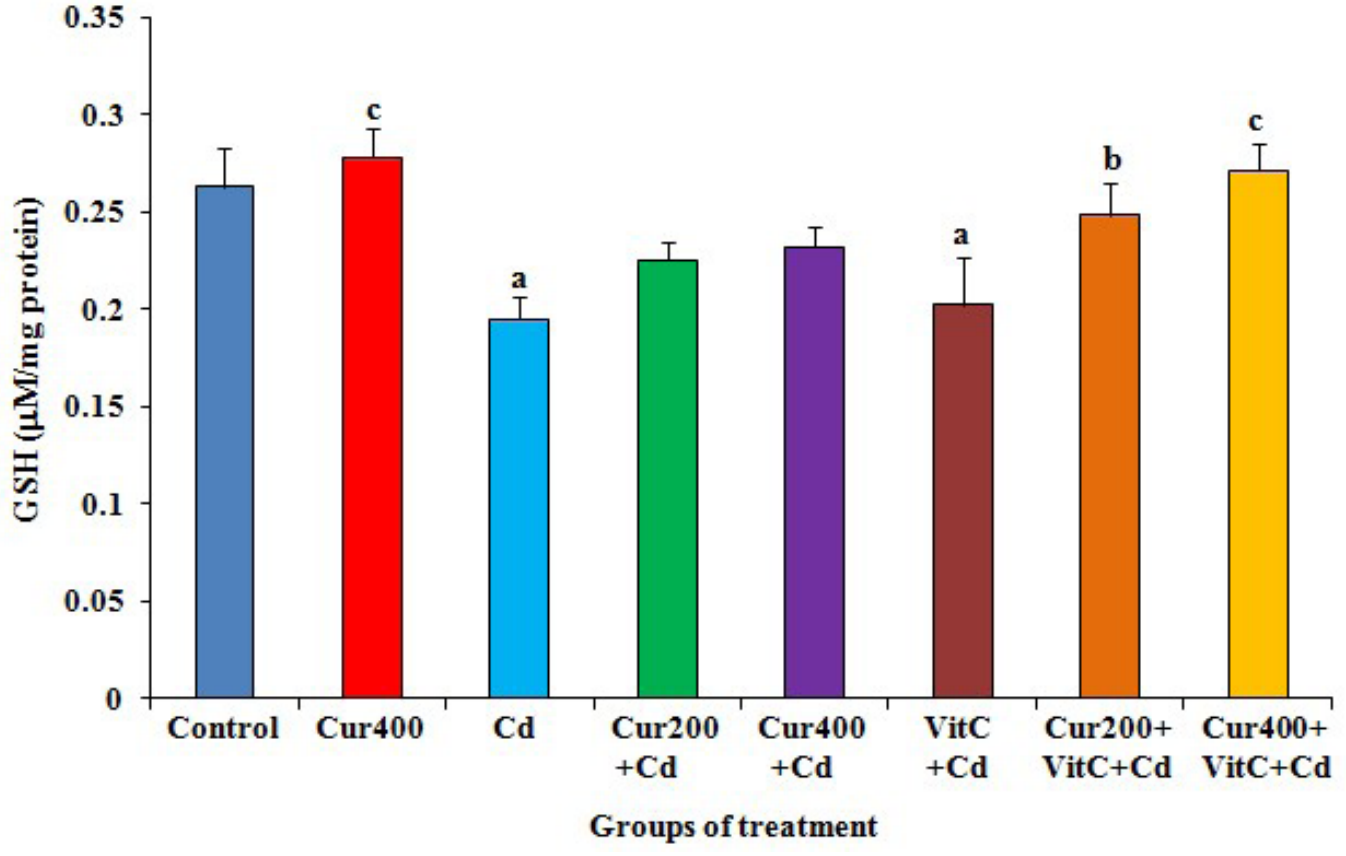
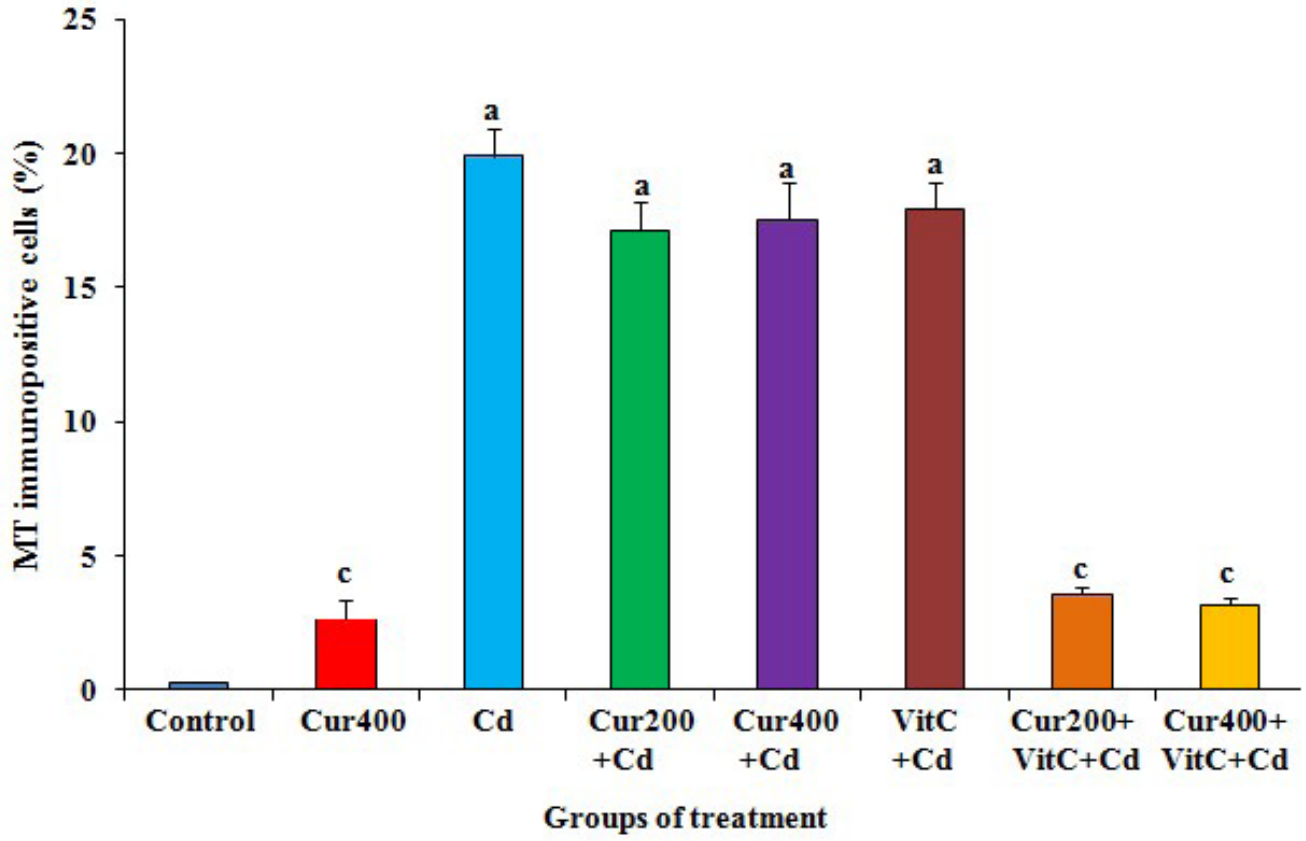


Figure 3: Effects of curcumin and vitamin C on MT immunostaining in rat liver tissue induced by Cd. Results were expressed as mean ± SEM from 8 animals in each group. [(a)]denote significantly different from control group at $p < 0.01$, [(c)]denote significantly different from Cd treated group at $p < 0.01$.



was carried out to test for any differences between the mean values of all groups. A *p*-value of <0.05 and 0.01 was considered statistically significant.

RESULTS

Effects of curcumin and vitamin C on MDA and reduced GSH levels in rat liver induced by cadmium

MDA levels in the liver tissue were used as a measure of lipid peroxidation. Figures 1 and 2 showed the changes of MDA and reduced GSH levels respectively in all groups. The MDA and reduced GSH levels were similar in the control and curcumin 400 mg/kg groups ($p>0.05$). Administration of Cd caused significantly increase of MDA levels and significantly decrease of reduced GSH levels as compared to the control group (p <0.01). The administrations of curcumin and vitamin C alone before Cd intoxication could not reverse the changes of MDA and reduced GSH levels as compared to the Cd treated group. The above changes were reversed in the combined pretreatment with curcumin 200 ($p < 0.05$) or 400 mg/kg ($p < 0.01$) and vitamin C when compared to the Cd treated group. Therefore, the combined pretreatment with curcumin particularly at the dose of 400 mg/kg and vitamin C could attenuate Cd intoxication in rat liver.

Effects of curcumin and vitamin C on immunohistochemistry of metallothionein and histological analysis in rat liver induced by cadmium

MT immunostaining in all groups were shown in Figure 4. Immunostained positive cells are very scarce in control and curcumin 400 mg/kg alone. In liver of Cd-treated rats, MT expressions were significantly increased ($p < 0.01$) as compared to the control group. The administrations of curcumin and vitamin C alone before Cd intoxication could not reduce such changes when compared to the Cd-treated group. The combined treatment with curcumin 200 or 400 mg/kg and vitamin C before Cd intoxication reduced significantly ($p < 0.01$) on MT expressions as compared to the Cd-treated group and reverse the changes almost similar to that of control.

The histological analysis of liver revealed that control (Fig. 5A) and curcumin-alone-treated rats (Fig. 5B) showed normal histoarchitecture. The administration of Cd in rats produced severe hepatic damage including the extensive degeneration of hepatocytes with necrosis, inflammation, cytoplasmic vacuolization and inflammatory cell infiltration (Figs. 5C) when compared to control rats (Fig. 5A). The cellular infiltrations and vacuolization were localized around the central vein.The administrations of curcumin and vitamin C alone before Cd intoxication showed a mild degree of lesions (Fig. 5D, 5E and 5F) when compared to the Cd-treated group. The combined treatment with curcumin 200 or 400 mg/kg and vitamin C before Cd intoxication reduced such changes and kept the organ almost similar to that of control (Fig. 5G and 5H). Results of the histological assessment support the outcome of the earlier studies by exhibiting Cd-induced necrosis in the liver tissue and its protection by combined pretreatment with curcumin and vitamin C.

DISCUSSION

In the present study, we found that subacute Cd intoxication induced liver damages, measured by increased lipid peroxidation and decreased reduced GSH. The damages were also associated with histopathological changes and immunohistochemistry of MT staining. The purpose of the present study is to evaluate the potential benefit of curcumin in combination with vitamin C on Cd-induced hepatotoxicity compared

Figure 4: Metallothionein (MT) immunostaining in rat liver at 100X magnification. Positively stained MT yielded a reddish brown-colored product in nuclei and cytoplasm of hepatocytes (A) control rat liver, (B) curcumin (400 mg/kg)-treated rat liver, (C) Cd (5 mg/kg)-treated rat liver, (D) curcumin (200 mg/kg) + Cd-treated rat liver, (E) curcumin (400 mg/kg) + Cd-treated rats liver, (F) vitamin C (100 mg/kg) + Cd-treated rat liver, (G) curcumin (200 mg/kg) + vitamin C + Cd-treated rat liver, (H) curcumin (400 mg/kg BW) + vitamin C + Cd-treated rat liver. Immunostained positive cells are very scarce in control and curcumin 400 mg/kg alone. MT was stained prominently in Cd, curcumin + Cd and vitamin C + Cd groups but reduced in combined treatment with curcumin and vitamin C.

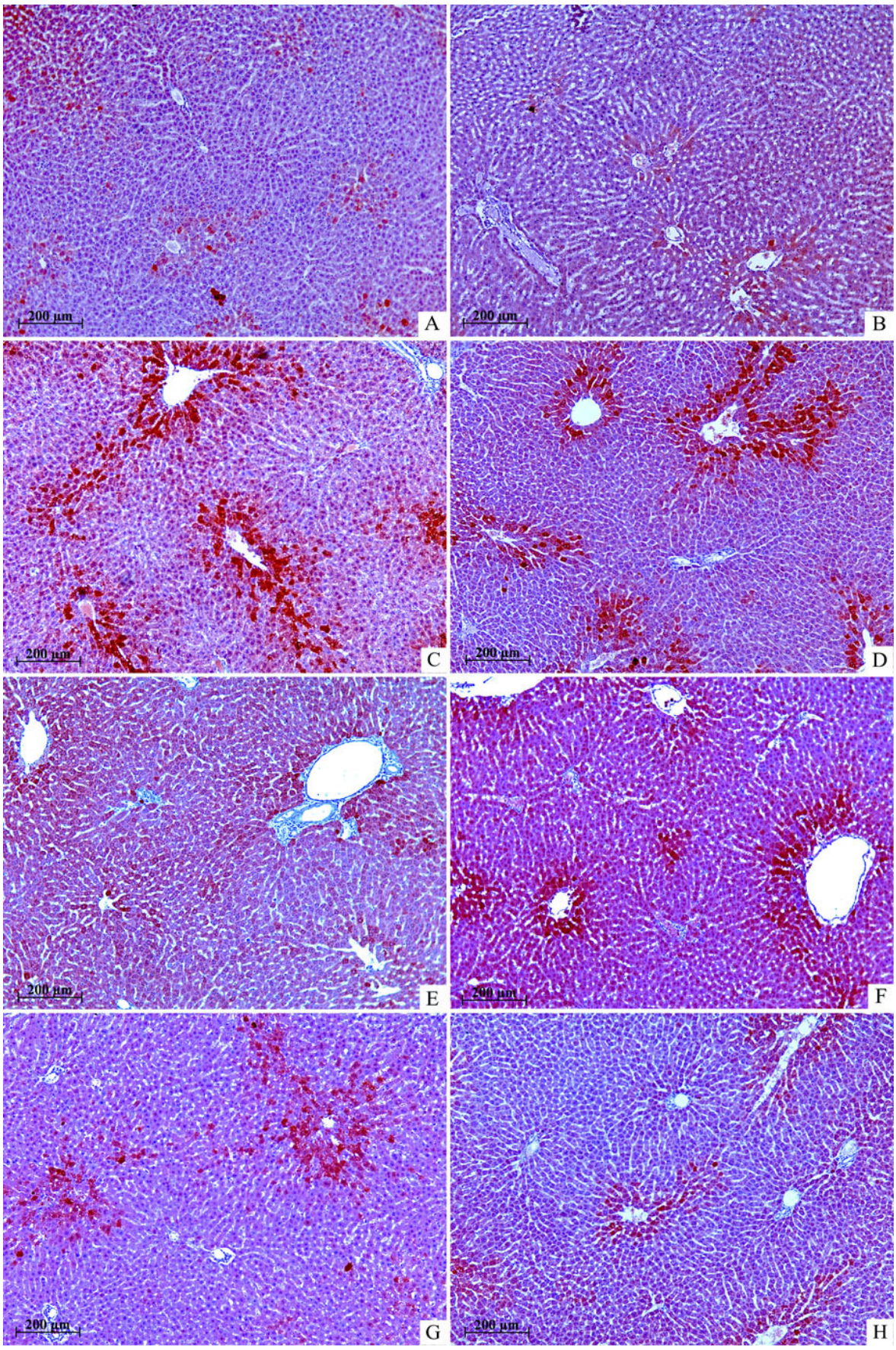
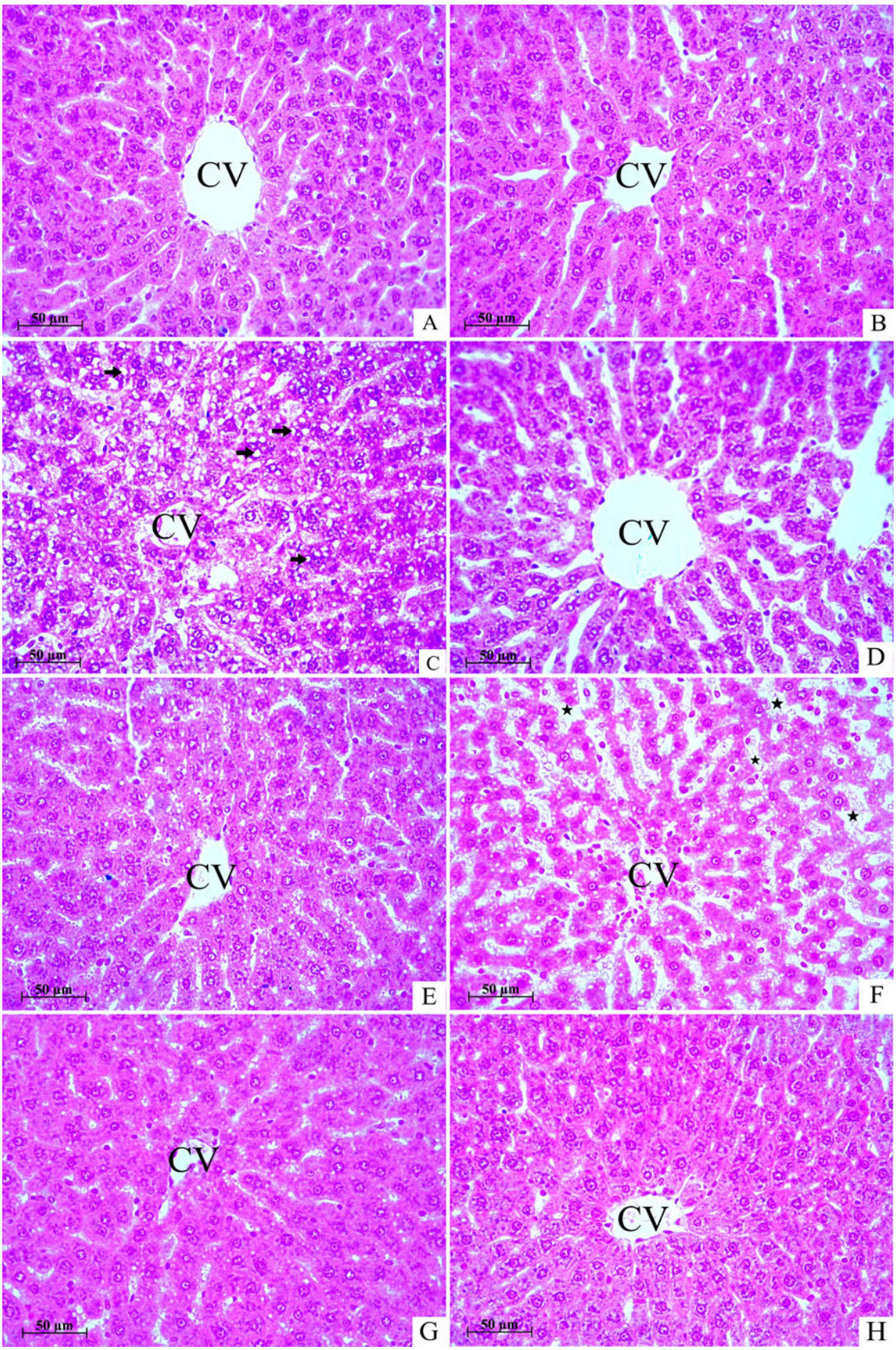


Figure 5: Representative photographs of rat liver by light microscope with H&E staining at 400X magnification. (A) control rat liver, (B) curcumin (400 mg/kg)-treated rat liver showing the normal appearance, (C) Cd (5 mg/kg)-treated rat liver showing extensive degeneration of hepatocytes with focal necrosis, vacuolated cytoplasm (arrow), inflammatory cell infiltration and damaged central vein, (D) curcumin (200 mg/kg) + Cd-treated rat liver, (E) curcumin (400 mg/kg) + Cd-treated rats liver showing a mild degree of lesions (F) vitamin C (100 mg/kg) + Cd-treated rat liver showing the dilated sinusoid (stars) (G) curcumin (200 mg/kg) +vitamin C + Cd-treated rat liver, (H) curcumin (400 mg/kg BW) + vitamin C + Cd-treated rats liver showing almost the normal appearance of hepatocytes around the central vein (CV).



to curcumin or vitamin C treatment alone in rats. Several lines of studies showed that curcumin prevent the arsenic, gentamicin and acetaminophen-induced oxidative stress in rats [12, 13, 14]. Vitamin C might also have a structural role in stabilizing membranes [24]. The present study has shown that administration of curcumin in combination with vitamin C significantly reversed the levels of MDA, reduced GSH, MT expressions and histopathological changes of liver tissues, which infers that the Cd induced alterations in the liver membrane might have been protected through membrane stabilization effects of curcumin and vitamin C.

Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of Cd.Cd induced oxidative stress by producing hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide [25]. Significant increase in the level of hepatic TBARS in Cd intoxicated rats could be possibly due to excessive formation of free radicals which leads to the deterioration of biological macromolecules [26]. Milton Prabu et al. [27] have reported that lipid peroxidation is considered a sensitive marker of Cd hepatotoxicity. Manca [28] have also reported the increased level of LPO in the various tissues of Cd treated rats. In the present study, we observed a marked elevation of hepatic LPO following Cd administration was in consistence with the other reports in Cd- intoxicated rats [29].

The impairment of the antioxidant defense system is considered as a critical event in Cd-induced hepatotoxicity. Exposure of Cd is characterized by the depletion of tissue and circulating non-enzymatic antioxidants including GSH [30]. GSH is a sulfhydryl peptide enormously present in all biological systems and participates in the maintenance of cytoplasmic and membrane thiol status. It forms the first line of defense against oxidative insult by acting as a non-enzymatic antioxidant by direct interaction of its sulfhydryl group with ROS or it can be involved in the enzymatic detoxification reaction of ROS as a cofactor or as a coenzyme. Cd binds exclusively to sulfhydryl groups of GSH leading to its inactivation [31]. In the present study, the reduced level of hepatic GSH by Cd could be probably due to either increased utilization of GSH by the cells act as scavengers of free radicals produced by Cd or increased utilization of GSH for the activity of GPx forming oxidized GSH (GSSG) due to increased generation of ROS [32]. Our findings are in consonance with the other published reports which quoted that GSH concentration is decreased during Cd intoxication [33].

Histopathological results also supported the biochemical findings. According the light microscopic examination, it was clearly demonstrated that Cd administration caused a significant abnormality of liver morphology (Fig. 5), showing the extensive degeneration of hepatocytes with necrosis, inflammation, vacuolization, inflammatory cell infiltration and fatty degenerative changes (panel C), as compared with control (panel A). Treatment with curcumin and vitamin C (panel G and H) before Cd administration resulted in the improvement of liver cell damages observed with curcumin or vitamin C alone, compare panel E and F with panel G and H. This histopathological analysis is in agreement with the observed result in the MDA, a biochemical indicator of necrosis. Similar changes in the hepatic tissue of Wistar rats have been reported by previous findings of Milton et al. [34]. These changes could be the results of membrane distribution induced by Cd. In fact, this metal promotes an early oxidative stress. Afterwards it contributes to the development of various pathological aspects in soft tissues including liver. The current study obviously shows that histopathologic changes in liver are associated with the increasing of LPO and reducing GSH in conjunction by previous reports [35]. Administration of curcumin in combination with vitamins C notably reduced the histological alterations evoked by Cd quite appreciably. It can be attributed to their antilipoperoxidative, antioxidant, and metal chelating properties, which significantly reduced the oxidative threat leading to reduction of pathological changes and restoration of its normal physiological function.

Metallothioneins (MT) are a group of low molecular weight cysteine-rich important antioxidant protein in the cellular defence against Cd toxicity. They have the ability to bind and sequestrate Cd. It is well established that Cd significantly enhances hepatic MT gene expression and MT synthesis [36]. In the present study, MT was stained prominently in hepatocytes of Cd-treated group might be related with accumulation side of this metal to prevent the free Cd ions from exerting their toxic effects. The results of MT immunostaining confirm the biochemical and histopathological changes. This indicated that Cd caused the hepatotoxicity by increasing of MT expressions and treatment with curcumin or vitamin C alone could not protect the hepatotoxicity induced by Cd.

Cellular damages caused by Cd exposure can be prevented by free radical scavengers or antioxidants, which further strengthens the hypothesis that free radicals play a key role in Cd toxicity. Curcumin, the most abundant curcuminoid compound in turmeric (*C. longa*), has multifunctional actions. The antioxidant mechanism of curcumin is due to its specific conjugated structure of two methoxylated phenols and an enol form of β-diketone. This structure is responsible for free radical trapping ability as a chain breaking antioxidant [37]. The ability of curcumin to chelate the toxic metals was shown by Daniel et al. [38]. They found that curcumin significantly protects against lipid peroxidation induced by heavy metals, lead and cadmium in the rat brain homogenate, as well as reduces lead-induced structural damage in the hippocampus. Curcumin has been reported to attenuate liver injury induced by diverse hepatotoxicants [39, 40, 41] via multiple mechanisms. The two doses of curcumin selected in this study were based on previous studies in which curcumin showed protective effect against oxidant damage in the heart [42], liver [43], and kidney [44]. Its efficacy in protecting against liver injury has been established by its antioxidant or free radical-scavenger action. In our study, we found that the treatment of Cd-exposed animals with curcumin (400 mg/kg) alone partially reversed Cd-induced increase in MDA and Cd-induced decrease in reduced GSH although no significantly effect. This result is supported by our previous study shown that curcumin partially protect against Cd-induced nephrotoxicity [45]. However, the treatment had done on shorter period (5 days) and lower doses of curcumin (250 mg/kg) used in the previous study.

Vitamin C (ascorbic acid) is an important dietary antioxidant, it significantly decreases the adverse effect of reactive species such as reactive oxygen and nitrogen species that can cause oxidative damage to macromolecules such as lipids, DNA and proteins which are implicated in chronic diseases including cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis. Ascorbic acid is a potent water soluble antioxidant capable of scavenging/neutralizing an array of reactive oxygen species viz., hydroxyl, alkoxyl, peroxyl, superoxide anion, hydroperoxyl radicals and reactive nitrogen radicals such as nitrogen dioxide, nitroxide, peroxynitrite at very low concentrations [46]. Vitamin C is an essential cofactor for many enzymes involved in diverse metabolic pathways [47]. Sahin et al. [48] found that serum activities of SGOT and SGPT were not influenced by dietary vitamin C. Also, Yousef et al. [49] and Yousef [50] found that rabbits treated with vitamin C did not show any changes in the activities of AST and ALT. This is in accordance with our results showing that vitamin C had no significant effects in prevention against liver damages in Cd intoxicated rats.

CONCLUSION

Therefore, it is new findings in the present study which is different from the previous study. The results of the present study demonstrated that

the curcumin in combination with vitamin C had a significant hepatoprotective action on Cd induced oxidative damage in the liver tissue of rat. Cd decreased the GSH level and disturbed the redox state of the cells. The observed data infers that increased lipid peroxidation and associated ROS generation, decline in antioxidant status might have culminated in collapse of membrane integrity that lead to liver damage in Cd intoxicated rats. Reversal of these abnormalities to a near normal status reflects the causal association of antioxidant/anti-radical properties of curcumin and vitamin C. Further the hepatic protection was maximum in the combined treatment of curcumin in combination with vitamin C than the curcumin or vitamin C alone in the Cd intoxicated rats. Curcumin and vitamin C might show their protective effects as a scavenger of free radicals. This feature might also contribute to their antioxidant activity. Nevertheless, this feature needs to be further investigated.

In conclusion, our findings indicate treatment of combined curcumin and vitamin C inhibits remarkably liver damages in Cd intoxicated rats. Combination form of antioxidants might be very useful in protection of liver against Cd toxicity.

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