

Protective effects of quercetin on cadmium fluoride induced oxidative stress at different intervals of time in mouse liver

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Quercetin, a member of the flavonoid family is a major antioxidant acquired in humans by food consumption, while Cadmium fluoride (CdF₂) is one of the naturally occurring chemicals having adverse effects. The protective effect of quercetin on time dependent oxidative damage induced in mice liver by CdF₂ was studied in the following groups of mice consisting of six mice each: (i) control group; (ii) mice treated with single i.p injection of 2 mg/kg bw CdF₂ for 24 h; (iii) mice treated with single i.p injection of 2 mg/kg bw CdF₂ for 48 h; (iv) mice treated with single i.p injection of quercetin (100 mg/kg bw); (v) mice treated with i.p injection of 100 mg/kg bw of quercetin followed by i.p injection of CdF₂ (2 mg/kg bw) for 24 h; and (vi) mice treated with i.p injection of 100mg/kg bw of quercetin followed by CdF₂ (2 mg/kg bw) for 48 h. Administration of quercetin two hours before CdF, significantly reduced the biochemical alterations in reduced glutathione, ascorbic acid, lipid peroxidation, super oxide dismutase, catalase and total protein ($p < 0.05$). Histopathology also showed the protective effect of quercetin. The livers treated with CdF₂ were atrophic, markedly nodular, inflamed and necrotic. However, this effect was reduced to a minimum in the mice pre-treated for two hours with quercetin.

Key words: cadmium fluoride, quercetin, oxidative stress, flavonoids, histology, adverse effect

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INTRODUCTION

Cadmium (Cd) is one of the naturally occurring metals usually present in the environment as a mineral combined with elements like oxygen, chlorine, fluorine or sulfur. Exposure to Cd, whether short-term or long-term, can cause serious health problems in both, experimental animals and humans, the target organs being kidney, liver and the vascular system (Zargar *et al.*, 2014; Ellis *et al.*, 2012). The main source of the human body exposure to Cd is food. It is absorbed at significant quantities from cigarette smoke, water and air contamination. Fluoride salts are an effective prophylactic for dental caries and bone health. However, fluoride has been reported to be cytotoxic in a concentration-dependent manner (Christen *et al.*, 1994). Cadmium Fluoride (CdF₂) is a water insoluble source of cadmium for use in oxygen-sensitive applications, such as metal production (Soni *et al.*, 1984). The hepatotoxic effects of CdF₂ appear to depend on its

elimination and detoxification. Although the liver is reported as a Cd-target organ, the mechanisms involved in its toxicity are not yet clarified because this metal exerts differential response, depending on concentration, time of exposure and cell type.

Many efforts are being made to minimize the severity of Cd toxicity by its elimination using different agents. However in chronic exposure, therapy is ineffective (Nordberg, 1984). As Cd exposure leads to oxidative stress, it is reasonable to use antioxidant therapy in case of cadmium intoxication (Rashid *et al.*, 2013; Siddiqi and Zargar 2014). Various antioxidant therapies have been reported to exert protective effects to Cd toxicity (Morales *et al.*, 2006a; Asagba *et al.*, 2007; Renugadevi and Milton Prabu, 2009). Quercetin (QE) is a bioflavonoid belonging to the class of flavonols. It is found in high concentration in fruits, vegetables and beverages like mulberry, apples, onions, potatoes, broccoli, soybeans, peanuts, tea and red wine. It has been reported to have antioxidant and cytoprotective effects (Choi *et al.*, 2003). Administration of QE prevents liver inflammation by inhibition of inducible form of nitric oxide synthase (iNOS), induced by various inflammatory stimuli (Pavanato *et al.*, 2003). Furthermore, beneficial effects of quercetin as antioxidant on a molar basis have been reported (Rice-Evans *et al.*, 1995). QE chelates transition metal ions, including iron, thus preventing iron-catalyzed Fenton reaction (Ferrali *et al.*, 2000). The present study was undertaken to elucidate the ameliorative effect of QE on CdF₂ induced hepatic oxidative stress.

MATERIALS AND METHODS

Chemicals. All the chemicals used, including quercetin, were purchased from Sigma Chemical C., St Louis, MO, USA.

Animals. Healthy 4-to-5-week-old mice (male/female) were obtained from the Animal Breeding Laboratory, King Saud University, Riyadh, Saudi Arabia and kept in the Central Animal House Facility of the institute. Mice weighing between 25–30g were housed in polypropylene cages at room temperature, 60±15% relative humidity, and with a 12-h light–dark cycle. Animals were provided standard laboratory chow and purified water ad libitum. The study was approved by the Institutional Animal Ethics Committee. Ethical guidelines for the care and use of laboratory animals in experiments were strictly followed.

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Abbreviations: AsA, ascorbic acid; CAT, catalase; CdF₂, cadmium fluoride; ip, intraperitoneal; w/v, weight/volume; SOD, superoxide dismutase; QE, quercetin

After one week of acclimatization, the mice were divided into different groups consisting of six mice each as described below.

Dose selection of CdF₂. Four groups consisting of six mice each were studied: (i) normal (control) mice; groups(ii, iii and iv) CdF₂-treated mice administered single ip injections of 1, 2 and 4 mg CdF₂/kg bw for 24 hours respectively (Adachi *et al.*, 2007); Group (ii) mice did not show any significant increase in oxidative stress and Group (iv) mice were found all dead within 2 hours of exposure to 4 mg CdF₂/kg bw, hence both groups were not included in the study and 2 mg CdF₂/kg bw was used in the present study.

Time-response toxicity of CdF₂ and experimental protocol. Six groups of mice consisting of six mice each were studied: (i) control group; (ii) mice treated with single i.p injection of 2 mg/kg bw CdF₂ for 24 h; (iii) mice treated with single i.p injection of 2 mg/kg bw CdF₂ for 48h; (iv) mice treated with single i.p injection of QE (100 mg/kg bw); (v) mice treated with i.p injection of 100 mg/kg bw of QE followed by i.p injection of CdF₂ (2 mg/kg bw) for 24 h; and (vi) mice treated with i.p injection of 100 mg/kg bw of QE followed by CdF₂ (2 mg/kg bw) for 48 h. Groups (i), (ii), (iv) and (v) were sacrificed by asphyxiation with carbon dioxide after 24 h while the rest of the groups were sacrificed after 48 hours.

Preparation of liver samples. The livers from the animals were dissected out, cleared of adhering tissues, weighed and homogenized in chilled normal saline (10% W/V). The homogenate was centrifuged at 13000 rpm for 10 min. The resulting clear supernatant was used for biochemical assays.

Biochemical analysis

Lipid peroxidation. Lipid peroxidation was done by method of Utley and coworkers (1967). Briefly, 1 ml of homogenate was incubated in a metabolic shaker at 37°C for one hour. 1.5 ml of 20% TCA was added to it and centrifuged at 600 g for 10 minutes. To 1 ml of supernatant was added 1 ml of freshly prepared Thiobarbituric acid (0.67%). The reaction was kept in water bath for 10 minutes. Upon cooling, absorbance was read at 535 using a reagent blank. Values were

expressed as nanomoles of malonaldehyde formed hour⁻¹mg protein⁻¹.

Ascorbic acid (AsA). Ascorbic acid was determined by the method of Jagota and Dani (1982). Homogenate, 0.2 ml, was treated with 0.8 ml of 10% TCA. After vigorous shaking, tubes were kept in ice cold bath for 5 min and centrifuged at 1200×g for 5 min. 0.2 to 0.5 ml of the supernatant were diluted to 2 ml with distilled water and 0.2 ml of Folin reagent (0.2 M) was added. After 10 min, the absorbance was read at 760 nm against a reagent blank. The amount of ascorbic acid was calculated from the standard graph. Values were expressed as µg of ascorbic acid µg protein⁻¹.

Reduced glutathione (GSH). The estimation was carried out by method of Beutler *et al.* Briefly, 0.4 ml of homogenate was mixed with 3.6 ml of double distilled water and treated with 0.6 ml of precipitating reagent (containing 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30.0 g of NaCl and made up to 100 ml with double distilled water).

The above reaction mixture was centrifuged at 600×g for 10 minutes. To 0.3 ml of supernatant, 2 ml of Na₂HPO₄ (0.3 M) and 0.25 ml of 5,5' dithio-bis-2-nitrobenzoic acid (0.4 % in 1% sodium citrate) were added, and volume was made up to 3 ml with DDW. OD was read at 412 nm against blank. Values were expressed as µg of reduced glutathione µg protein⁻¹.

Superoxide dismutase (SOD). Superoxide dismutase was determined by the method of Nishikimi and coworkers (1972). Briefly, the reaction mixture contained 0.1 ml of sodium pyrophosphate (0.1 mM), 0.1 ml of NBT (0.3 mM), 0.1 ml of NADH (0.47 mM), 0.05 ml of PMS (0.93 µM) and 0.1 ml of enzyme in a total volume of 1 ml. Rate of change of absorbance was measured at 560 nm. Values were expressed as units of enzyme min⁻¹ mg protein⁻¹.

Catalase (CAT). Catalase activity was estimated in the whole homogenate by the method of Aebie (1984). The reaction mixture in a total volume of 3 ml contained 0.4 M sodium phosphate buffer pH 7.2, 1.2 ml of H₂O₂ and suitably diluted enzyme. The reaction was started by adding H₂O₂ and reading the change in absorbance at 240 nm for two minutes. One unit of CAT activity was defined as micromoles of H₂O₂ decomposed per min using molar absorbance of H₂O₂ (43.6 M⁻¹ cm⁻¹).

Table 1. Effect of quercetin and cadmium fluoride induced alterations alone and in combination, on the levels of enzymatic and non-enzymatic antioxidant contents in liver of control and experimental mice.

Treatment Groups	Control	CdF ₂ (2 mg/kg) 24 h	CdF ₂ (2 mg/kg) 48 h	Control+QE (100 mg/kg)	QE+ CdF ₂ (24 h)	QE+ CdF ₂ (48 h)
Lipid Peroxidation (mmoles of malonaldehyde formed/hour/mg protein)	1.15 ± 0.57 ^{bc}	3.23 ± 0.57 ^{adef}	3.80 ± 0.71 ^{adef}	0.37 ± 0.12 ^{bef}	0.98 ± 0.17 ^{bef}	2.13 ± 0.40 ^{bcde}
Ascorbic acid (µg of ascorbic acid/µg protein)	1.73 ± 0.05 ^{bc}	8.65 ± 1.30 ^{acdef}	4.00 ± 1.44 ^{abde}	0.05 ± 0.01 ^{bef}	0.06 ± 0.02 ^{bef}	3.88 ± 1.01 ^{bde}
Reduced glutathione (µg of GSH/mg protein)	5.30±0.80 ^{bc}	2.28±0.4 ^{adef}	1.45±0.93 ^{adef}	6.16±0.33 ^{bce}	4.23±0.75 ^{bcdf}	6.55±1.77 ^{bce}
Catalase (Units/mg protein)	4.89 ± 2.14 ^{bcdef}	0.33 ± 0.06 ^{ad}	0.38 ± 0.22 ^{ae}	1.33 ± 0.23 ^a	1.08 ± 0.46 ^a	2.50 ± 0.22 ^{ab}
Superoxide dismutase (Units/mg protein)	35.40 ± 1.56 ^{bef}	8.12 ± 3.83 ^{acd}	20.02 ± 4.12 ^a	28.52 ± 9.29 ^{bf}	24.69 ± 6.45 ^{ce}	9.66 ± 4.50 ^{acd}

CdF₂ (2 mg/kg b.w) was administered intraperitoneally for 24 and 48 h. Quercetin(100 mg/kg b.w) was administered intraperitoneally alone and 2 h before CdF₂ injection for each time interval. Data are representative of mean ±S.D. of three independent experiments, each group containing 6 mice. *significant (p<0.05)when compared to control. ^bsignificant (p<0.05)when compared to 2 mg/kg bwCdF (24 h) group. ^csignificant (p<0.05) when compared to with the 2 mg/kg bwCdF (48 h) group. ^dsignificant (p<0.05)when compared to quercetin 100 mg/kg bw (24 h) group. ^esignificant (p<0.05)when compared to quercetin + CdF (24 h) group. ^fsignificant (p<0.05)when compared to quercetin + CdF (48 h) group. ^asignificant (p<0.05) when compared to control.

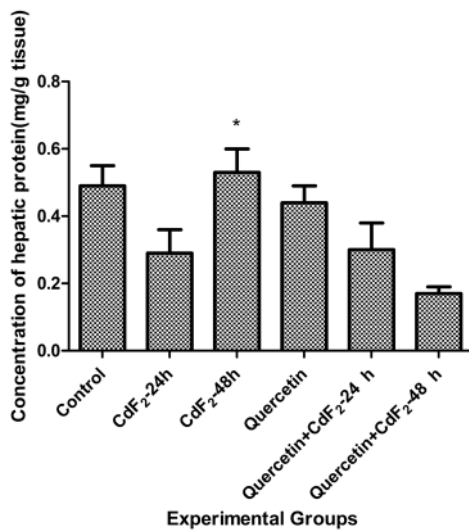


Figure 1. Effect of QE and CdF₂ alone and together at different time intervals on hepatic protein content in mice.

* $p < 0.05$ when compared to control and QE + CdF₂ treated groups after 48 hours (Independent Sample T-Test between the control and the treated groups in liver).

Protein. The protein content in the sample was measured by the modified method of Markwell and coworkers (1978) using bovine serum albumin as the standard. The amount of protein was calculated from a standard curve. Protein values are expressed as mg/gram fresh tissue.

Histopathology. For histopathology, the left lobes of the excised liver were cut and processed for microscopy. The processing was done as follows: fixing the specimens in a 10% neutral buffered formalin solution, block preparation in paraffin, cutting sections of 5–6 μm thick, and the sections stained with haematoxylin–eosin stain. The sections were photographed and analyzed by an ex-

pert pathologist not being informed about the sample assignment to the experimental groups.

Statistical analysis. Values were expressed as mean \pm S.D. The data were statistically representative in terms of number, mean and standard deviation (S.D.). Comparison between different groups was done using Independent sample T-Test for comparing two groups and One-way ANOVA Test was used for comparison between more than two groups with (LSD) as multiple comparison. Correlation between various variables was done using Pearson correlation coefficient (R) with graphic representations using linear regression. A probability value (p value) less than or equal to (0.05) was considered significant. All statistical calculations were done using computer program SPSS (Statistical Package for Social Science) version (11.0).

RESULTS

The effect of oxidative stress induced by CdF₂ (2 mg/kg bw) alone was time dependent and there was found a significant protection by QE at 24 h and 48 h time intervals with respect to changes in the levels of lipid hydroperoxides, non-enzymatic and enzymatic antioxidants in control and experimental mice (Table 1). The levels of lipid hydroperoxides and AsA were significantly ($p < 0.05$) increased in CdF₂-treated mice at both 24 and 48 hr when compared to control and QE alone groups. The pretreatment of mice with QE resulted in significant recovery of lipid peroxidation and AsA levels. Treatment with CdF₂ was found to significantly decrease the reduced glutathione at both 24 and 48 h ($p < 0.05$). Administration of QE (100 mg/kg b.w) before 2 h to CdF₂ significantly decreased the levels close to normal and QE alone groups ($p < 0.05$). The results presented in table 1 revealed that CdF₂ at both time intervals significantly decreased the activities of enzymatic antioxidants (SOD and CAT; $p < 0.05$). Administration of QE 2 hrs before injection with CdF₂ significantly increased the lev-

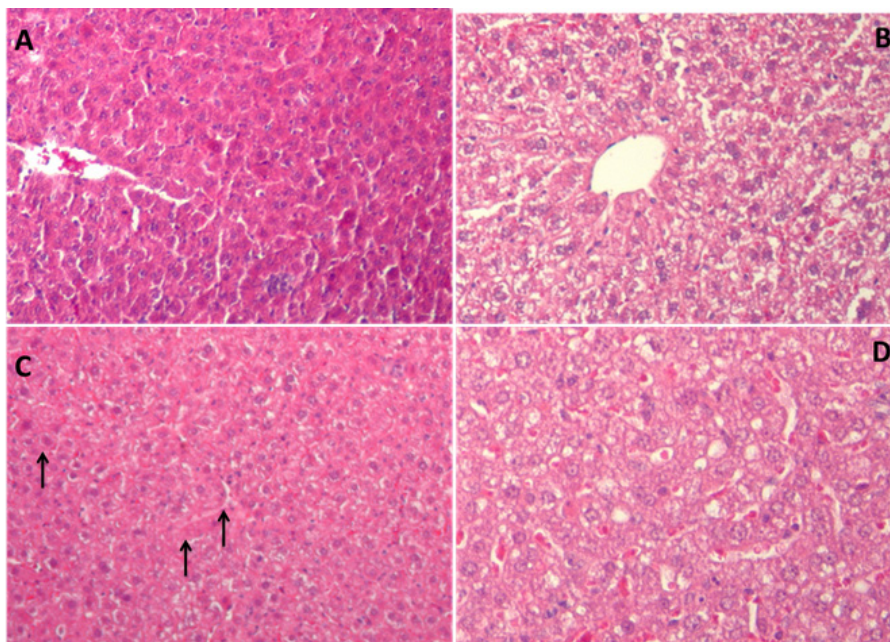


Figure 2. Liver histology. Liver sections are shown from control group (A), the quercetin treated group (B), the CdF₂ (2 m/kg bw for 48 h) group (C), and the group of mice receiving QE 2 hours before administration of CdF₂ for 48hr (D). Normal hepatocytes with well-distinguished cytoplasm and nucleus can be seen in (A), and also in (B). Severe centrilobular congestion, massive necrosis, and inflammation is seen in (C). In (D), only mild morphological changes and less centrilobular congestion could be seen. Haematoxylin–eosin, 40 \times .

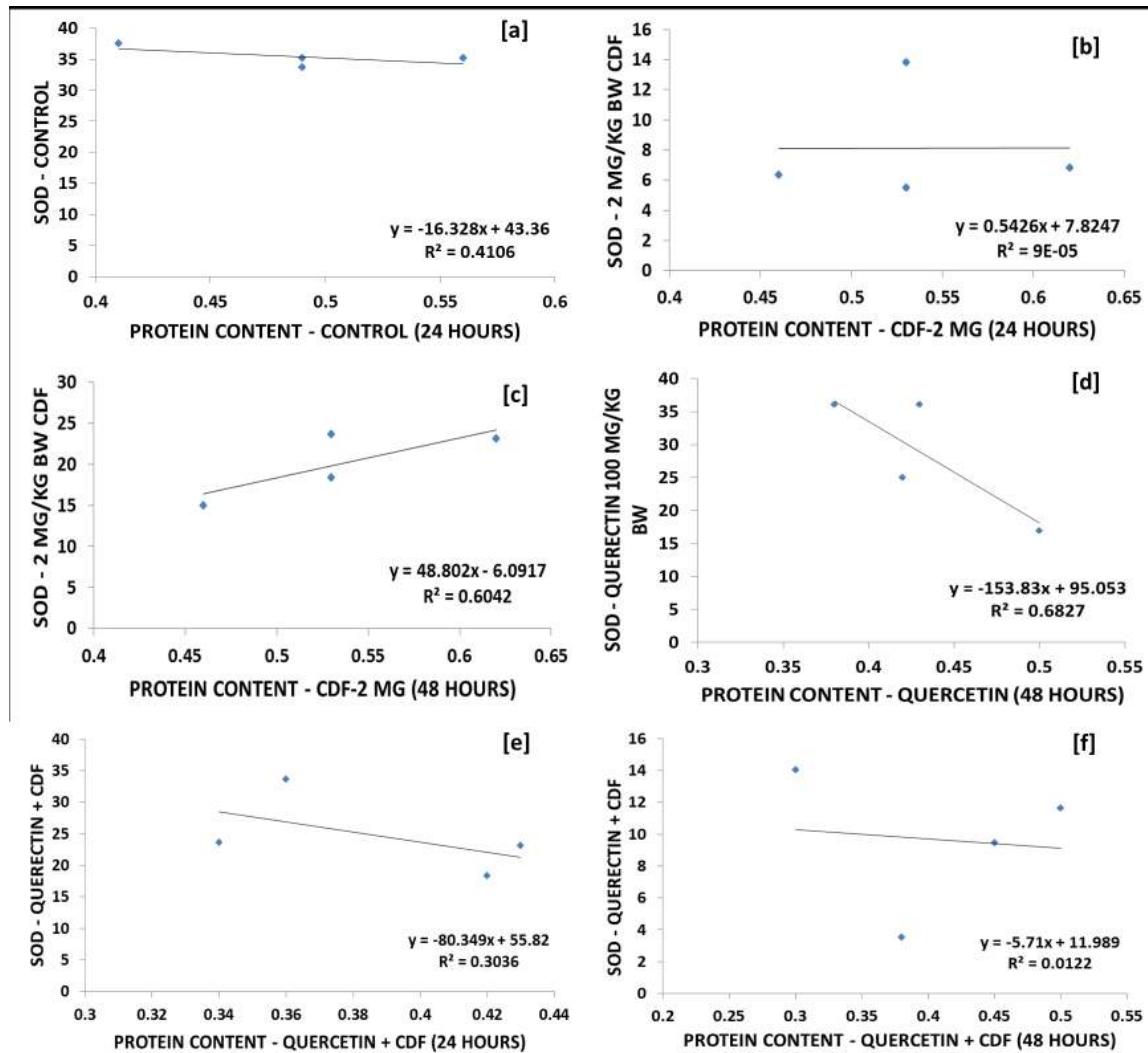


Figure 3A. Correlations between protein content and superoxide dismutase activity at all selected doses in liver. Positive correlation is visible in Fig. 3A on (c).

(a) Correlation between protein content and the SOD activity in control group in liver with best fit line curve (negative correlation); (b) Correlation between protein content and the SOD activity with 2 mg/kg body weight CdF₂ for 24h in liver with best fit line curve (positive correlation); (c) Positive correlation between protein content and the SOD activity with 2 mg/kg body weight CdF₂ for 48h in liver with best fit line curve (positive correlation); (d) Correlation between protein content and the SOD activity with 100 mg/kg body weight QE in liver with best fit line curve (negative correlation); (e) Correlation between protein content and the SOD activity with 100 mg/kg bw QE followed by 2 mg/kg body weight CdF₂ in liver for 24 h with best fit line curve (negative correlation); (f) Correlation between protein content and the SOD activity with 100 mg/kg bw QE followed by 2 mg/kg body weight CdF₂ in liver for 48 h with best fit line curve (negative correlation).

els of enzymatic antioxidants in mice livers nearing that of control groups at both time intervals ($p < 0.05$). Administration of QE modulates the activity of enzymatic and non-enzymatic antioxidants and enhances the defense against reactive oxygen species-generated damage induced by CdF₂ alterations in mice livers.

Figure 1 shows the effect of CdF₂ and QE alone as well as together at different time intervals on hepatic protein content in mice in all groups. With the exposure to 2 mg CdF₂, mice showed a significant increase in protein content in liver tissue ($p < 0.05$) when compared to control at 48 h although protein content decreased at 24 h non-significantly. QE, when administered 2 hours before CdF₂ in 48 h exposure group decreased the protein content below all the observed values. The results in Fig. 1 showed that CdF₂ exposure decreased protein content at 24 h but the difference being non-significant when compared with control. Quercetin alone as well as along with CdF₂ did not show any significant difference

in hepatic protein content when compared to control at both time intervals showing its protective effect to protein degradation by CdF₂.

Figure 2 shows histopathological changes elicited by CdF₂ and reduction of these changes by QE pretreatment. Sections from the control mice (Fig. 2A) or from the mice receiving QE alone (Fig. 2B) showed normal hepatocytes with well-positioned cytoplasm and nucleus. Massive inflammation, necrosis, and centrilobular congestion were observed in CdF₂ treated mice (48 hours) (Fig. 2C). These changes were well reduced in mice receiving QE (100 mg/kg bw) followed 2hr later with the introduction of 2 mg/kg b.w CdF₂ for 48 hours (Fig. 2D). This group exhibited only mild morphology changes and less centrilobular congestion when compared to control.

Figure 3A shows Pearson correlations between protein content and the SOD activity in livers of all treated groups of mice. All groups were found to have weakly

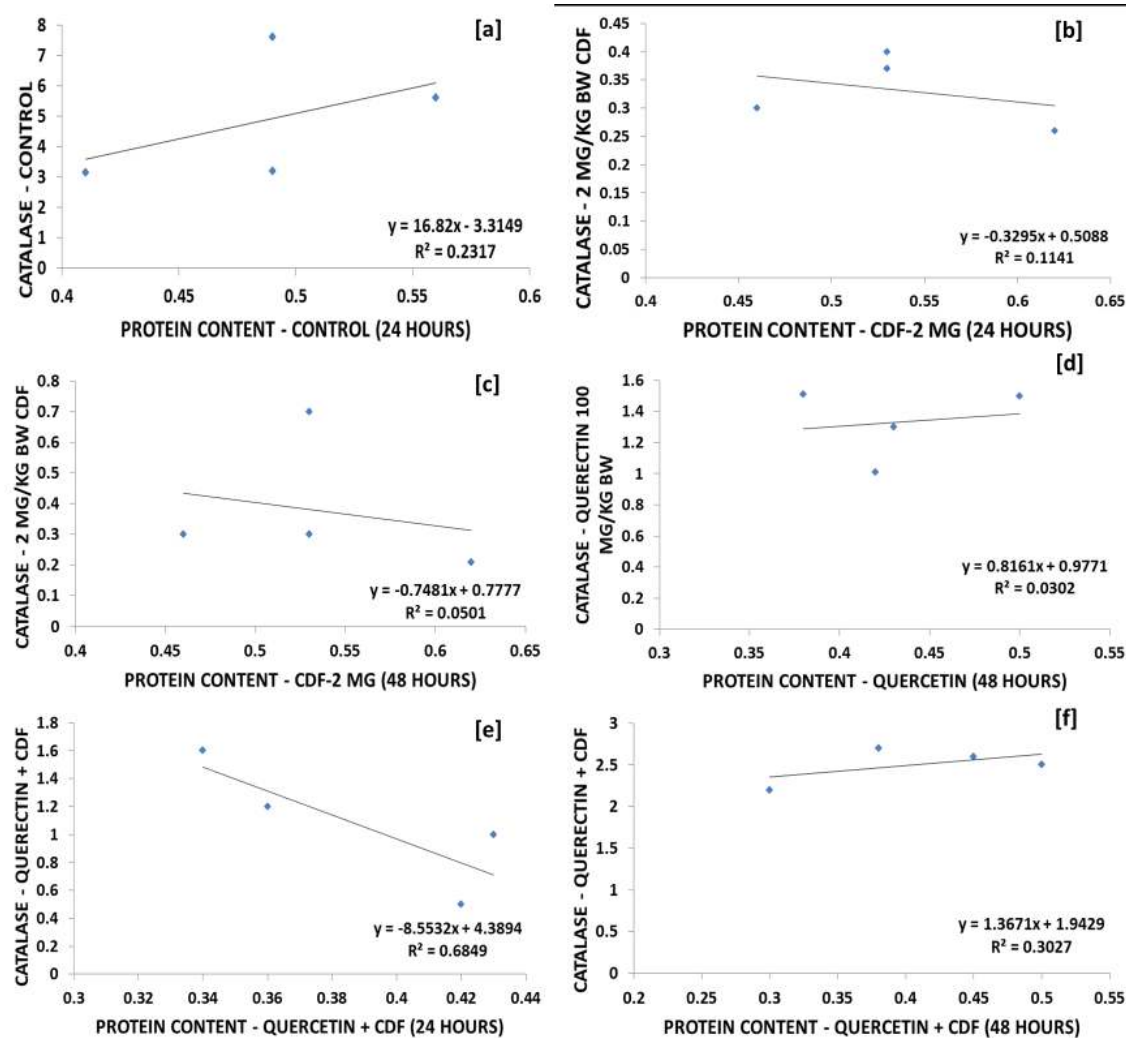


Figure 3B. Correlations between protein content and CAT activity at all selected doses in liver. Positive correlation is visible in Fig. 3B on (d) and (f).

(a) Correlation between protein content and the CAT activity in control group in liver with best fit line curve (positive correlation); (b) Correlation between protein content and the CAT activity with 2 mg/kg body weight CdF₂ for 24 h in liver with best fit line curve (negative correlation); (c) Correlation between protein content and the CAT activity with 2 mg/kg body weight CdF₂ for 48 h in liver with best fit line curve (negative correlation); (d) Correlation between protein content and the CAT activity with 100 mg/kg body weight QE in liver with best fit line curve (positive correlation); (e) Correlation between protein content and the CAT activity with 100 mg/kg bw QE followed by 2 mg/kg body weight CdF₂ in liver for 24 h with best fit line curve (negative correlation); (f) Correlation between protein content and the CAT activity with 100 mg/kg bw QE followed by 2 mg/kg body weight CdF₂ in liver for 48 h with best fit line curve (positive correlation).

negative correlations between protein content and SOD activity except the CdF₂ group sacrificed at 48 hours after exposure which showed significant positive correlation ($p < 0.05$). Figure 3B shows positive correlations between protein content and the CAT activity in liver of control, QE alone group ($p < 0.05$) and QE+CdF₂ group at 48 hours and negative correlation in the rest of the experimental groups of mice including the group treated with CdF₂ alone ($p < 0.05$).

DISCUSSION

Cadmium fluoride is a toxic pollutant in industrialized countries because it forms a complex with metallothionein, a cysteine rich liver protein (Klaassen *et al.*, 1999). CdF₂ induces oxidative stress which is a causative factor in many diseases and underlying pathologies as it plays important roles in cellular signaling, inflammatory,

and genotoxic and proliferative responses (Schins, 2003; Knaapen *et al.*, 2004; Van *et al.*, 1999; Zhong *et al.*, 1997; Borm *et al.*, 2004). The potentially toxic free radicals are usually scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids. The present study focused on investigating the antioxidant role of QE on CdF₂ intoxication as indicated by restraining the significant oxidative stress biomarkers and histology of liver.

In the present study, the ascorbic acid levels increased and GSH levels decreased in CdF₂ exposed mice livers both at 24 and 48 h significantly (Table 1). It has been observed that under stress conditions, the ascorbic acid (AsA) requirement increases to maintain normal physiological conditions. Under conditions of oxidative stress, the GSH consumption is enhanced markedly. GSH synthesis and export is the major role of liver in the antioxidant defense. Glutathione, a tripeptide, eliminates reactive oxygen and nitrogen species produced dur-

ing normal physiological activities and during oxidative stress (Lushchak, 2012). The decrease of hepatic GSH observed after the exposure to CdF₂ is thought to result from increased GSH-peroxidase activity in livers of exposed mice. The decreased GSH is thought to be associated with increased AsA synthesis and lipid peroxidation (Bánhegyi *et al.*, 1996; Martensson *et al.*, 1992). It was further reported that glutathione depletion induces glycogenolysis dependent, ascorbate synthesis in isolated murine hepatocytes (Braun *et al.*, 1996). Ascorbate is the only endogenous antioxidant that can completely protect the lipids from detectable peroxidative damage induced by aqueous peroxy radicals. Ascorbate is a much more effective antioxidant than the protein thiols, α -tocopherol, bilirubin, or urate. The antioxidant and metal chelating property of QE was able to bring back the levels nearly to normal, which was significant at 24 h, but the values were non-significant at 48 hours; again the reason may be a lower quantity of QE in the body of animal to show antioxidant defense.

The significant decrease of levels of antioxidant enzymes like CAT and SOD at both 24 and 48 hours CdF₂ was elevated near to normal by QE supplementation at 24 hours, but it was nonsignificant at 48 hours in the experimental group (Table 1). CAT is the enzyme responsible for dissipation of hydrogen peroxide formed during oxidative stress. It has been reported that CAT and SOD constitute a mutually supportive team of defense against ROS. Because of the high activity of antioxidant enzymes in the liver and decreased content of GSH, the CdF₂ induced oxidative stress turns to be severe; the inbuilt mechanism of the body fails to alleviate the damage. The decreased activity of SOD in liver in CdF₂-treated mice may be due to the enhanced lipid peroxidation or inactivation of the antioxidant enzymes. The non-significant values of antioxidant defense of QE at 48 hrs may be due to metabolism of QE. At 48 h, the QE levels in plasma of animals might have decreased due to digestion of this compound and thus there would be hardly any free QE present.

In this study, 2 mg CdF₂ caused a significant increase in protein content in liver tissue when compared to control at 48h ($p < 0.05$). QE, when administered i.p 2 hours before CdF₂, decreased the protein content significantly when compared with CdF₂ alone group at 48 hours. Increase in protein and decreased antioxidant content, like CAT and SOD may contribute to more damage. Positive correlation between QE alone and control groups shows that QE tries to prevent protein oxidation in CdF₂ treated groups at both 24 and 48 hours by increasing the antioxidant enzymes. Increase in protein content may also be due to increased amount of cysteine groups upon exposure (Fig. 1). This condition could be related with cachexia. In cachexia, rates of muscle protein synthesis may be elevated because there is increased availability of amino acids resulting from accelerated muscle protein degradation (Wolfe *et al.*, 2001). Quercetin administration decreased the protein values non-significantly at 24 hours. At 48 hours, protein levels were non-significantly below normal. The reason could be that this tissue is deficient in protein synthesis. The reason again may be scarcity of QE levels due to its metabolism at this time interval. There were no significant changes in liver weight to bodyweight ratio in any experimental groups when compared to each other (not shown). The non-significant increase in liver/bw ratio in all groups can be attributed to the normal growth. Any alteration in the architecture of liver, as it is involved in the detoxification functions, is likely to have an impact on its func-

tion. Histopathology of liver tissues done here depicted the protective effects of QE on liver morphology which is consistent with biochemical studies.

Strong negative correlations between protein content and the enzyme activities in the livers of all treated groups of mice except the control and QE group treated for 48 hours may show that duration and severity of protein damage might parallel the decreased enzyme activity.

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Declaration of interest

The authors report no declarations of interest

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