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Research Article
Cadmium-induced Hepatotoxicity and Oxidative Stress in Rats: Protection by Roflumilast via NF-κB and HO-1 Pathway

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Abstract

Background and Objective: Oxidative injury is reflected as an early sign of cadmium (Cd) toxicity and has been linked with hepatotoxicity. The present study was conducted to investigate the ameliorative effect of roflumilast (ROF), a phosphodiesterase-4 inhibitor against Cd-induced hepatotoxicity in Wistar albino rats. **Materials and Methods:** Twenty four rats were assigned to four groups (n = 6). Group 1 served as normal control and the second group was administered with cadmium chloride (3 mg kg^{-1}, i.p.) for seven consecutive days. Third and fourth group rats were treated with Cd with concomitant administration of ROF at doses of 0.5 and 1.5 mg kg^{-1} (p.o.), respectively for 7 days. At the end of the study, blood samples were collected for the estimation of liver functions biomarker (AST and ALT). Followed by blood collection, all rats were sacrificed and hepatic tissues isolated for assessment of oxidative stress markers (MDA, GSH, SOD and CAT) and histopathological studies. **Results:** The treatment of rats with Cd results in increased serum AST and ALT levels and lipid peroxidation, depleted hepatic GSH content and inhibited activities of SOD and CAT significantly compared to the normal control group. The histological assessment showed vacuolar degeneration of liver cells with focal necrosis in Cd treated rats. ROF administration at both doses preserved the biochemical and histological changes in dose-dependent manner caused by Cd toxicity to near-normal levels. **Conclusion:** The present study concludes that ROF might be acting as an antioxidant and protect the liver from Cd-induced oxidative stress.

Key words: Roflumilast, cadmium, hepatotoxicity, HO-1, NF-κB p65

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cadmium (Cd) is a naturally occurring serious environmental toxicant with harmful effects on health in both animals and humans. Cd is nearly absent in newborns but known to accumulate in multiple organ systems gradually more specifically in liver and kidney, which might be up to levels of 75% of the total Cd present in thebody[1,2]. Cd produces toxic effects even at low doses, due to its accumulation in the body with a long biological half-life (>20 years)[3]. Smoking is the major source of Cd exposure in humans. One cigarette could have 6.67 µg Cd and 40-60% of it generally passes through the pulmonary epithelium into systemic vasculature. Among the affected organs, Cd exposure exhibited evident liver injury with focal hepatic necrosis[4]. Hyder *et al.*[5] reported the relation between increased urinary Cd levels and enzymatic markers of hepatic inflammation among men and suggested an increased risk of mortality with chronic Cd exposure, specifically, a threefold increased risk of liver disease mortality. In the human liver, Cd causes apoptosis of the hepatoblastoma cell line HepG2, a cell line known to express metallothioneins and heat shock proteins essential for detoxification[6]. There is a lack of data related to the effect of Cd on the human liver because it is difficult to obtain human hepatocytes for toxicological studies. Cd also reported to enhance caspase-9 and caspase-3 activities in human hepatocytes[7].

Animal studies have demonstrated that Cd first accumulates in the liver, so acute exposure to toxic doses of Cd results primarily in hepatic damage. In the liver cells, Cd makes complexes with small peptides and proteins via sulfhydryl groups, including glutathione (GSH)[8]. Cd exposure to the animals at higher doses resulted in membrane lipid peroxidation and a reduction in glutathione levels in kidney and liver[9] followed by oxidative damage to DNA and proteins, increased genes encoding heat-shock proteins and decreased antioxidant enzyme activities[10]. Cd induces toxicity by producing excess reactive oxygen species (ROS) which results in oxidative damage to cell organelles[11] but the molecular mechanism elaborating Cd-induced free radicals generation is not revealed so far.

The cGMP and cAMP are the fundamental second messengers that play a critical role in the regulation of multiple cellular metabolisms. Intracellular cAMP levels are maintained by its organized synthesis through adenylate cyclase enzyme and its deprivation via phosphodiesterase (PDEs) enzymes. Elevated cAMP level activates a signaling mechanism that resulted in the regulation of various protein activities and gene expression. The cAMP level is also controlled by PDE enzymes through the degradation of cAMP. Therefore, any modifications in PDE activities will have a direct effect on cAMP signaling. Out of 3 cAMP-specific PDEs family (PDE3, PDE4 and PDE7), the PDE4 is the main and most abundant[12,13]. The role of PDE4 in the pathogenesis of inflammation and hepatic injury reported in rats by Gobejishvili *et al.*[14]. Furthermore, the beneficial role of PDE inhibitors also has been reported in experimental hepatic injury[14,15].

Therefore, the present investigation was aimed to elucidate the potential of test compound (ROF) against stress induced by Cd and liver toxicity in rats.

MATERIALS AND METHODS

The study was carried out from January-April, 2019 in the Department of Pharmacology, College of Pharmacy, Prince SATTAM Bin Abdulaziz University, Al-kharj, Kingdom of Saudi Arabia.

Chemicals and reagents: For induction of hepatotoxicity, cadmium salt was procured from Sigma (St. Luis, USA). For western blot analysis, primary and secondary antibodies were purchased from Santa Cruz (Dallas, USA). Nitrocellulose membrane purchased from BIO-RAD Laboratories (Hercules, USA). Chemiluminescent HRP substrate (Western blot detection kit) was purchased from Millipore (Billerica, USA). All other chemicals used for biochemical analysis were analytical grade reagents.

Animals: In the present study, 24 male Wistar rats (aged 6-8 weeks old) weighing 200-250 g, obtained from Prince Sattam Animal Care Unit at College of Pharmacy, PSAU, Kingdom of Saudi Arabia and used in the present investigation. Rats were provided free access to purified drinking water *ad libitum* and standard commercially available pellets diet. Rats were kept in standard, hygienic and controlled environmental conditions at a temperature of 22±2°C, relative humidity 50±10% and a light and dark cycle of 12h in each. The study was approved by the Ethical Review Committee, College of Pharmacy, Prince Sattam Bin Abdulaziz University, KSA (approval ref number HAP-01-KJ-050). Guidelines of the Institute of Laboratory Animal Resources, National Research Council were followed during whole experimental periods[16].

Experimental design: The experiment was designed by following the study done by Yadav *et al.*[17] with some modifications. In the present research, 24 male rats were

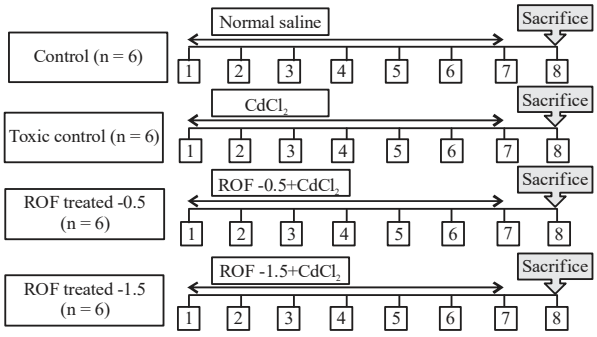

Fig. 1: Timeline of the experiment

Animals in the control group received normal saline orally, for 7 days. Toxic control rats received $CdCl_2$ (3 mg kg^{-1}) intraperitoneally daily, for 7 days, group 3 and 4 rats received $CdCl_2$ in the same schedule as toxic group as well as ROF (after 1 h of $CdCl_2$) in the 2 doses 0.5 and 1.5 mg kg^{-1} orally, for 7 days

assigned into 4 groups (Control, toxic control and ROF treatment groups). The animals in the control group received normal saline for 7 days. Toxic control rats received 3 mg kg^{-1} Cd ($CdCl_2$) intraperitoneally (i.p.) daily for 7 days. Group 3 and 4 rats received $CdCl_2$ in the same schedule as a toxic group as well as ROF in the 2 divided doses 0.5 and 1.5 mg kg^{-1} (p.o.) for 7 days. The timeline is mentioned in Fig. 1.

$CdCl_2$ was administered daily 1 h before ROF treatment. At the end of the study, all the rats fasted for 12 h and blood samples were collected from retro-orbital plexus into tubes without adding anticoagulant. The tube containing blood sample were centrifuged at 5000 rpm for 10 min to separate serum which was stored at -20°C until further biochemical analysis for liver function test (AST and ALT).

After a successful separation of serum, all animals were sacrificed by dislocation of cervical vertebrae. The liver was weighed and washed with cold saline. For histopathological assessment, a small portion of tissue was preserved in formalin solution (10%) and the remaining tissues were kept at -80°C for biochemical estimation of oxidative stress parameters (such as MDA, total glutathione, SOD and CAT) and protein expression.

Serum biochemical estimations: The serum levels of liver function marker (AST and ALT) were measured using standard kits obtained from BioSystem (Barcelona, Spain)[18].

Liver tissue biochemical estimations: 10% w/v phosphate buffer (0.1M, pH-7.4) were used to mince and homogenized liver tissue followed by centrifugation at 12000 rpm for 30 min at 4°C). The supernatants obtained were used for biochemical estimation of oxidative stress parameters such as MDA[19], total glutathione[20], SOD[21] and CAT[22].

Western analysis: A small piece of liver tissues from all the rats separately homogenized in the tube containing protein lysis buffer having cocktail protease inhibitor followed by centrifugation at 14000 rpm, for 10 min at 4°C[23]. Total protein content in the homogenates was estimated using UV spectrophotometry by an established method[24]. A mixture of 1:1 ration of protein sample and sample buffer containing 20 µg proteins were loaded in each well of SDS-polyacrylamide gel (10%) followed by electrophoresis and after completing the electrophoresis run, the separated protein on the gel was then transferred to nitrocellulose membrane. After (3-5) washing, nitrocellulose then incubated overnight with primary targeted antibody (GST, NF-κB p65 and HO-1) followed by 1-2 h incubation with the secondary antibody against each primary antibody at room temperature with mild shaking. Nitrocellulose membrane after washing (3-5 times) were incubated for 5 min in Chemiluminescent HRP substrate to visualize band on the membrane for imaging using C-Digit scanned (LI-COR, Lincoln, USA). The visualized band image was analyzed by the ImageJ® image processing program (National Institutes of Health, Bethesda, USA) and the intensity band was compared against the band intensity of β-actin bands.

Histopathological studies: For assessment of liver histopathology, the tissues were preserved for 48h in 10% buffered formalin, dehydrated, cleared in xylene and embedded in paraffin blocks. Sections (5-6 µm thick) of the tissue were stained with hematoxylin and eosin (H and E) dye, Masson's trichrome (MT) and Periodic Acid-Schiff (PAS) stain which was mounted for microscopical observations[25].

Statistical analysis: All the data were presented as mean±SEM (n = 6) with 95% confidence intervals (CI). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and the individual comparisons were obtained by Tukey's test. Values were considered statistically significant when $p<0.01$.

RESULTS

Effect on serum diagnostic marker enzymes: The serum AST and ALT levels of all the animals were determined and showed in Fig. 2. Significantly increased levels of serum diagnostic

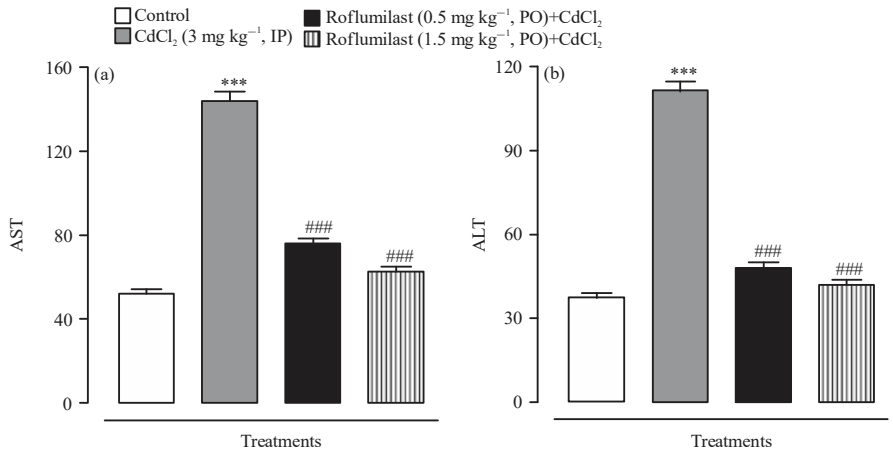

Fig. 2(a-b): Effect of ROF on serum marker enzymes against $CdCl_2$-induced hepatotoxicity in rats, (a) Aspartate transaminase (U mL^{-1}) and (b) alanine transaminase (U mL^{-1})

***p<0.001, show comparison of $CdCl_2$ with control (Unpaired t-test), ###p<0.001, show comparison of treated (Roflumilast, 0.5 and 1.5 mg kg^{-1}) with $CdCl_2$ group (One-way ANOVA followed by Tukey test), each bar represents Mean±SEM (n = 6)

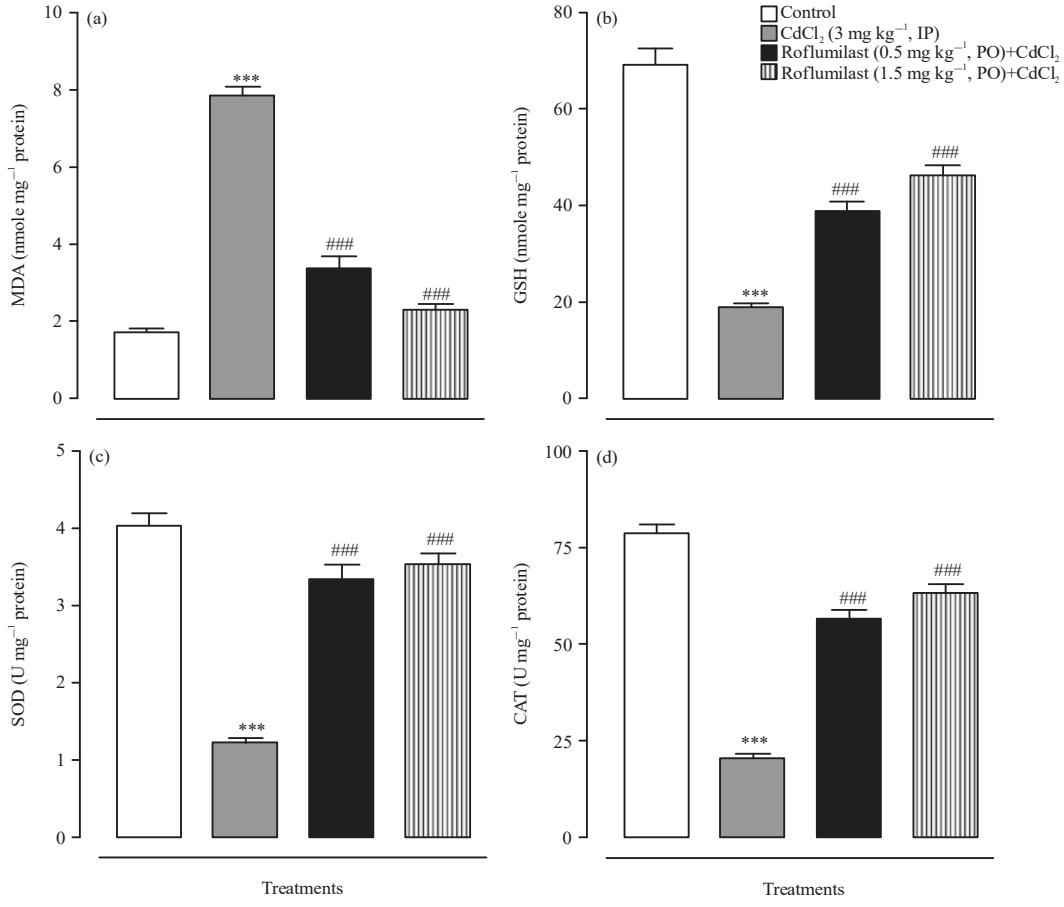

Fig. 3(a-d): Effect of ROF on hepatic oxidative stress markers against $CdCl_2$-intoxicated rats, (a) Malondialdehyde, (B) Total glutathione, (c) superoxide dismutase and (d) catalase

***p<0.001, show comparison of $CdCl_2$ with control (Unpaired t-test), ###p<0.001, show comparison of treated (Roflumilast; 0.5 and 1.5 mg kg^{-1}) with $CdCl_2$ group (One-way ANOVA followed by Tukey test), each bar represents Mean±SEM (n = 6)

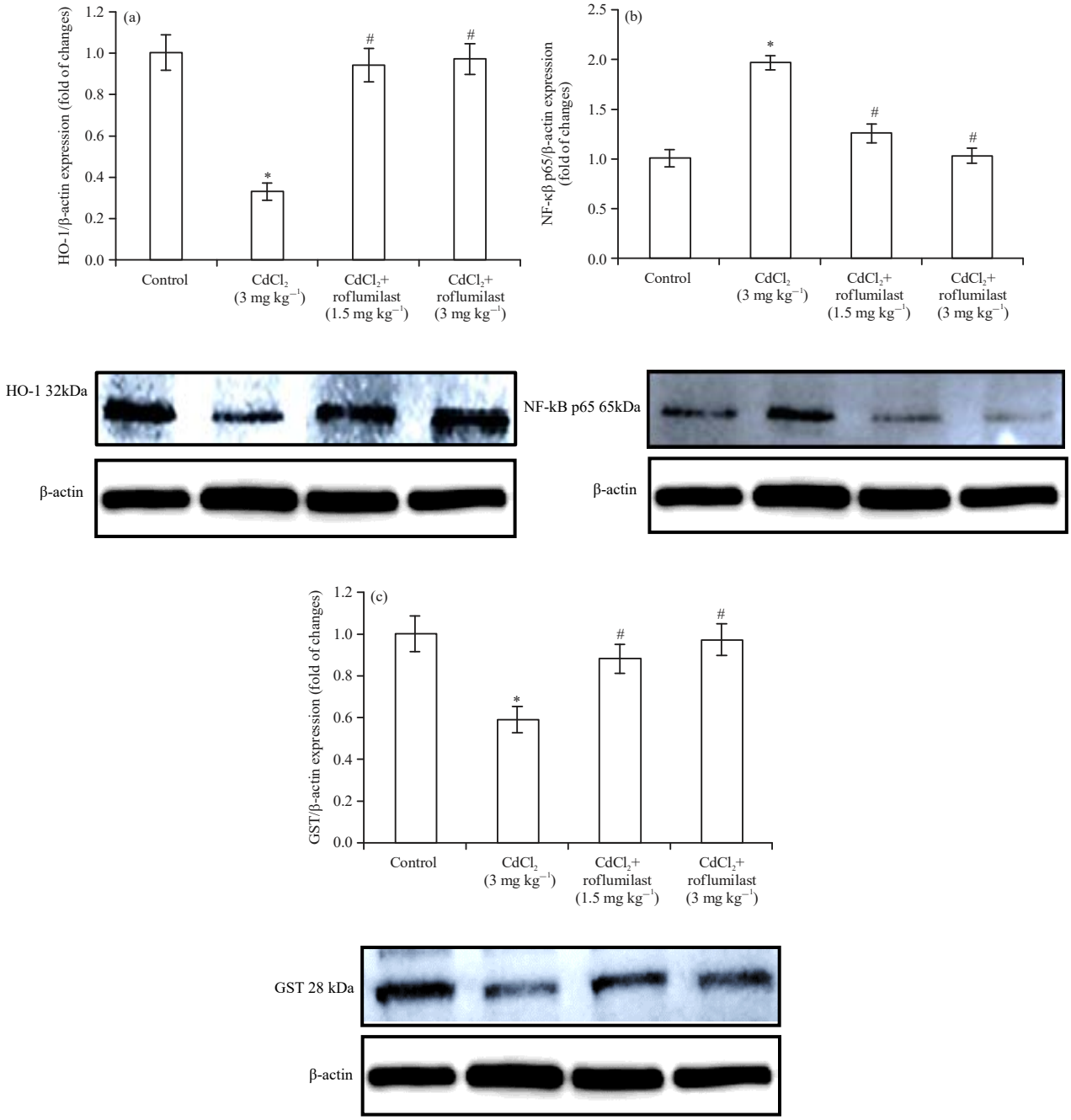

Fig. 4(a-c): Effect of ROF on HO-1, NF-κB and GST protein expression in liver tissues of $CdCl_2$-intoxicated rats, (a) Heme oxygenase-1, (b) Nuclear factor kappa-light-chain-enhancer of activated B cells and (c) glutathione-S-transferase

*p<0.001, show comparison of $CdCl_2$ with control (Unpaired t-test), #p<0.001, show comparison of treated (Roflumilast; 0.5 and 1.5 mg kg^{-1}) with $CdCl_2$ group (One-way ANOVA followed by Tukey test), each bar represents Mean±SEM (n = 6)

marker enzymes were observed in Cd-intoxicated rats as compared to normal control rats. This increased level of serum AST and ALT levels due to Cd toxicity were significantly ($p<0.05$) and dose-dependently restored upon the treatment with ROF.

Effect on hepatic oxidative stress markers: The levels of oxidative stress markers in the liver tissue of all rats are measure and shown in Fig. 3. The levels of lipid peroxidation products viz., MDA significantly ($p<0.001$) elevated in Cd treated rats as compared to normal control rats. Cd-induced

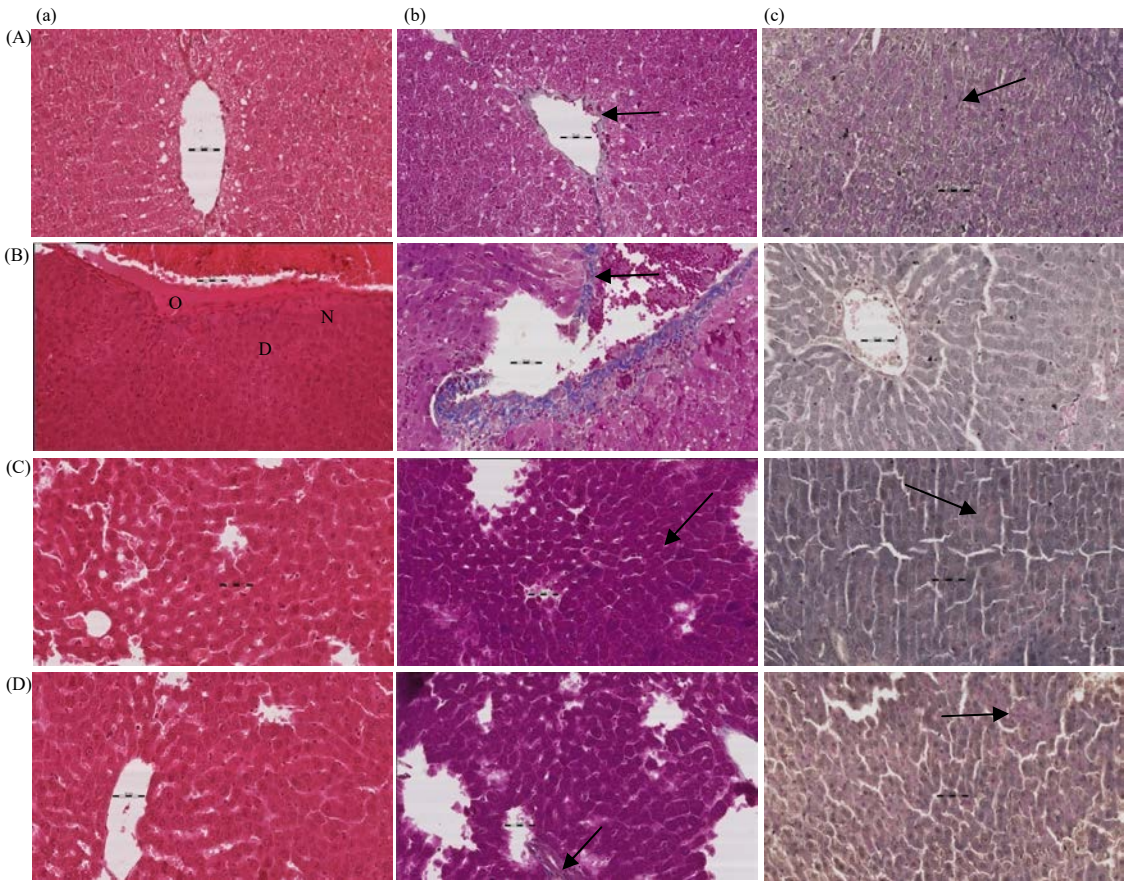

Fig. 5(a-c): Effect of ROF on histopathological features of $CdCl_2$-intoxicated liver tissues with (a) Hematoxylin and Eosin (H and E, 300X) (scale bar: 50 μ), (b) Masson Trichrome (MT, 300X) (scale bar: 50 μ) (arrow indicates area of blue collagen fibers) and (c) Periodic Acid-Schiff (PAS, 300X) (scale bar: 50 μ) (arrow indicates area of PAS positive material) staining

Photomicrographs of liver tissues of (A) normal control rats, (B) $CdCl_2$- intoxicated rats, (C) ROF treated at low dose (0.5 mg Kg^{-1}), (D) ROF treated at high dose (1.5 mg Kg^{-1}) N: Necrosis, D: Degeneration, O: Occluded central vein, CF: Collagen fibres

toxicity also resulted in significant ($p<0.001$) depletion of total GSH levels accompanying the diminished activity of SOD and CAT enzyme in liver tissues when compared with normal control rats. ROF administration afforded significant ($p<0.001$) protection against Cd-induced alteration of lipid peroxidation, total GSH levels along with SOD and CAT enzyme activity

Effect on western blot analysis: Western blot analysis was done to examine the expression of NF-κB p65 as it has been reported earlier that activation of NF-κB plays an important role in the production of inflammatory mediators. The results from the present study showed a significant elevation of NF-κB p65 protein expression in Cd-intoxicated rats when compared with the normal control group. Furthermore, a significant ($p<0.001$) depletion in the levels of HO-1 and GST protein expression also recorded as compared to the normal group (Group No. 1). Administration of ROF at increasing doses resulted in significant restoration of NF-κB p65, GST and HO-1 expression as compared to the Cd-intoxicated rats (Fig. 4).

Histological examination of liver tissue: Photomicrographs of the liver tissues of the normal control and experimental animals with different staining are shown in Fig. 5. Histopathological investigations of the liver tissues from normal control rats stain with Hematoxylin and Eosin (H and E) exhibited typical liver architecture and histology. Histopathological investigations showed that Cd-treatment produced pathological changes of necrosis, degeneration and accumulation of hyaline materials at the sinusoidal spaces of the liver. The histopathological alterations induced by Cd were protected in the liver of rats administered with ROF and

showed clear improvement and almost normal histological features. Masson trichrome (MT) staining of the liver tissue of the normal control group presented a normal distribution of collagen fibers. However, Cd treatment resulted in a remarkable deposition of collagen fibers (blue color) within liver parenchyma and surrounding occluded central vein. Relatively clear improvement and almost normal distribution of collagen fibers were observed in ROF treated groups. Histological liver section of the normal control group shows normal hepatocyte function of producing and storing PAS-positive materials particularly glycogen (magenta color). Liver sections of the Cd-treated group show a complete absence of PAS-positive materials which indicates hepatocyte's loss of function and could not produce or store glycogen. ROF treated group shows clear and better improvement as hepatocytes start producing and storing PAS-positive materials particularly glycogen.

DISCUSSION

Almost every organ of the human body is affected as a result of the exposure with metals and metalloids. Among metals, cadmium (Cd) is of major concern because of its growing incidence as an environmental contaminant and exposed to most of the population through various sources especially tobacco smoke, Cd-rich foods and ambient air, predominantly in the industrial area and also been used extensively in consumer products[26,27]. Previous literature reported that prolonged exposure of Cd resulted in damage to various tissues, including liver injury[28]. The present investigation was aimed to evaluate the protective effect of roflumilast in rats against the oxidative injury and liver toxicity induced by cadmium. The results from the present investigation demonstrate significant changes in the development of Cd-induced liver toxicity as compared with normal control rats. However, co-administration of ROF with Cd in rats for 7 consecutive days significantly ameliorates the Cd-induced hepatotoxicity and oxidative injury. These findings are supported by biochemical and histological findings.

It has been reported earlier that chronic Cd exposure causes changes in the biochemical functions markers which ends in serious toxicological effects. Accumulation of Cd in different organs after chronic exposure could produce oxygen free radicals (OFR) from peroxidation of membrane polyunsaturated fat. The OFR resulted in the generation of ROS that ends up with cellular antioxidant defense system inhibition and development of end organ pathology[29,30].

Cd-induced injury and oxidative stress may alter the activities of some antioxidant and reliable diagnostic injury marker enzymes by elevating the expression of different stress genes[31,32]. Cd-induced liver injury is a well-established model confirmed by the elevated levels of serum marker enzymes (AST and ALT) specifying the cellular leakage and loss of functional integrity of hepatic membrane[33]. In the present investigation also, Cd-induced toxicity resulted in elevated levels of AST and ALT therefore, corroborate with the previous findings. However, ROF treatment attenuated Cd-induced liver toxicity confirmed by the decreased level of marker enzymes thus proposing protection against Cd toxicity in rats possibly by stabilizing the cell membrane in liver damage.

Different mechanisms of Cd-induced toxicity and oxidative stress have been proposed, including increased lipid peroxidation by diminishing glutathione levels or antioxidant enzymes activities and interaction with membrane components[34,35]. In the present study, Cd administration resulted in increased LPO, possibly due to the overproduction of OFR and diminished antioxidant defense. Therefore, supplementation of antioxidants could ameliorate Cd-induced toxicity[36]. The results from the present investigation also showed that ROF treatment in Cd treated rats effectively diminish the LPO exerted by Cd, revealing its anti-lipid peroxidative and antioxidant effects.

Antioxidant enzymes preserved the cellular integrity from oxidative damage caused by OFR. Cd exposure is considered as the reduction of enzymatic and non-enzymatic antioxidants[37]. Total GSH acting as a non-enzymatic antioxidant and measured as the first line of defense against oxidative stress or it can play an important role in the enzymatic detoxification reaction of ROS. SOD and CAT constitute a mutually supportive group of antioxidant defense against ROS by inhibiting the overproduction of OFR[30,38]. Cd intoxication leads to the inactivation of glutathione[39] and prominently decreased the hepatic glutathione content, which defines the excess use of glutathione for the protection of Cd-induced oxidative injury. In the present study also Cd treatment exhibited a significant reduction in the total GSH levels and activity of hepatic antioxidant enzymes (SOD and CAT) could be due to the over generation of ROS[29,40]. Treatment with ROF in Cd intoxicated rats could restore the depleted levels of total GSH, confirming the role of ROF in the mechanism of glutathione metabolism which results in increased total glutathione levels and intracellular antioxidant power[41]. Furthermore, ROF also resulted in significant restoration of hepatic antioxidant enzymes confirming that hepatic injury caused by Cd might be prevented by the ROF.

Cytosolic NF-κB is known to be stimulated in response to various inflammatory reactions, atmospherically pollutants, pro-oxidants, cancer-causing agents, stress, etc[42]. Excess

production of ROS could initiate the cascade of stimulation of NF-κB signaling[43]. Previous studies reported that NF-κB stimulation may initiate Cd-induced apoptosis in rats[44]. In the present investigation also, it was observed that Cd-induced oxidative stress in rat liver resulted in stimulation of NF-κB protein expression and reduced expression of GST and HO-1. Whereas, ROF treatment found to be suppress the Cd-induced oxidative injury through the decreased expression of NF-κB and improved expression of GST and HO-1. The observed findings are supported by the earlierstudies[45].

Histopathological findings supported the biochemical results and suggested the ameliorative effect of ROF against Cd-induced liver injury. Histological assessment of the liver of Cd-intoxicated rats showed the pathological appearance of necrosis, degeneration and accumulation of hyaline materials at the sinusoidal spaces of the liver. However, the hepatic oxidative damage caused by Cd in rats was significantly reduced by the ROF treatment at both doses. Previous studies suggested an anti-inflammatory and antioxidant effects of ROF[46,47].

CONCLUSION

The results from the present study demonstrated and confirmed that Cd could effectively induce noticeable oxidative stress as well as diminish the antioxidants defense system. Administration of ROF could potentially protect the liver by preserving its normal physiology against hepatotoxicity and oxidative damage related to Cd-exposure. These findings suggested the therapeutic potential of ROF to be considered in the future for the protective and curative purposes of associated liver diseases especially caused by heavy metals.

SIGNIFICANCE STATEMENT

This study confirmed that the roflumilast could prevent the cadmium-induced liver function markers, oxidative stress and inflammatory markers, which can be beneficial for the liver diseases. This study will help the researcher to design further studies to explore the molecular mechanism. Thus, a new approach for the treatment of liver disorders may be arrived.

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