Cadmium-Induced Hepatotoxicity in Mice – Prophylactic Supplementation of Quercetin Exerts Hepatoprotective Effect by Modulating PI3K/Akt/NF-κB Signaling Pathway

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Summary

This current study seeks to examine the pre-protective function of Quercetin in Cadmium (Cd)-induced liver damage, along with its modulation of the PI3K/Akt/NF-κB signaling pathway. A total of 60 male C57BL/6J mice were randomly assigned to four groups: control (C), quercetin (Q, 100 mg/kg/day), Cd (Cd, 2.5 mg/kg/day), and quercetin and Cd (Q+Cd). Before receiving Cd treatment, quercetin was administered intragastrically for 4 weeks. In the present study, liver markers, oxidative stress parameters, pro-inflammatory cytokines, liver histopathology, apoptotic markers and PI3K/Akt/NF-κB signaling molecules were examined. We observed that the body weight of the Cd-treated mice dramatically rise after 4 weeks of quercetin pre-administration, and the Cd concentration was significantly decreased. Liver function markers like alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were significantly reduced in quercetin treatment in Cd-induced mice. Additionally, we observed that quercetin reduced Cd-mediated liver injury in mice by assessing the level of malondialdehyde (MDA), and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) concentrations and the histological alterations. By monitoring tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), quercetin successfully reduced the inflammatory cytokines that the Cd metal caused in the liver. Additionally, in the liver tissues of Cd-mediated, quercetin could enhance the expression of Bcl-2 and decrease the expression of p-Akt, p-PI3K, Bax, Caspase-9, Caspase-3, NF-κB. In conclusion, quercetin protects against Cd-induced liver injury *via* several pathways, including oxidative stress, inflammation and apoptosis, and its protective effect correlates with antioxidant activity.

Key words

Cadmium • Quercetin • PI3K/AKT signaling • Oxidative stress • Hepatotoxicity

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Introduction

Cadmium (Cd), a commonly prevalent environmental contaminant, poses a significant risk to human health. Because of its widespread use in anthropogenic and industrial activities, Cd production, utilization, and release to the atmosphere, land, and aquatic environments have steadily grown. Unlike organic contaminants, Cd cannot be chemically destroyed by microbes; instead, organisms may accumulate it [1,2]. Cd-contaminated dust particles, aerosols, cigarette smoke inhalation, and ingestion of Cd-polluted drinks or food are all potential sources of Cd exposure [3]. As a multi-organ toxin, Cd severely affects multiple organs, like the liver, kidneys, testes, bone, and spleen. After acute or chronic Cd exposure, the liver is the main target organ because it has the largest depot of Cd accumulation in soft tissues [4]. Administration of Cd to liver tissue *via* membrane transporters found in the membranes of liver sinusoidal endothelial cells [5]. When Cd enters the hepatic tissue, it inhibits the function of antioxidant enzymes and produces ROS [6]. As a result, higher ROS levels induce lipid peroxidation, damage to cellular macromolecules such as

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DNA, proteins, and lipids, and eventually cause systemic oxidative stress [7].

Considerable research studies have been conducted on detoxifying harmful heavy metals with dietary nutrients. Quercetin (3,3',4',5,7-Penta-hydroxyflavone), a polyphenolic flavonoid molecule found in high levels in vegetables, fruits, and tea, has been revealed to have medicinal value in the treatment of many illnesses, including hepatoprotection and suppression of liver fibrosis [8,9]. In a typical human diet, quercetin consumption is estimated to be up to 25 mg/day [10]. It has a phenolic hydroxyl structure, which confers antioxidant activities and has therapeutic efficacy for a variety of disorders, including liver diseases, ischemic heart disease, atherosclerosis, malignancy, and renal damage [11-13].

Quercetin is gaining popularity for its pharmacological and biological effects, such as anti-oxidant [14], anti-inflammation [15], and anti-apoptosis [16], among others, and is thought to protect the liver against heavy metal toxicity. Though the actual mechanism of Cd-induced hepatotoxicity is still unidentified, it is widely assumed that the probable mechanism involves the metal's capability to produce free radical damage. Furthermore, earlier research has shown that oxidative stress induced by excessive reactive oxygen species (ROS) generation and persistent stimuli of inflammation lead to hepatocyte apoptosis [17].

The PI3K/Akt signaling pathway is one of the major signal transduction pathways that regulate numerous cellular activities *in vivo*. The main functions of this pathway include the induction of stem cell differentiation and metastasis, promotion of cell proliferation, inhibition of apoptosis, and regulation of tissue inflammation, tumor growth, and invasion [18,19]. Considering the effectiveness of quercetin in regulating PI3K/Akt and NF-κB related pathways in hepatotoxicity cells, while the precise mechanism by which quercetin pre-administration controls the PI3K/Akt and NF-κB-related pathways in Cd-induced hepatotoxicity cells is not understood yet. Consequently, the current study had two goals: (1) To determine whether quercetin pre-administration is associated with reducing Cd-induced hepatotoxicity. (2) To investigate whether quercetin's hepatoprotective effects are associated with the PI3K/Akt/NF-κB signaling pathways.

Materials and Methods

Ethical approval

The Committee for the Care and Use of Laboratory Animals at Wuxi No.2 People's Hospital (Wuxi, China) evaluated and approved the methodology of experiments, Approved No. 2020006.

Animals and treatment

The inbred 6-8 weeks old male C57BL/6J mice with 20 g initial body weight were used in experiments. The mice were kept in a standard environment, with 22±1 °C ambient temperature, 50 % relative humidity (RH), and a 12/12 h dark/light cycle. Mice were unrestricted to access food and water. The Medical Ethics Committee of Wuxi No.2 People's Hospital approved the animal experiment protocol (Approval No. 2020006). All experimental procedures were carried out following the International Laboratory Animal Care and Use Guidelines.

After 7 days of acclimatization, the mice were assigned randomly to 4 groups, each with 8 mice (Fig. 1). The study took place for 13 weeks. (1). Animals in the control group were administered just saline solution. (2) Quercetin group: the animals were administered 100 mg/kg/day of quercetin [20] (Sigma-Aldrich, Steinheim) intragastrically, five times per week. (3) Cd group: Beginning in the fifth week, the animals were intragastrically administered the $CdCl_2$ (Sigma-Aldrich, Steinheim) 2.5 mg/kg/day [21], five times per week. (4) Quercetin + Cd group: these animals were given both quercetin and Cd in the above doses. After a four-week quercetin pre-administration, quercetin and Cd were administered simultaneously. The experiment took 13 weeks to complete. Following the experiments, all mice were sedated with sodium pentobarbital (50 mg/kg intraperitoneal injection) and slaughtered *via* cervical dislocation.

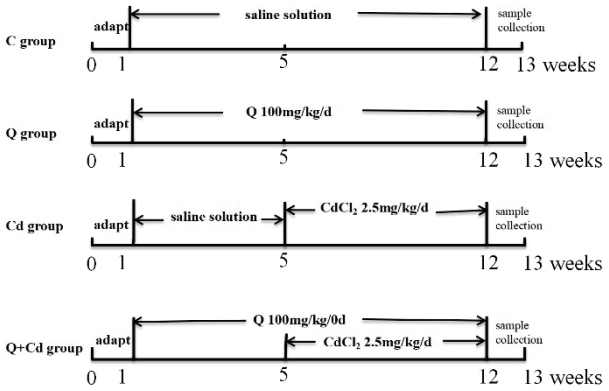

Fig. 1. Schematic diagram of animal experimental design.

Sample preparation

Blood was collected from the posterior orbital venous plexus at the end of the trial. After collecting blood samples, the liver tissues of the animals in the different groups were dissected. We measured the serum concentrations of the alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). A histopathological examination of the liver tissues was also conducted. Oxidative stress indicators such as malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) were measured in the liver tissues samples. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), three inflammatory cytokines, were evaluated in the serum and liver tissues. Moreover, the body weights were also recorded.

Assessment of Cd concentrations

Cd concentration in the liver tissues and serum was determined as described earlier [21]. The samples of each wet liver tissue and serum were obtained (selected randomly) in all groups and digested in HNO_3 to remove any bio-organic debris. The concentration was then determined using an inductively coupled plasma mass spectrometry (ICP-MS) approach (ELAN-6000 model, Perkin-Elmer, Sciex, Toronto, Canada). The detection limit was 0.08 µg Cd/l.

Histological examination

The liver tissues were processed for histological examination, fixed in 4 % paraformaldehyde, and followed by paraffin embedding. Then, 5 µm thick sections were cut from the tissues. The tissue slides were subjected to hematoxylin and eosin (H&E) staining to evaluate live tissue morphology [22]. Slides were observed and photographed using a light microscope (DM1000-3000, Leica, Germany).

Biochemical assays

The colorimetric assay kits were used for the determination of liver markers aspartate aminotransferase (AST-C010-2-1), alanine aminotransferase ALT (C009-2-1) activities were determined according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) [23]. 5 µl of buffer solution, 20 µl of substrate solution and 2 µl of 2 mmol/l sodium pyruvate solution were added and mixed well to be added to 5 µl of serum and then incubated at 37 °C for 30 min. After incubation, 20 µl of chromogenic agent was added, mixed with 10 s and incubated at 37 °C for 20 min. 200 µl of alkali reagent was added and mixed well; then, stand for 15 min at RT and measure the OD value of each well with microplate reader at 510 nm. Alkaline phosphatase (ALP-A059-2-2) was determined by 1 ml of buffered substrate, 3 ml of deionized water and 0.1 ml of serum were incubated 15 min at 37 °C. Then 2.0 ml of color reagent was added to all the tubes. The control tubes received the enzymes after the addition of color reagent. The reaction mixture was read at 510 nm. The following kit procedure estimated Lactate dehydrogenase (LDH-A020-2-2). 1.0 ml of buffered substrate was mixed with 10 µl of serum and the tubes were incubated at 37 °C for 15 min. 0.2 ml of coenzyme solution (NAD) was then added and again incubated for 15 min. The reaction was stopped by the addition of 1.0 ml of 2,4-dinitrophenylhydrazine. The tubes were incubated at 37 °C for another 15 min, and 5.0 ml of 0.4 N NaOH was added to each tube and mixed well. The intensity of the color was measured at 520 nm.

Inflammatory markers

According to the manufacturer's instructions, the pro-inflammatory cytokines TNF-α (H052-1-2), IL-6 (H007-1-2), and IL-1β(H002-1-2) were measured in the serum and liver tissues using the relevant ELISA diagnostic kits (Biological Technology Company Ltd., Shanghai, China) [24]. All samples were evaluated in standardized units per liter.

Liver oxidative stress markers analysis

The level of MDA, GSH, GPx, CAT, and SOD and the effects of quercetin were assessed in the liver tissue samples of mice suffering from Cd-induced liver damage. The standard colorimetric test kits MDA (A003-1-2), GSH(A005-1-2), GPx (H545-1-2), CAT (A007-1-1) and SOD (A001-3-2) were used to assess oxidative stress indicators according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) [24]. To level of Malondialdehyde (MDA) content was estimated by the following protocol. 200 µl of sample and added 600 µl of TBA solution to generate 800 µl of MDA-TBA adduct then incubated at 95 °C for 1 h; placed with an ice bath for 15 min. After incubation, the reaction mixture was measured at 532 nm on the microplate reader.

GSH was estimated by a commercial kit (Nanjing Jiancheng Bioengineering Institute, China).

5 µl of homogenate was pipetted out and precipitated with 2.0 ml of 5 % TCA. 2.0 ml of supernatant was taken after centrifugation, and added 1.0 ml of Ellman's reagent and 4.0 ml of 0.3 M disodium hydrogen phosphate. The yellow color developed was read at 412 nm. The known amount of enzyme preparation assayed that the glutathione peroxidase activity (GPX) was allowed to react with H_2O_2 and glutathione (GSH) for a specified time. Then, the GSH content remained after measuring the reaction. The following procedure assayed Catalase (CAT) activity. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in hydrogen peroxides (H_2O_2). The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H_2O_2 for various periods. The reaction was stopped at different time intervals by adding a dichromate-acetic acid mixture and the remaining H_2O_2 as chromic acetate was determined microplate reader. SOD is based on inhibition of the formation of NADH-phenazine metho sulphate, nitroblue tetrazolium formazon. The reaction is initiated by the addition of NADH to the assay mixture. After incubation for 90 s, glacial acetic acid was added to stop the reaction. The color developed at the end of the reaction was extracted into the n-butanol layer and measured at 520 nm.

Western blotting analysis

Total protein from mice liver tissues was isolated using ice-cold radio-immunoprecipitation assay buffer (RIPA) (Beyotime Institute of Biotechnology, Haimen, China). The protein concentrations were measured by BCA (Bicinchoninic acid) assay (Thermo-Fisher Scientific, Inc., Pierce) [25]. Protein (30 µg) was isolated on a PVDF membrane using a 12 % SDS-PAGE. Nonfat milk (5 %) was used as a blocking solution at room temperature for 2 h, followed by incubation with PI3K, Akt, p-Akt, p-PI3K, Bcl-2, Bax, Caspase-9, Caspase-3, NF-κB, p-NF-κB, and β-actin (each at 1:1000) at 4 °C overnight. All the antibodies used were obtained from Abcam (Cambridge, MA, USA). Following TBST washing thrice, anti-mouse HRP-conjugated secondary antibodies (1:10000) (cat. no. BM3873, Wuhan Boster Biological Technology, Ltd., Wuhan, China) were employed on the blots at room temperature for 1 h. The bands were observed by applying a chemiluminescent reagent, as directed by the SuperSignal^{TM} West Dura Extended Duration Substrate kit (Pierce, Thermo-Fisher Scientific, Inc.). The band signals were quantified using a VICTORZ 1420 multi-label counter (PerkinElmer, Inc., Waltham, MA, USA). Image J software (v.1.46) was used to calculate the results, representing density relative to β-actin.

Statistical analysis

Using information acquired from repeated (at least three times) experiments, statistical analyses were performed using SPSS (v. 20.0) software. The statistical results are presented as mean ± standard deviation (x ± SD). One-way Analysis of Variance (ANOVA) was employed to validate the groups' statistical differences, followed by the Tukey test in pair-wise repeated comparisons. Comparative data between groups were analyzed using the analysis of variance. GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA) was used for data analysis and graphing. The data were statistically significant when a $p<0.05$.

Results

We initially prepared 4 weeks of pre-administration of quercetin, which was given concurrently with Cd. 13 weeks were needed to complete the trial. Following that, changes in mouse weight were noted, Cd concentrations in liver tissues and serum were assessed, biochemical serum assays were performed, and an examination of inflammatory cytokines in the liver was completed. In order to investigate the regulation of quercetin on Cd-induced proteins, Western blotting detection was carried out. Finally, we discovered that the hepatoprotective actions of quercetin pre-administration are associated with PI3K/Akt/NF-κB signaling pathways.

Body weight, Cd concentrations and MT expression of the mice

The main aim of this study was to examine the mechanism through which quercetin attenuates liver injury caused by Cd. As shown in Figure 2, the mice in the group with Cd-induced liver injury experienced a gradual decrease in body weight throughout 0 to 13 weeks after Cd induction, with a significant decline observed particularly from the 9th week onward ($p<0.01$). However, the administration of quercetin intervention for 4 weeks showed the capacity to alleviate the weight loss observed in the mice. The observed phenomenon demonstrated a correlation between the concentration of Cd in the serum of mice induced by Cd and the duration of exposure. However, the administration of quercetin resulted in a progressive decrease in serum

Cd concentration over time. Particularly noteworthy is the significant divergence in body weight and serum Cd concentration between the quercetin group and the Cd-induced liver injury model group in the 13th week ($p<0.05$). As a result, samples for subsequent experiments were collected from mouse tissues in the 13th week. However, after 4 weeks of quercetin pre-administration, the level of Cd in the liver in the Q+Cd group reduced, with no significant difference. These results suggest that quercetin pre-administration may reduce Cd concentration in a Cd environment.

Furthermore, we investigated the Cd-induced mice have significantly increased metallothionein (MT) protein expression in liver tissue. In contrast, pre-administration of quercetin significantly reduced the MT expression when compared to Cd-induced mice (Suppl. Fig. 1).

Quercetin attenuated liver functions in Cd-induced hepatic damage

In the present study, the concentration of Cd in the liver was significantly increased in mice exposed to Cd. The concentrations of serum ALT, ALP, and AST, the significant biomarkers of liver injury, were measured to validate the protective effect of quercetin against Cd-induced hepatic damage. As illustrated in Figure 3, serum ALT, ALP, and AST concentrations were significantly greater in the Cd group compared to the control group ($p<0.01$). However, compared to the Cd group, pre-administration with quercetin in Cd-treated mice substantially lowered the ALT, ALP, and AST levels in serum ($p<0.01$). The Cd group showed a significant rise in serum LDH levels compared to the control group. However, the combination of quercetin and Cd therapy dramatically reduced serum LDH levels compared to the Cd group ($p<0.05$). These findings showed that quercetin has a pro-effective effect against Cd poisoning and aids liver function recovery.

Effect of quercetin on Cd-induced oxidative stress in mice

ROS surpassed the capacities of the antioxidant defense system, thereby intensifying oxidative stress. To determine if quercetin enhances the enzymatic activities linked to the antioxidant defense system, we subsequently assessed the concentrations of pivotal antioxidants. The findings showed that Cd treatment significantly lowered liver SOD, GSH, CAT, and GPx concentrations compared to the control (Fig. 4, $p<0.01$). SOD, GSH, CAT, and GPx activities were increased when quercetin was combined with Cd treatment ($p<0.05$). The exposure to Cd resulted in a notable increase in MDA level in the liver when compared to the control group (Fig. 4, $p<0.01$). However, the pre-administration of quercetin effectively reduced the MDA level in the liver of Cd-treated mice to a significant extent (Fig. 3, $p<0.01$).

Quercetin mitigated Cd-induced liver histopathological modifications

We investigated the histological alterations in the liver tissues of mice from various groups to see whether quercetin might pre-protect them against Cd-induced liver damage. Histopathological arrangements of the normal control mice liver exhibited regular vein structure, clearly arranged hepatocytes and healthy cell nuclei. In both the control and quercetin-treated groups, the central veins (CV) were of normal size, as illustrated in Figure 5. The Cd-treated hepatocytes have dilated and congested CVs and infiltration of inflammatory cells in the portal regions (Fig. 5). The microphotograph from the quercetin combined with Cd-treated group showed modestly congested CVs and typical polyhedral hepatocyte cords with distinct nuclei and abundant cytoplasm (Fig. 4). Compared to the Cd-treated group, quercetin pre-administration significantly lower score of the histological lesions in Cd-treated mice substantially (Fig. 5, $p<0.01$).

Quercetin attenuated Cd-induced pro-inflammatory cytokines

As shown in Figure 6, the ELISA test was used to assess TNF-α, IL-1β, and IL-6, the pro-inflammatory cytokines, in serum and liver tissue samples. It was found that the Cd-induced mice model showed an increase in these cytokines compared with control mice ($p<0.05$). Quercetin pre-administration decreased the amounts of pro-inflammatory cytokines brought on by Cd in a way similar to quercetin when compared to the Cd group ($p<0.05$).

Effects of quercetin on the levels of PI3K/Akt/NF-κB signaling pathway in the liver

According to several studies, Cd stimulates the PI3K/Akt pathway, stimulating the NF-κB pathway. To see whether quercetin inhibits this signaling pathway in Cd-induced hepatotoxicity, we used Western blotting to assess the protein levels such as PI3K, Akt, p-PI3K, NF-κB, and p-NF-κB. (Fig. 7). When subjected to Cd, p-PI3K, PI3K, Akt, NF-κB, and p-NF-κB were all higher

than the control group, whereas the levels of these proteins were lowered in comparison to the Q+Cd group ($p<0.05$). These findings showed that pre-administration with quercetin reduced NF-κB -mediated inflammation in Cd-induced hepatotoxicity, most likely through suppressing the PI3K/Akt pathway.

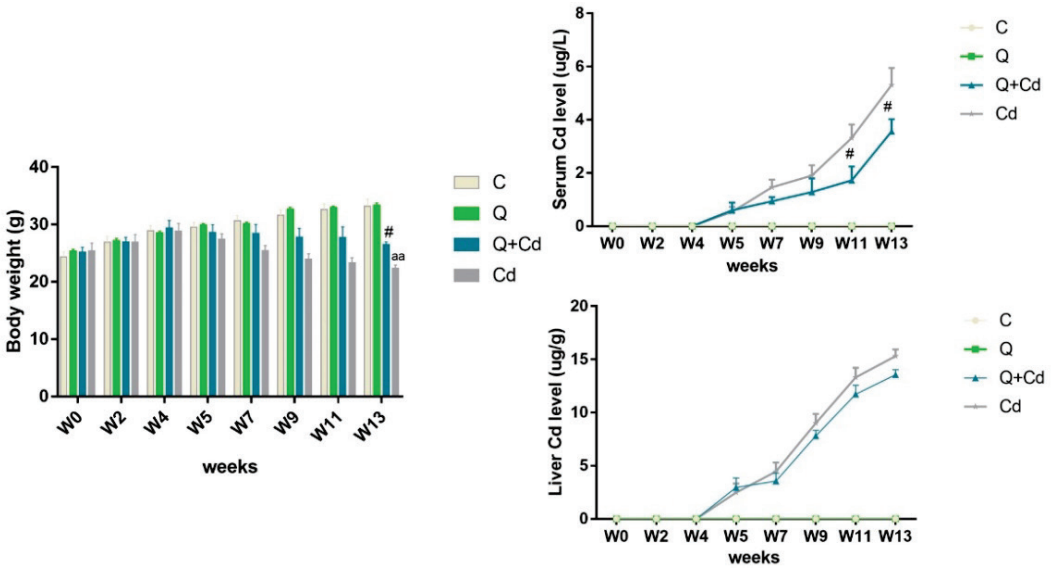

Fig. 2. Body weight and Cd concentration of the Mice. Cd concentration in liver and serum of mice treated for 9 weeks to Cd (WO, W2, W4, W5, W7, W9, W11, and W13). Control group (C), Quercetin group (Q), Quercetin + Cd group (Q+Cd) and Cd group. The data represented as mean ± SD, * $p<0.05$ vs. W4 in the same group. # $p<0.05$ vs. Cd group.

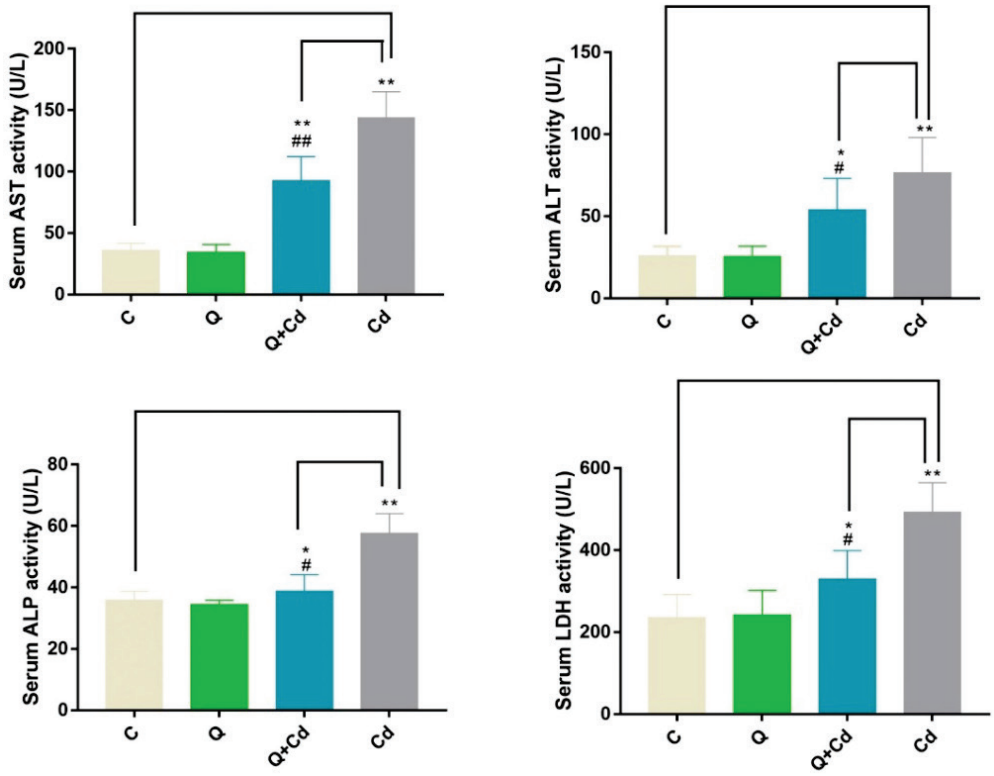

Fig. 3. Effect of quercetin on serum AST, ALT, ALP, and LDH levels due to Cd administration. Data represented as mean ± SD (n=8). Significance considered by * $p<0.05$, ** $p<0.01$ vs. C group, # $p<0.05$, ## $p<0.01$ vs. Cd group.

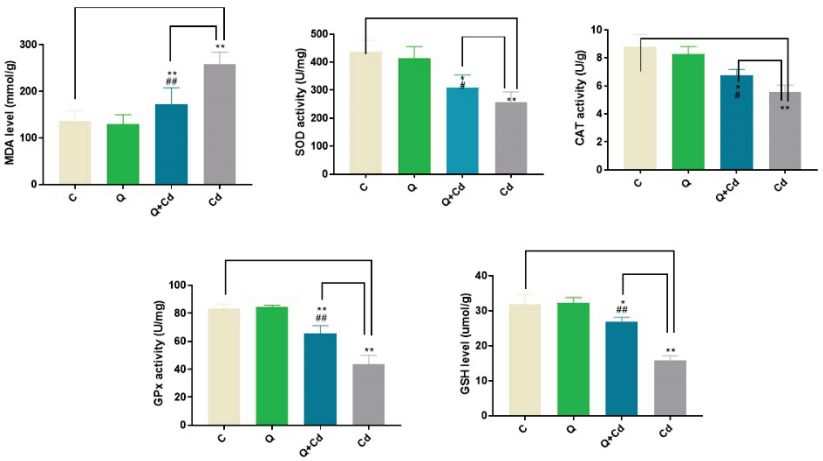

Fig. 4. Effect of quercetin on oxidative stress markers MDA, GSH, SOD, CAT, and GPx. Data represented as mean ± SD (n=8). Significance considered by * p<0.05, ** p<0.01 vs. C group, # p<0.05, ## p<0.01 vs. Cd group.

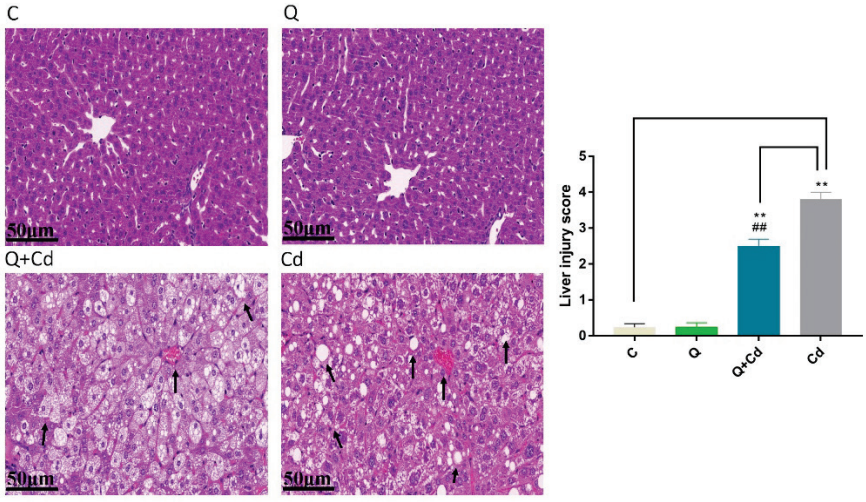

Fig. 5. Effect of quercetin on histopathological changes in Cd-induced liver tissues. For pathological verification, H&E stains were applied to the tissues and observed at 100× magnification. A blinded pathologist assigned damage ratings on a scale of 0 to 4, with 0 denoting no changes, 1 denoting minor changes, 2 denoting moderate pathological changes, 3 indicating poor injury and pathological changes, and 4 denoting severe injury and inflammation. Black arrows show the injured hepatocytes owing to necrosis/apoptosis, infiltration of inflammatory cells, and loss of sinusoidal gaps. Data represented as mean ± SD (n=8). Significance considered by * p<0.05, ** p<0.01 vs. C group, # p<0.05, ## p<0.01 vs. Cd group.

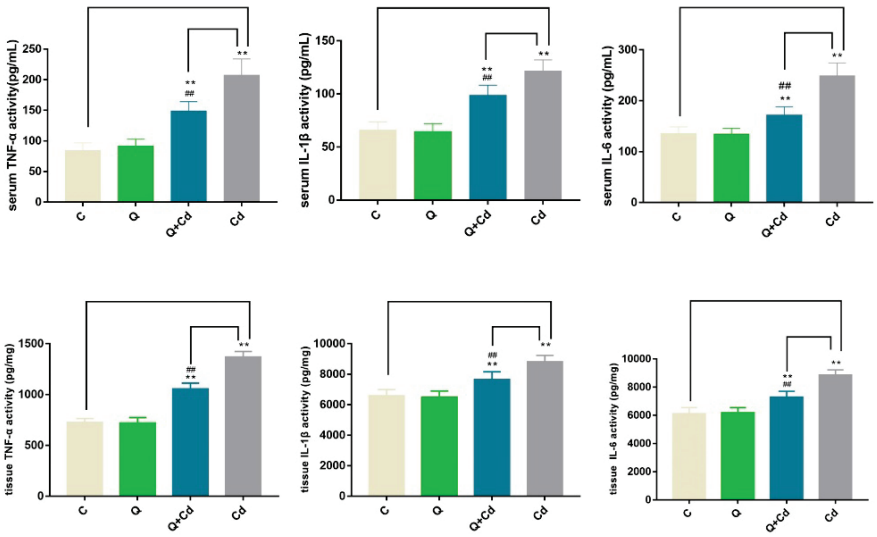

Fig. 6. Effect of quercetin on pro-inflammatory cytokines triggered by Cd. The levels of TNF-α, IL-1β, and IL-6 pro-inflammatory cytokines in the Cd-induced mice were determined through the ELISA test. Data represented as mean ± SD (n=8). Significance considered by * p<0.05, ** p<0.01 vs. C group, # p<0.05, ## p<0.01 vs. Cd group.

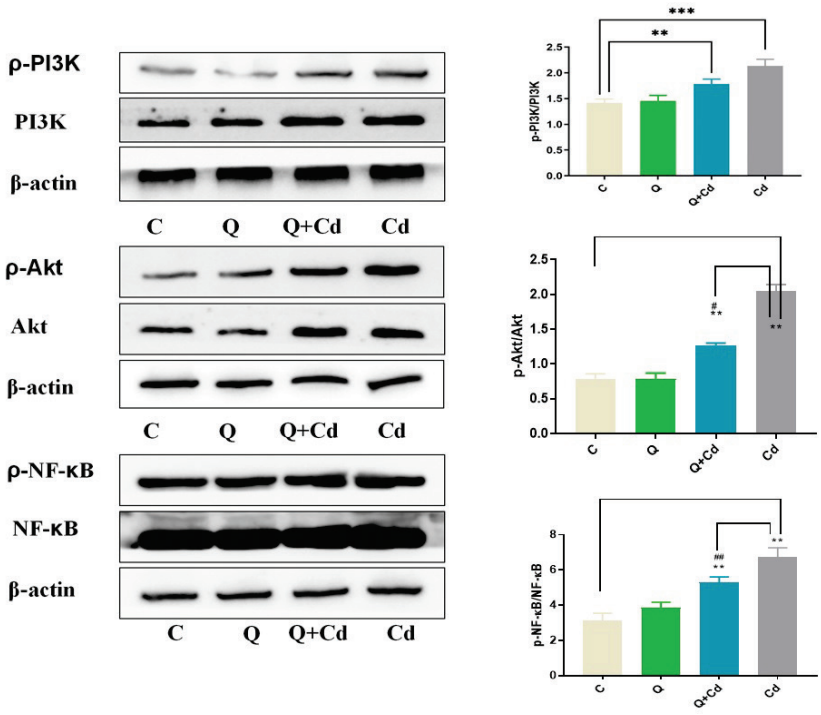

Fig. 7. Effect of quercetin on PI3K/Akt and NF-κB signaling pathways due to Cd administration. Expressions of PI3K, Akt, and NF-κB were determined using Western blot analysis, and β-actin was used as a loading control. The relative protein expressions were quantified through densitometry analysis. Data represented as mean ± SD (n=8). Significance considered by * $p<0.05$, ** $p<0.01$ vs. C group, $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs. Cd group.

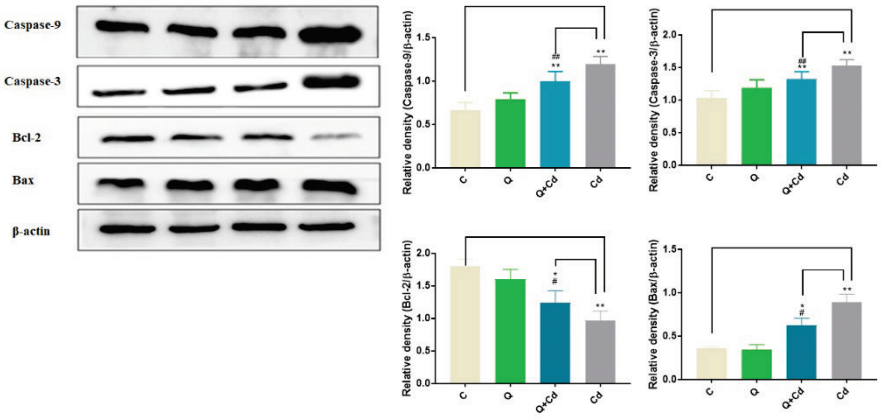

Fig. 8. Effect of quercetin on apoptosis signaling pathways in Cd-induced liver injury. Protein expressions of Caspase-9, Caspase-3, Bcl-2, and Bax, were measured using Western blot analysis, and β-actin was used as a loading control. The relative protein expressions were quantified through densitometry analysis. Data represented as mean ± SD (n=8). Significance considered by * $p<0.05$, ** $p<0.01$ vs. C group, $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs. Cd group.

Quercetin altered Cd-induced apoptosis in liver injury

Western blot was employed to assess the apoptotic regulators caspase-9, caspase-3, Bax, and Bcl-2 in liver tissues. The findings demonstrated that caspase-3, caspase-9, and Bax expressions were considerably higher in the Cd-induced model in comparison to the control mice (Fig. 8, $p<0.05$). However, when compared to the Cd-induced model group, pre-administration of quercetin with enhanced Bcl-2 level and reduced levels of caspase-9, caspase-3, and Bax significantly reversed the expression of these apoptotic regulators ($p<0.05$).

Discussion

Cd overdose or continuous use has been linked to serious hepatotoxicity. In animals, the liver is the primary site of xenobiotic processing and is a target organ for xenobiotic-induced toxicity [26]. As shown by the

increase in serum hepatic enzymes in the current investigation, Cd treatment causes liver damage. Elevated activities of AST, ALT, ALP and LDH enzymes indicate the disordered state of hepatic tissues and the resultant hepatic dysfunction [27]. The over-generation of ROS affects the performance of cell organelles (mitochondria and endoplasmic reticulum) and DNA, thereby affecting the protein formation [28]. Furthermore, Cd-induced mice showed histological alterations in the liver, including structural impairment, hepatocellular necrosis, leukocyte infiltration, and extensive bleeding. The findings of this study supported the notion that Cd produced significant liver damage [7,27,29]. Quercetin is a flavonoid that is abundant in nature. Quercetin has been found safe and tolerable to animals, even at large dosages [30]. Vicente-Sánchez *et al.* [21] showed that quercetin did not improve the effects of Cd on plasma marker enzymes ALT, AST, ALP, ACP, LDH, and GGT. This might be owing to differences in the liver damage model, Cd treatment techniques, quercetin treatment, and dosage, all of which result in distinct treatment effects of quercetin on Cd toxicity. The dosage and period of Cd modelling intervention in this experiment were consistent with those reported by Vicente-Sánchez *et al.* The distinction in this experiment is that animals were given quercetin for four weeks before Cd induction and treated similarly. Notably, we found that quercetin pre-administration effectively protected against Cd-induced liver damage by lowering increased serum ALT, AST, ALP, and LDH activities and alleviating the liver's histological alterations, indicating that quercetin has a pre-protection activity. These findings demonstrated that quercetin might pre-protect mice against Cd-induced hepatic dysfunction and histological impairment.

Metallothionein (MT) is widely recognized as a sensitive and reliable biomarker for heavy metal contamination due to its crucial functions in regulating redox potentials, essential metal homeostasis, and sequestration of non-essential metals for excretion. In this study, Cd exposure induced the increased MT protein expression in mice liver tissues. In contrast, pre-administration of quercetin significantly reduced the MT protein expression when compared to Cd-induced mice (Suppl. Fig. 1). The metallothionein protein expression might be one of the main mechanisms through which pre-administration of quercetin alleviates Cd toxicity. Subsequent research will delve into the molecular mechanisms underlying the antioxidant properties of quercetin pretreatment in the context of cadmium-induced hepatotoxicity, with a focus on determining the levels of metallothionein in the liver.

Even though people are suffering from Cd-induced liver damage, few therapies are available to recover. It has been found that inflammation and oxidative stress show essential roles in the pathological alterations in the liver caused by Cd exposure [29,31,32]. Therefore, anti-inflammatory and antioxidant therapy may effectively treat liver impairment caused by Cd poisoning. Major antioxidant levels were well observed in the model mice where antioxidant defense GSH and enzymes GPx and CAT were depleted. In contrast, the MDA level increased due to peroxidation of the cell lipid membrane [31,33]. These conditions are caused by an abundance of free radicals in the cellular environment, which depletes the basic defense of GSH. Because of the inactivity of GSH and the continual free radicals generation, the functions of antioxidant enzymes would be reduced [33]. Peroxidation of the lipid membrane will continue until an external source of antioxidants donates an electron to end the scavenging effect [34]. In recent years, antioxidant compounds have been considered for numerous pathologic conditions, including the therapy of oxidative stress-related disorders. Quercetin is a flavonoid with multiple bioactivities, including antioxidant properties [33] and the ability to regulate inflammation [34]. Quercetin effectively prevents oxidative stress by replenishing the antioxidant GSH, sustaining antioxidant enzyme activities, and reducing lipid peroxidation [35,36]. The data presented in this study indicate that exposure to Cd resulted in a significant decrease in the levels of antioxidative indices, including SOD, GPx, CAT, and GSH. However, administration of quercetin partially attenuated this decrease in Cd-treated mice.

Additionally, the levels of hepatic MDA, a marker of lipid peroxidation, was notably increased in the Cd-treated group compared to the control group. Nevertheless, quercetin administration in Cd-treated mice significantly inhibited this increase in MDA level. Taken together, these findings suggest that quercetin may safeguard against Cd-induced redox imbalance by augmenting the efficacy of the antioxidant defense system, thereby facilitating the restoration of hepatic injury.

Several investigations have shown that quercetin reduces oxidative stress, improves morphological performance, and inhibits the PI3K and NF-κB pathways in heavy metal-induced hepatocellular injury [37-39]. The PI3K/Akt signaling pathway is a crucial regulatory

pathway that governs diverse cellular functions and holds significant relevance in numerous physiological processes and pathological responses. Activation of the PI3K/Akt signaling pathway occurs in the initial phases of liver injury, wherein it assumes pivotal roles in anti-apoptosis, anti-inflammatory, anti-oxidative stress, and autophagy regulation. However, the effects of quercetin pre-administration on the pathological mechanisms of Cd-induced liver injury, such as oxidative stress, inflammatory response, and cell apoptosis, are not fully understood. Inflammation is interrelated to oxidative stress, where pro-inflammatory cytokines are initially triggered to support the inflammatory response. Cd administration has been shown to activate the NF-κB pathway, which causes the transcription of inflammation-related genes and the upregulation of inflammatory proteins [38,39]. Pro-inflammatory cytokines TNF-α, IL-6 and IL-1β are continuously produced once the NF-κB protein translocates into the cell nucleus and triggers the transcription process of the pro-inflammatory genes. These characteristics were found to be true in the Cd-induced group [40,41]. Inflammatory cell infiltration was also seen in the histopathological findings. Quercetin pre-administration for 4 weeks has anti-inflammatory effects on Cd-induced hepatic damage and inflammation. The concentrations of pro-inflammatory cytokines were significantly decreased in the ELISA test, and quercetin suppressed the production of NF-κB protein, as revealed by Western blot data analysis.

The PI3K/Akt signaling pathway is a well-established antiapoptotic pathway in cellular biology. The activation of the PI3K/Akt signaling pathway leads to the inhibition of caspase 3-mediated cell death through the phosphorylation of the Bcl-2/Bcl-XL-associated death promoter (Bad) by Akt. And apoptosis is a cellular suicidal mechanism that is initiated during severe inflammatory conditions or when the cells are under oxidative stress. Apoptosis plays a vital role in the pathophysiology of liver damage [42]. Typical apoptosis in hepatic cells can be detected with features of fragmented DNA, contracted hepatocytes, and activated apoptosis-mediator proteins [43,44]. Animal study finding revealed that Cd-induced hepatotoxicity causes apoptosis in liver cells. During stress, mitochondria send out apoptotic signals that activate caspase-9 and caspase-3, triggering pro-apoptotic signals that are then carried on by downstream targets [45]. Apoptotic regulatory protein Bcl-2 has anti-apoptotic functions by stabilizing the effect of pro-apoptotic protein Bax [46]. Apoptosis was activated in the livers of Cd-induced group mice, as the expressions of cleaved caspase-3 and caspase-9 were significantly increased, as was pro-apoptotic Bax protein, but anti-apoptotic Bcl-2 proteins were reduced. The quercetin pre-administration significantly inhibited the expressions of pro-apoptosis proteins, whereas the anti-apoptotic Bcl-2 protein was enhanced. The anti-apoptotic activity of quercetin can be evidenced by the upregulation of phosphorylated PI3K/Akt anti-apoptotic signaling proteins. By regulating apoptosis under oxidative stress and inflammatory conditions, the PI3K/Akt signaling pathway is an essential target for liver protective mechanisms [25,47]. The regulatory effect of quercetin on apoptosis signaling proteins can be evidenced in the past study on cervical cancer cells by suppressing the anti-apoptotic Akt protein. This research supports the idea that quercetin is selective in normal and cancer cells, which might help to maintain a healthy balance in the regulation of apoptosis-related proteins [46]. The liver protective action against Cd toxicity is explained by quercetin's involvement in regulating the apoptotic and inflammatory signaling pathways PI3K/Akt/NF-κB.

Conclusions

The present study's data provides the initial confirmation of the hepatoprotective properties of quercetin in mitigating liver damage induced by Cd. Quercetin exhibits potential as a regulator in countering Cd-induced liver injury. Our findings demonstrate that Cd induces a notable reduction in hepatic antioxidant mechanisms, an elevation in serum enzymatic activities, and disruption of inflammatory and apoptotic-mediated signaling pathways. Moreover, further inquiries ought to establish a systematic timetable and/or composition of these nutraceuticals to counteract cadmium at various cellular and subcellular tiers. The emergence of hepatoprotective compounds, such as quercetin, presents promising prospects for additional therapeutic interventions targeting diverse hepatic disorders (Fig. 9). However, a noteworthy constraint of the current study is the absence of inhibitors or knock-out models employed to impede the molecular mechanisms implicated in Cd-induced hepatotoxicity, thereby verifying the hepatoprotective efficacy of quercetin. Consequently, this necessitates additional investigations to substantiate the existing findings.

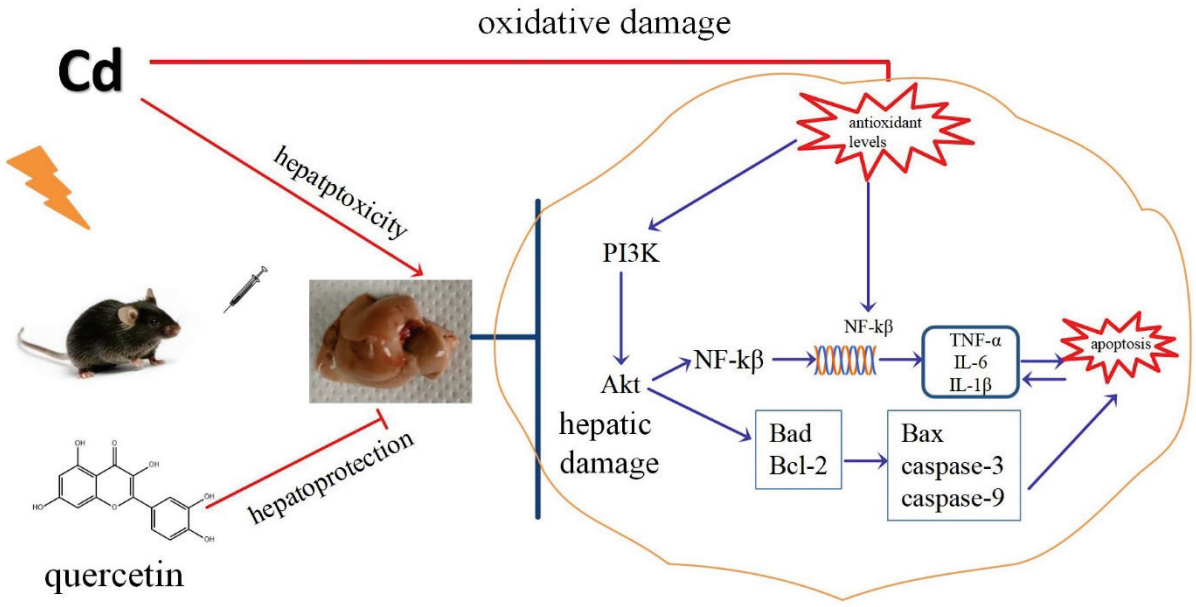

Fig. 9. Schematic representation of hepatoprotective mechanism of quercetin in Cd-induced liver injury. As depicted, quercetin inhibited the PI3K/Akt/NF-κB signaling pathways by Cd-generated hepatic damage. The → indicates activation or induction, and ⊣ indicates inhibition or blockade.

Conflict of Interest

There is no conflict of interest.

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