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The potential hepatoprotective effect of royal jelly against cadmium chloride-induced hepatotoxicity in mice is mediated by suppression of oxidative stress and upregulation of Nrf2 expression

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ABSTRACT

This study was aimed at investigating the possible protective mechanism of royal jelly (RJ) against hepatotoxicity induced by cadmium chloride ($CdCl_2$). The study included four groups: the control group received saline (0.9% sodium chloride), $CdCl_2$ group received 6.5 mg/kg $CdCl_2$ for seven days, RJ group received 85 mg/kg standardized RJ containing 6% 10-hydroxy-2-decenoic acid equivalent to 250 mg crude RJ, and finally, the fourth group received RJ 2 h before $CdCl_2$ injection daily for 7 days. Oxidant/antioxidant markers of liver function estimation and histopathology were determined. The results revealed that RJ significantly ameliorated the hepatotoxic side effects of Cd. Furthermore, RJ inhibited oxidative stress, inflammation, and hepatic tissue injury; normalized enzymatic and nonenzymatic antioxidant molecules; and enhanced nuclear-related factor-2 (Nrf-2) expression. Our results provide new insights into the hepatoprotective property of RJ and revealed that RJ prevented hepatic injury, oxidative stress, and inflammation by upregulating Nrf2 and the anti-apoptotic protein Bcl-2. Hence, RJ can be used as a hepatoprotective agent against the toxic effects of $CdCl_2$.

1. Introduction

Increased industrial pollution due to a rapid growth in the human population has exponentially worsened environmental problems. Heavy metal ions are toxic chemicals released into the soil, water, and air, which then enter the food chain. Cadmium (Cd) is a heavy metal, which has no known biological role [1]. Cd exposure has increased the risk of harmful effects on human health, which may cause conditions such as renal failure, anemia, cancer, and bone fragility [2]. Cd is a cytotoxic, mutagenic, and carcinogenic heavy metal [3]. There is evidence of a relationship between liver cancer in humans and rodents and Cd exposure [4]. The exact underlying mechanisms of the adverse effects of Cd exposure are rather complex and have not yet been fully elucidated [5], with the most recognized modality being oxidative stress. Acute exposure to Cd enhances reactive oxygen species (ROS) production and contributes to robust oxidative stress. Inflammation is also a key marker for Cd-induced tissue damage [6].

The first approach to alleviate Cd intoxication is to facilitate Cd excretion by chelation therapy; however, side effects are unavoidable with this approach. Fortunately, there is another approach that can be used to alleviate the Cd-induced oxidative stress, *i.e.*, the use of antioxidants [7]. Royal jelly (RJ) is a honeybee product secreted from the mandibular and hypopharyngeal glands of *Apis mellifera* (nurse bees). This secretion is produced from sap and other plant juices and is a valuable source of antioxidants [8]. RJ, which is produced by the incomplete digestion of honeydew in the stomach of worker bees, is essential for the development of the queen bee [9]. RJ is a blend of glucose, lipid, protein, minerals, vitamins [10], aspartic acid, phosphorous compounds, sterols, gel, nucleic acids, several trace ingredients, and acetylcholine, which are crucial for the nutritional and healing properties of RJ [11]. The composition of RJ is 10% monosaccharides, 12% raw protein, and 65% water. It also contains an ether-soluble fraction of fatty acids [12]. RJ has a wide range of pharmacological applications including use as an immune-stimulant [13], a potent antioxidant [10], and a hepatoprotective agent [14]. Furthermore, RJ has antitumor, hypoglycemic, antibacterial, antihyperlipidemic, and anti-inflammatory properties [15].

The aim of this study was to evaluate the protective effect of RJ

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administration on male mice with liver injury, oxidative stress, and inflammation due to Cd exposure.

2. Material and methods

2.1. Chemical and materials

Capsulated pure royal jelly was purchased from Pharco pharmaceuticals Co. Egypt, contains 6% 10-HDA. Whereas, $CdCl_2$ was supplied by Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used for the experiments were of analytical grade.

2.2. Grouping of animals

Twenty-eight male adult Swiss mice weighing 22–27 g were acquired from the Egyptian Organization for Biological Products and Vaccine. Seven mice were accommodated per cage; the animals were given a free access to water *ad libitum* and a commercial diet. The mice were housed in the animal facility of the Zoology Department at Helwan University (Cairo, Egypt) under the following conditions: a temperature of 22–25 °C with a 12-h artificial light/12-h dark cycle. The animals were treated per criteria of Investigations & Ethics for Laboratory Animal Care at Department of Zoology, Faculty of Science, Helwan University (approval no, HU2017/Z/03). One week after acclimatization, the mice were randomly divided into four equal groups of seven mice each.

Group I was the control group; the animals in the group were intraperitoneally (i.p.) injected with 0.9% NaCl (physiological saline) on a daily basis for 7 days. Group II was i.p. administered with 6.5 mg/kg $CdCl_2$ dissolved in physiological saline daily for 7 days. Group III received RJ (85 mg/kg equivalent to 250 mg crude RJ dissolved in saline) by oral gavage for 7 days. Group IV was pre-administered with 85 mg/kg RJ 2 h before the injection of 6.5 mg/kg $CdCl_2$ i.p. daily for 7 days. Twenty-four hours after the last treatment, the mice were killed and the liver was quickly removed, weighed, and directly homogenized in ice-cold 10 mM phosphate buffer (pH 7.4) to prepare a 10% (w/v) homogenate for a biochemical investigation.

2.3. Body weight

The final body weight of the mice was measured as described previously [9].

2.4. Determination of Cd concentration

Cd concentration was measured according to the method described by Jihen et al. [16]. Liver tissue was weighed and dried in an oven for 4 h at 250 °C. Then, the dried samples were digested with 2 M nitric acid and 2 M hydrochloric acid for 5 h at 150 °C. The samples were diluted with deionized water to 50 ml. Cd concentrations were determined by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer 3100) at 283.3 nm. The Cd concentrations are expressed as μg/g of wet liver tissue.

2.5. Biochemical parameters

2.5.1. Liver functions tests

The activities of alanine transaminase (ALT) and aspartate transaminase (AST) enzymes were determined with a colorimetric method [17]. Total bilirubin (TB) in serum was assessed per the method described by Schmidt and Eisenburg [18].

2.5.2. Determination of malondialdehyde and nitrite/nitrate

Malondialdehyde (MDA) was analyzed per the technique described by Ohkawa et al. [19]. MDA was determined using 1 ml of 0.67% thiobarbituric acid and 1 ml of 10% trichloroacetic acid and then heated in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by absorbance at 535 nm, and then expressed in terms of MDA formed. The amount of nitrite/nitrate [nitric oxide (NO)] was assessed using the method described by Green et al. [20]. In this method, sulfanilic acid is converted to a diazonium salt by reaction with nitrite in an acid medium. The formed salt is then coupled to N-(1-naphthyl)ethylenediamine forming an azo dye. The absorbance of the resultant azo dye with its bright reddish-purple color was read at 540 nm.

2.5.3. Enzymatic and nonenzymatic antioxidant molecules

Catalase (CAT) activity was determined per the method described by Luck [21]. Superoxide dismutase (SOD) activity was verified according to the method described by Sun et al. [22]. Glutathione reductase (GSH-R) activity was determined according to the techniques described by Factor et al. [23], wherein the capability of the enzyme to catalyze the oxidation of glutathione in the presence of NADPH was measured indirectly. The associated decline in absorbance was measured at 340 nm. Glutathione peroxidase (GSH-Px) activity was measured indirectly based on the ability of the enzyme to oxidize GSH into oxidized GSH (GSSG) [24]. The GSSG was recycled again to its reduced state by GSH-R in the presence of NADPH, the disappearance of which was measured at 340 nm, while glutathione (GSH) was assayed using the method of Ellman [25].

2.5.4. Determination of inflammation markers interleukin-1ß and TNF-α level

Quantitative measurements of IL-1ß (IL-1β; Cat. no. EM2IL1B, ThermoFisher Scientific) and TNF-α (TNF-α; Cat. no. EZMTNFA, Millipore) levels were performed using enzyme-linked immunosorbent assay (ELISA) kits specified for mice according to the protocol provided with each kit.

2.5.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was separated from liver tissues by using RNeasy Plus Minikit (Qiagen, Valencia, CA). cDNA synthesis was performed using the Script™ cDNA synthesis kit (Bio-Rad, CA). Real-time PCR was performed using Power SYBR® Green (Life Technologies, CA) on an Applied Biosystems 7500 Instrument. The thermal profile for the PCR reaction was 95 °C for 4 min, followed by 40 cycles at 94 °C for 60 s and 55 °C for 60 s. After PCR amplification, the $^{\Delta}$Ct was determined. PCR primers for SOD, CAT, GSH-P × 1, GSH-R, Bax, Bcl-2, Cas-3, iNOS, IL-1β, TNF-α and Nrf2 genes were prepared by Jena Bioscience GmbH (Jena, Germany) by using the Primer-Blast program from NCBI. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was employed as an internal control and appeared to be unchanged by the different therapies. The primer pairs used are listed in Table 1.

2.6. Histopathological examination

Liver tissue samples were fixed for 24 h at 25 °C in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned (4–5 μm). The deparaffinized sections were stained consistently with hematoxylin and eosin for light microscopy. Images were recorded at an original magnification of 400× (Nikon Eclipse E200-LED, Tokyo, Japan).

2.7. Immunohistochemistry analysis

To investigate apoptosis-related proteins, the prepared liver sections (4-μm thickness) were blocked with 0.1% hydrogen peroxide containing methanol for 15 min to damage the endogenous peroxidase. After blocking, the sections were incubated with a rabbit polyclonal Bcl-2, Bax, or caspases-3 antibody at 4 °C overnight. Thereafter, the

Table 1
Primer sequences of genes analyzed in real time PCR.

Name	Accession number	Forward primer (5'—3')	Reverse primer (5'—3')
GAPDH	NM_001289726.1	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA
SOD2	NM_013671.3	GCCCAAACCTATCGTGTCCA	AGGGAACCCTAAATGCTGCC
CAT	NM_009804.2	CCGACCAGGGCATCAAAA	GAGGCCATAATCCGGATCTTC
GSH-Px1	NM_001329527.1	CAGCCGGAAAGAAAGCGATG	TTGCCATTCTGGTGTCCGAA
GSH-R	NM_010344.4	TGGCACTTGCGTGAATGTTG	CGAATGTTGCATAGCCGTGG
Nrf2	NM_010902.4	CCTCTGTCACCAGCTCAAGG	TTCTGGGCGGCGACTTTATT
iNOS	NM_001313922.1	CGAAACGCTTCACTTCCAA	TGAGCCTATATTGCTGTGGCT
IL-1β	NM_008361.4	TGCCACCTTTTGACAGTGATG	TTCTTGTGACCCTGAGCGAC
TNF-α	NM_013693.3	AGAGGCACTCCCCCAAAAGA	CGATCACCCCGAAGTTCAGT
Bcl-2	NM_009741.5	GACAGAAGATCATGCCGTCC	GGTACCAATGGCACTTCAAG
Bax	NM_007527.3	CTGAGCTGACCTTGGAGC	GACTCCAGCCACAAAGATG
Caspase 3	NM_001284409.1	GAGCTTGGAACGGTACGCTA	CCGTACCAGAGCGAGATGAC

The abbreviations of the genes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SOD2, superoxide dismutase 2 mitochondrial (MnSOD); CAT, catalase; GSH-P × 1, glutathione peroxidase 1; GSH-R, glutathione reductase; Nrf2, nuclear factor erythroid 2-related factor 2; iNOS, inducible nitric oxide synthase; IL-1β: Interleukin 1 beta; TNF-α: Tumor necrosis factor; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-like protein 4.

sections were rinsed with phosphate-buffered saline and incubated with biotinylated goat anti-rabbit immunoglobulins, followed by incubation with streptavidin–peroxidase complexes at 30 °C for 30 min for the third time. The peroxidase activity was developed using diaminobenzidine (DAB)-hydrogen peroxide. Images were recorded at an original magnification of 400× (Nikon Eclipse E200-LED, Tokyo, Japan).

Regarding the immunohistochemical sections, the color intensity for each protein was evaluated in a semi-quantitative fashion. The intensity was categorized as + (weak immunoreactivity), + + (moderate immunoreactivity), + + + (strong immunoreactivity), and + + + + (very strong immunoreactivity).

2.8. Statistical analysis

Data are presented as the mean ± standard error of mean values. Data from various evaluations were investigated by one-way analysis of variance and Tukey's post hoc test using a statistical package program (SPSS version 17.0); p values < 0.05 were assumed statistically significant.

3. Results

The final weights of animals injected with $CdCl_2$ for 7 days decreased compared to those in the control group (Fig. 1). Furthermore, the final weights of the RJ-pretreated animals exposed to $CdCl_2$ were greater than those of the $CdCl_2$-injected animals without RJ pretreatment. The animals treated with RJ alone showed the same effect on weight as those shown by the animals in the control group.

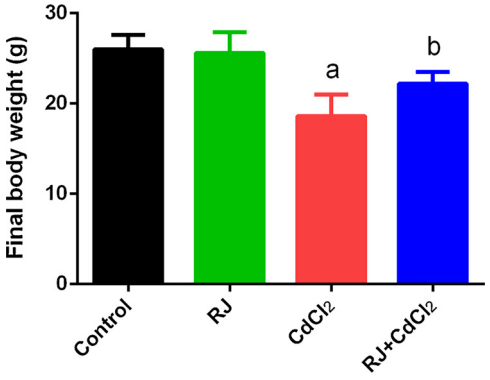

Fig. 1. Effects of royal jelly (RJ) on body weight in mice treated with cadmium chloride ($CdCl_2$).
Data are expressed as mean ± SD values (n = 7). $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

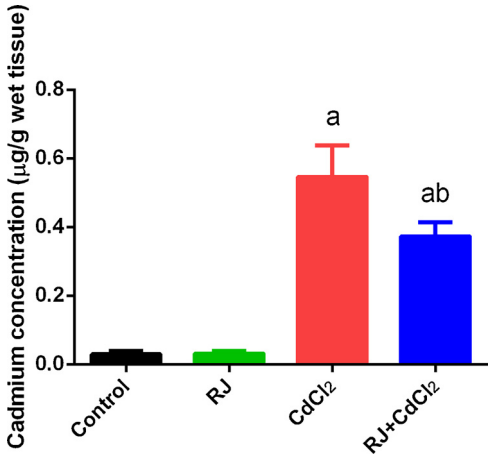

Fig. 2. Effects of royal jelly (RJ) on the cadmium concentration in the liver of mice treated with cadmium chloride ($CdCl_2$).
Data are expressed as mean ± SD values (n = 7). $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

Cd concentrations by groups are shown in Fig. 2. Compared to the control group, the group treated with $CdCl_2$ alone and the RJ + $CdCl_2$ group showed higher concentrations of Cd in the liver tissue. The Cd concentrations in the RJ group were the same as those in the control group. RJ pretreatment affected Cd concentrations in the animals treated with $CdCl_2$, decreasing the Cd concentration compare to that in the $CdCl_2$-injected animals without RJ pretreatment.

As shown in Fig. 3, RJ did not significantly affect liver enzyme (ALT and AST) function and TB. In contrast, AST, ALT, and TB levels significantly increased in mice that were administered $CdCl_2$ alone and in the Cd-injected mice with RJ pretreatment compared to the corresponding levels in the control group. The Cd-injected mice with RJ pretreatment showed significantly decreased AST, ALT, and TB levels compared to the corresponding levels in the $CdCl_2$-injected mice.

Fig. 4 shows increased MDA and NO levels in the $CdCl_2$ and RJ + $CdCl_2$ compared to the corresponding levels in the control group. $CdCl_2$-exposed animals treated with RJ showed significantly decreased ($p < 0.05$) MDA and NO levels in the animals' liver tissue compared to other Cd-exposed animals that were not administered any other treatment. Compared to the control group, the animals treated with RJ showed no significant effects. These results indicate that RJ has antioxidant properties. Furthermore, a change in GSH content was observed in the $CdCl_2$-intoxicated mice wherein the GSH content in hepatic tissue depleted significantly compared to that in the normal control group. Pretreatment with RJ significantly ($p < 0.05$) restored the content of

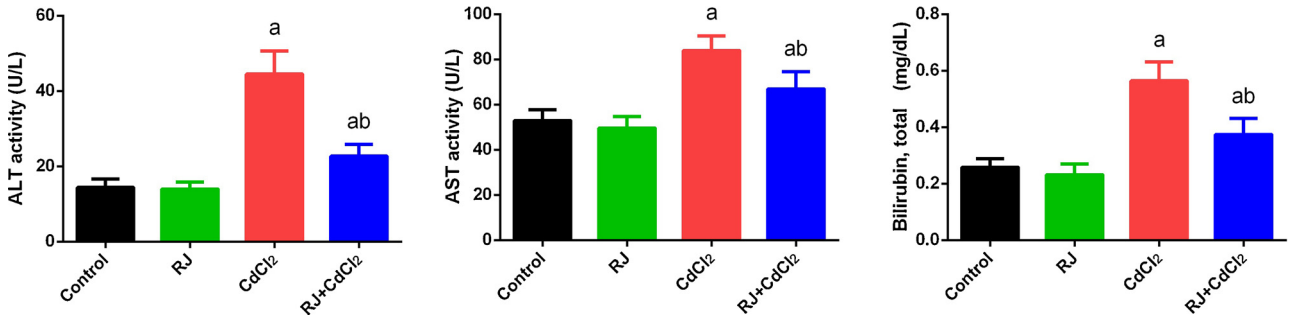

Fig. 3. Effects of royal jelly (RJ) on the liver function parameters alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin in the plasma of mice treated with cadmium chloride ($CdCl_2$).
Data are expressed as mean ± SD values (n = 7). $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

GSH to near normal levels compared to that in the Cd-treated mice.

$CdCl_2$ significantly reduced the levels of enzymatic antioxidants (SOD, CAT, GSH-Px, and GSH-R) compare to the corresponding levels in the control group, whereas pretreatment with RJ and $CdCl_2$ treatment significantly enhanced the activity of the enzymatic antioxidants compared to that in the $CdCl_2$ group. The group that was administered RJ alone showed no significant change in the activity of SOD, CAT, GSH-Px, and GSH-R compare to the normal group (Fig. 5).

qRT-PCR assays showed that superoxide dismutase mitochondrial (MnSOD), CAT, GSH-P × 1, and GSH-R gene expression (Fig. 6) downregulated in Cd group compared to that in the control group. On the other hand, the expression of the same genes increased in the Cd with RJ group compared to that in the control group.

There was a substantial increase ($p < 0.05$) in the levels of IL-1β and TNF-α in Cd-treated mice compared to that in the control mice; however, pretreatment with RJ prompted a significant reduction in the levels of those markers (Fig. 7). Consistent with the results obtained by ELISA method, the qRT-PCR results revealed that the mRNA expression of TNF-α and IL-1β was markedly upregulated in the liver of the $CdCl_2$-treated mice. However, RJ pretreatment downregulated the expression of these genes. Furthermore, $CdCl_2$ injection upregulated inducible nitric oxide synthase (iNOS) expression in hepatic tissue, indicating that Cd promoted NO production in liver tissue. Interestingly, RJ pretreatment effectively downregulated the expression of iNOS induced by the Cd treatment.

To further investigate the potential hepatoprotective roles of RJ against Cd-induced oxidative stress and inflammation, we determined Nrf2 expression in hepatic tissue. The results show that the Nrf2 mRNA expression in hepatic tissues significantly downregulated after Cd intoxication compared to that in the control mice (Fig. 8). However, RJ pretreatment significantly upregulated Nrf2 expression in the Cd-treated mice.

As shown in Fig. 9, the hepatic tissue of the control mice and mice treated with RJ alone showed a normal liver architecture with a normal lobular pattern. The hepatocytes showed well-defined central nuclei and abundant cytoplasm. An examination of the liver tissue of the Cd-treated mice revealed congestion and dilation of the central vein while the portal area was expanded due to bile duct proliferation and edema. Furthermore, inflammatory aggregates were observed, while pretreatment with RJ limited the pathological changes caused by Cd exposure in this group.

Immune activity of Bax and caspase-3 in the liver of the $CdCl_2$-treated animals was stronger than that in the control animals (Fig. 10), whereas the immune activity of Bcl-2 was lower than that in the control animals. RJ pretreatment modified apoptosis-related proteins, in that Bcl-2 immunoreactivity increased while that of Bax and caspase-3 decreased. Consistent with the immunohistochemistry results, qRT-PCR findings revealed that the mRNA expression of Bax and caspase 3 significantly upregulated in the liver of the $CdCl_2$-injected mice while that of Bcl-2 was downregulated. However, RJ pretreatment prevented Cd-induced apoptosis in the liver (Fig. 11).

4. Discussion

Cd is a highly poisonous metal and an environmental toxin with an escalating associated risk of cancer. It is transported through the blood and distributed widely in the body, with predominant accumulation and hence toxicity in the kidney, reproductive organs, lungs, immune system, liver, and bones [26]. Previous studies have reported that inflammation and oxidative stress induced by Cd cause toxicity in these tissues [5]. Core procedures of Cd-induced cell lesions which contains number of aspects such as genotoxic harm [27], oxidative damage [5], and interference with metabolism and the levels of essential trace elements [28].

Cd that enters the body combines with metallothionein and then accumulates in soft tissue along with solid organs, specifically the liver,

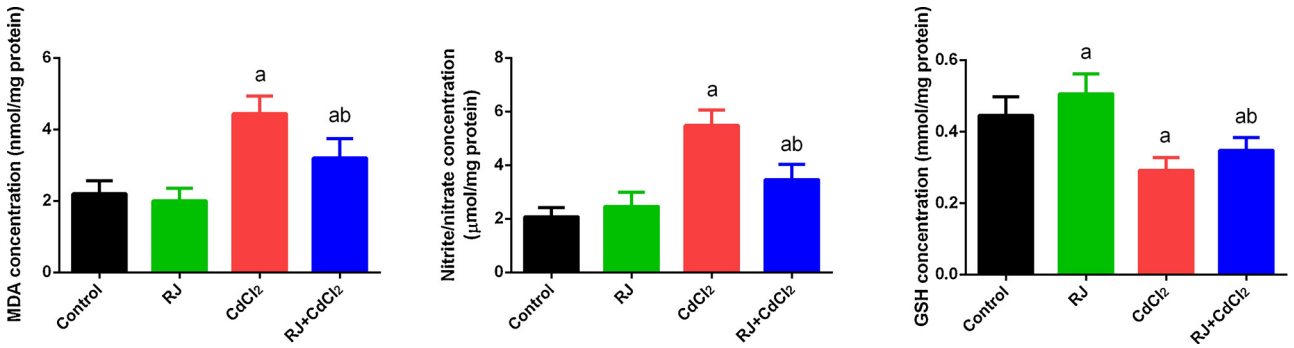

Fig. 4. Effects of royal jelly (RJ) on malondialdehyde (MDA) and nitric oxide (NO) levels and glutathione (GSH) content in the liver of mice treated with cadmium chloride ($CdCl_2$).
Data are expressed as mean ± SD values (n = 7). $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

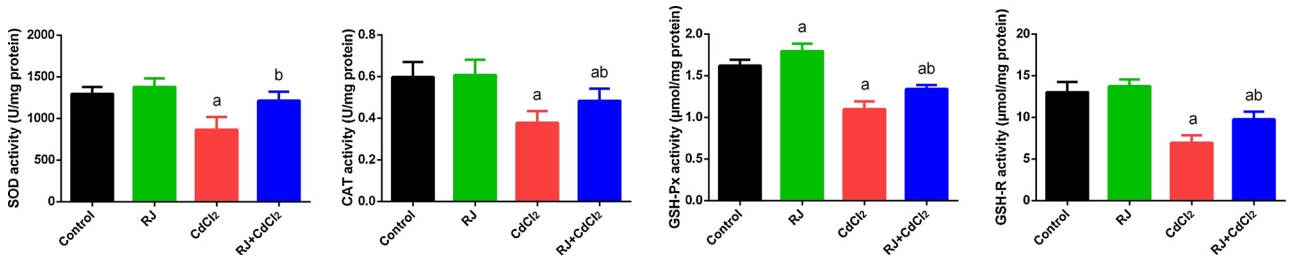

Fig. 5. Effects of royal jelly (RJ) on the enzyme activity of superoxide dismutase (SOD) catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-R) in the liver of mice treated with cadmium chloride ($CdCl_2$).
Data are expressed as mean ± SD values (n = 7). $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

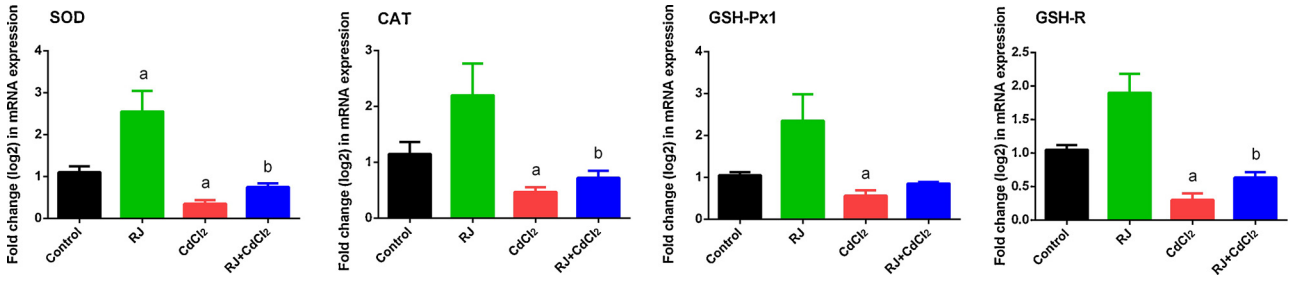

Fig. 6. Effects of royal jelly (RJ) on gene expression of superoxide dismutase mitochondrial (MnSOD), catalase (CAT), glutathione peroxidase 1 (GSH-P × 1), and glutathione reductase (GSH-R) in the liver of mice treated with cadmium chloride ($CdCl_2$).
Results (means ± SD values from triplicate assays) were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold induction (log2 scale), relative to the mRNA level in the control. $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

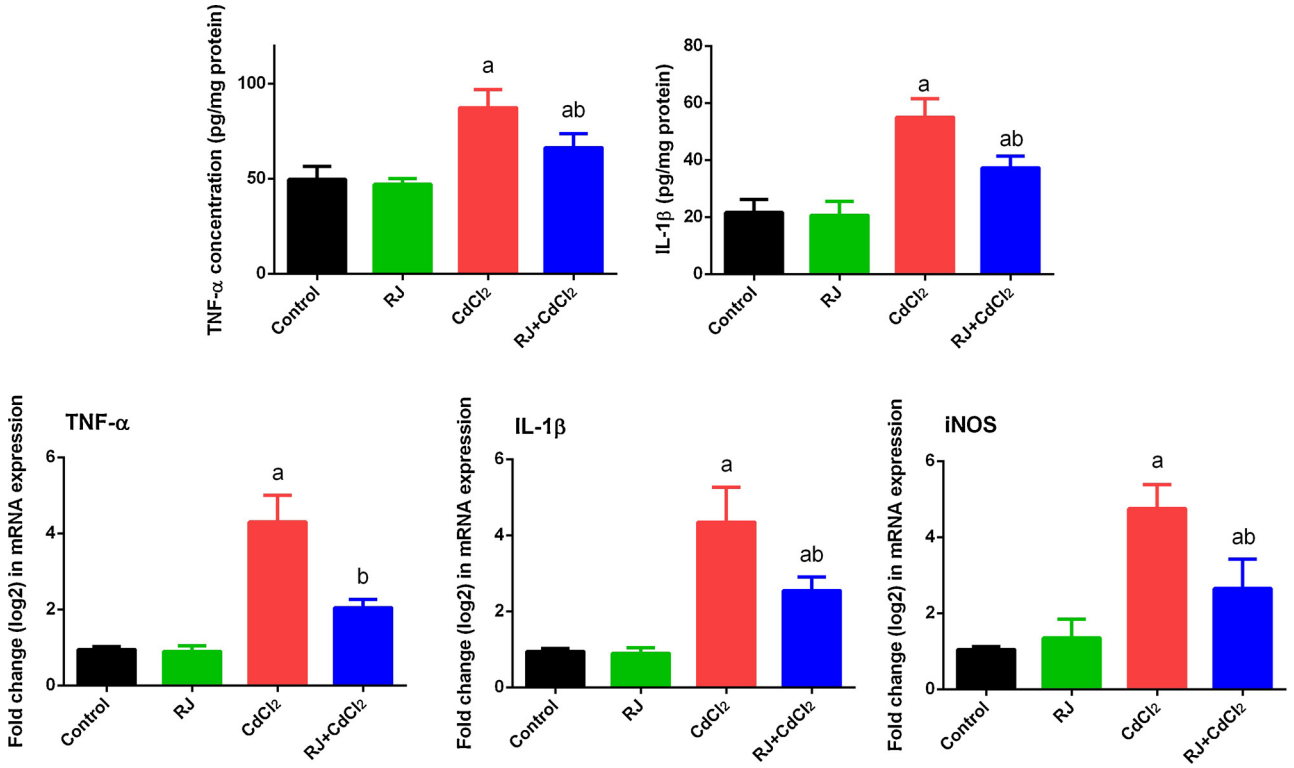

Fig. 7. Effects of royal jelly (RJ) on the levels of tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) and on the expression of TNF-α, IL-1β, and inducible nitric synthase (iNOS) in the liver of mice treated with cadmium chloride ($CdCl_2$).
Data of ELISA findings are expressed as the mean ± SD of 7 mice, whereas data of mRNA expression (mean ± SD of triplicate assays) were normalized to those of GAPDH and expressed as fold induction (log 2 scale), relative to the mRNA level in the control. $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

which is the primary organ affected by exposure to many poisonous chemicals, as it is the organ responsible for the metabolism of external toxic ingredients [29]. ALT is a specific enzyme marker of liver damage [14]. In the present study, administration of Cd led to a sharp increase in ALT levels. Glucoprotein is a 57-KDa glycoprotein found in RJ; it plays an important role in stimulating liver regeneration and

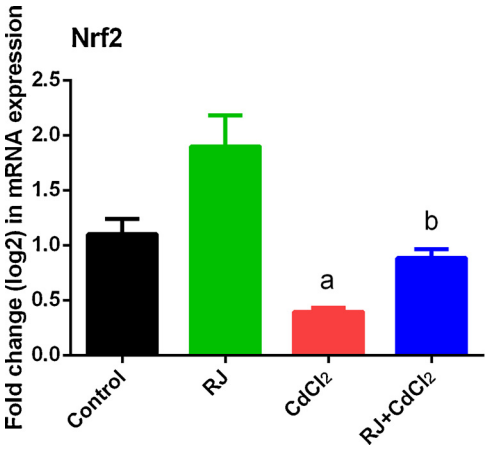

Fig. 8. Effects of royal jelly (RJ) on the gene expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in the liver tissue of mice treated with cadmium chloride ($CdCl_2$).

Results (means ± SD values from triplicate assays) were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold induction (log 2 scale), relative to the mRNA level in the control. $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

hepatocyte development. Studies have shown that RJ has a strong hepatoprotective effect against chemicals that lead to liver damage [14,30]. The hepatoprotective effects of RJ observed in this study are consistent with those reported by Mahesh et al. [31] who confirmed that honey decreased the incidence of liver damage and oxidative stress.

Cd has toxic effects *via* the production of ROS through an imbalance in the antioxidant–oxidant status causing oxidation of biological molecules, such as proteins, lipids, and DNA [16]. Cd exposure induces acute hepatotoxicity, apoptosis, necrosis, inflammatory infiltration, and peliosis [32]. It also causes DNA–protein crosslinks, DNA single-strand breaks, and chromosome aberrations (CAs) [33]. Free radicals trigger the development of many diseases and cause harmful effects that cause peroxidation of biomembranes and DNA, which is the reason for tissue destruction. Antioxidants may prevent the body from several diseases caused by the destructive effects the free radicals [34]. Antioxidants are radical scavengers; they have the ability to inhibit lipid peroxidation and other free radicals that facilitate the progression of many diseases [35]. Previous studies showed that RJ proteins after the hydrolysis using protease P have antioxidant activity against lipid peroxidation through scavenging hydroxyl radicals [36] and the authors attributed this activity to three dipeptides containing tyrosine residues at their C-terminal. Furthermore, Kanbur et al. [37] found that the antioxidant activity of RJ not only due to the hydroxyl radicals-scavenging activity but also due to the indirect effect of RJ based on the suppression of enzymes that enhance the peroxidation of endogenous lipids as well as cytochrome P450 expression, which is one of the intracellular source of oxygen radicals.

Reduced GSH is a cofactor of GPx and the most abundant antioxidant in mammalian cells, which are affected by Cd toxicity. Cd has a high affinity for sulfhydryl groups and can decrease the levels of SOD, GSH, GSH-Px, and CAT in soft tissues [38]. However, pretreatment with RJ minimizes this effect. RJ is a substantially effective antioxidant, has free radical-scavenging activity [39], and is used for reducing the toxic effects of chemical agents [40]. RJ markedly increased GSH and reduced MDA levels. In a previous study, rats administered RJ showed a decrease in MDA levels and an increase in GSH-Px and SOD activity [40]. The free amino acids namely, proline, cysteine and cystine of RJ might responsible for this effect by enhancing glutathione biosynthesis and scavenging free radicals [41]. Furthermore, the previous studies reported that RJ enhanced antioxidant enzyme activity in different models [36,42].

Redox-sensitive Nrf2 is a fundamental transcription factor controlling the cellular antioxidant guards and maintaining the redox homeostasis. In case of heavy metals [43], and few antioxidants [44], Nrf2 transforms in the nucleus, and controls ARE genes, which protect the cells from oxidative stress [45]. The overexpression of Nrf2 inhibits apoptosis induced by Cd in some cells due to the high sensitivity of Nrf2 to Cd [46]. Interestingly, RJ protected against Cd-induced liver toxicity by inducing Nrf2 expression. The effect of RJ on Nrf2 was confirmed by Inoue et al. [47] they found that 4-hydroperoxy-2-decenoic acid ethyl ester (HPO-DAEE), a fatty acid derivative in RJ, increased the expression of heme oxygenase-1 (HO-1) mRNA, an antioxidant enzyme, through increasing the translocation of Nrf2 to bind to ARE (antioxidant response element). AREt subsequently activates the transcription of genes encoding many of antioxidative and electrophile detoxification enzymes.

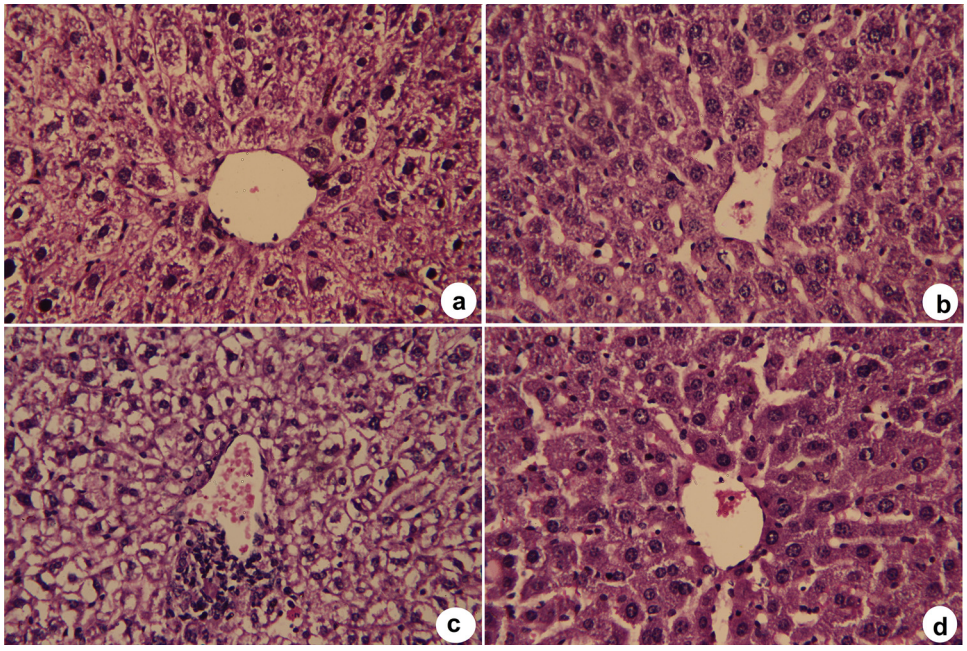

Fig. 9. Photomicrographs of mouse liver. Liver tissues from the control and royal jelly (RJ)-treated groups (a and b, respectively) showing a healthy architecture. Tissues from the cadmium chloride ($CdCl_2$)-treated mice (**c**) showed severe inflammation and apoptosis. Pretreatment with RJ (d) markedly reduced all hepatocellular damage caused by cadmium. Hematoxylin and eosin staining, magnification ×400.

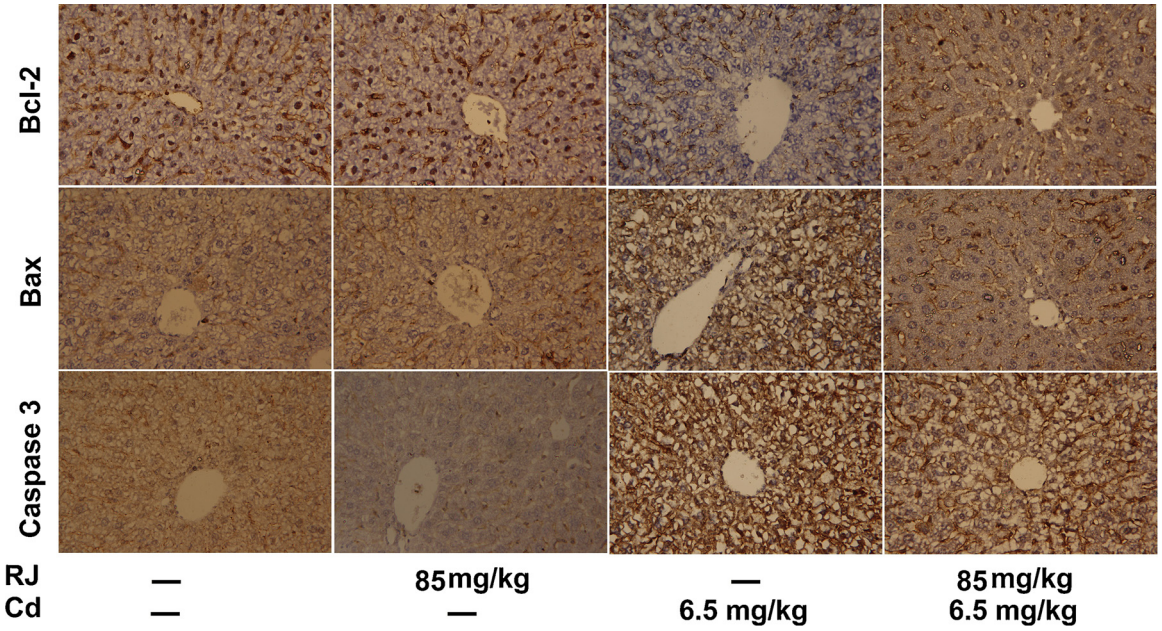

Fig. 10. Photomicrographs showing changes in Bcl-2, Bax, and caspase-3 expression in the liver tissue of mice following treatment with royal jelly (RJ) and cadmium chloride ($CdCl_2$). (magnification, 400×).

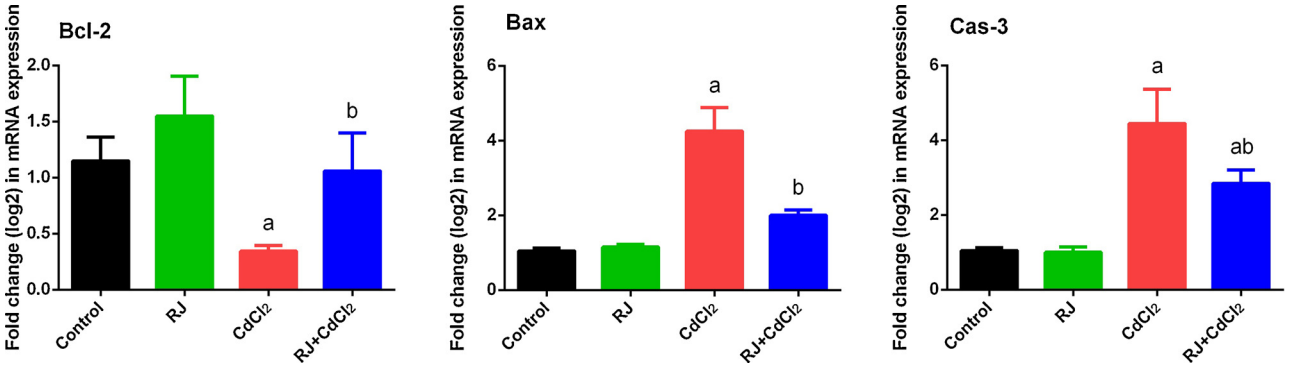

Fig. 11. Effects of royal jelly (RJ) on the expression of Bcl-2, Bax, and caspase-3 in the liver of mice treated with cadmium chloride ($CdCl_2$). Results (means ± SD values from triplicate assays) were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold induction (log 2 scale), relative to the mRNA level in the control. $^{a}p < 0.05$ *vs.* the control mice; $^{b}p < 0.05$ *vs.* the $CdCl_2$-treated mice, using the Tukey's post hoc test.

The resident macrophages of the liver, called Kupffer cells, play an important role in the mechanism of Cd-induced hepatotoxicity [48]. Kupffer cells express iNOS during stress or inflammation [49], which explains the overexpression of iNOS in the hepatic tissue of $CdCl_2$-treated mice. Furthermore, Cd exposure in the present study induced an increase in the level of IL-1ß, consistent with the finding reported by Wang et al. [44] who studied the level of secreted interleukin-1ß in the liver and kidney tissue following Cd exposure. However, RJ pretreatment resulted in a significant reversal of Cd-induced inflammation in the liver tissue. Our results in accordance with the earlier report of Aslan et al. [50]. The authors found that RJ supplementation prevented renal inflammation in rats induced by ethylene glycol. Chen et al. [51] also found that trans-10-hydroxy-2-decenoic acid (10-H2DA), 10-hydroxydecanoic acid (10-HDAA), and sebacic acid (SEA), three fatty acids in RJ, reduced inflammatory responses by suppressing mRNA expression of pro-inflammatory cytokine genes through attenuating NF-κB (nuclear factor-kappa B) translocation.

In the present study, we observed that Cd intoxication induced apoptosis by inducing caspases-3 and Bax and inhibiting Bcl-2 expression. The activation of caspase-3 occurs through the release of cytochrome c into the cytosol from the mitochondria, which might lead to apoptosis [52]. Bcl-2 family proteins play a role in mitochondrial-dependent apoptotic pathways. Bcl-2 is anti-apoptotic protein that defends cells from apoptosis while pro-apoptotic proteins such as Bax enhance apoptosis [53]. Moreover, apoptosis induced by Cd has been confirmed to be correlated to oxidative stress in various types of cells [54]. Interestingly, RJ pretreatment inhibited significantly apoptosis. Amiri et al. [55] found that RJ supplementation increased Bcl-2 expression and decreased Bax expression and the authors suggested that this improvement might be due to antioxidant activity and the free radicals scavenging ability of RJ. Furthermore, the anti-aging effect of RJ is associated with restrained ROS formation that in turn prevents the oxidative damage of the cell and improves the apoptosis-related genes [56].

5. Conclusion

RJ used in this study was useful in improving the hepatotoxicity induced by $CdCl_2$, and the results of this study will contribute vital knowledge, which may help successfully overcome the side effects of $CdCl_2$ exposure.

Conflicts of interest

The authors have no conflicts of interest to declare.

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