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Original Article

Royal jelly protects dichlorvos liver-induced injury in male Wistar rats

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Abstract

Background and purpose: Dichlorvos, an organophosphate insecticide, induces side effects on normal tissues. On the other hand, Royal jelly (RJ) with antioxidant activities has many medical benefits including liver toxicity. In this study, we investigated the role of RJ in improving dichlorvos adverse impact on the liver of male rats.

Experimental approach: Forty-eight male rats were randomly divided into 8 groups (n = 6); receiving by gavage normal saline (0.09%), dichlorvos (4 mg/kg/day), RJ (50, 100, 150 mg/kg/day; RJ 1, 2, 3) or dichlorvos + RJs, daily for 28 consecutive days. At the end of experiments, histopathology alterations, apoptosis induction, and biochemical factors related to the liver were evaluated.

Findings/Results: There was a significant reduction in the number of hepatocytes and total antioxidant capacity (TAC) levels in the dichlorvos group compared to the control group, whereas these parameters in the dichlorvos + RJs groups, were significantly increased compared to the dichlorvos group. Central vein diameter, liver enzymes (aspartate transaminase, alanine transaminase, and alkaline phosphatase) serum levels of nitric oxide, and apoptotic index were significantly higher in the dichlorvos group than in the control, while these parameters were decreased in the dichlorvos + RJs groups versus the dichlorvos group.

Conclusion and implications: RJ at 50 mg/kg protected dichlorvos-induced liver damage in rats. Dichlorvos-hepatitis mechanism could be oxidative induction as long as antioxidant reduction leads to apoptosis in this organ, while RJ due to its antioxidant potential suppresses this hazardous cellular and molecular process.

Keywords: Antioxidants; Apoptosis; Dichlorvos; Liver.

INTRODUCTION

Organophosphates which are used as pesticides are classified in the WHO class IB as highly hazardous chemicals (1). Two mechanisms involved in the poisonous effects of these compounds are inhibition of the cholinesterase enzymes, phosphorylation of the active site of the enzyme, and damage to cell membranes that leads to the production of free radicals. Also, they are involved in disrupting the antioxidant system (2,3). In this respect, the role of antioxidants, specifically natural ones become essential to relieving the destructive effects of such toxins.

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Among the wide majority of pesticides, dichlorvos (2, 2-dichloroethenyl dimethyl phosphate, $C_4H_7Cl_2O_4P$) is one of the most important organophosphate compounds, commonly used as an agricultural insecticide. It is metabolized in the liver and excreted by the kidneys (4). Dichlorvos is rapidly absorbed through the skin, gastrointestinal, and respiratory tracts and reduces the activity of antioxidant enzymes such as superoxide dismutase and glutathione S-transferase. Dichlorvos induces apoptosis through the mitochondrial pathway in the brain and liver tissues (5). Studies have shown that dichlorvos is a potential cause of cancer induction in workers (6-8).

The antioxidant activity of royal jelly (RJ), one of the products of the honey bee, prevents oxidative stress, lipid peroxidation, and protection of the DNA from oxidative damage. The results of studies showed that RJ is a highly potent antioxidant and acts as an anti-inflammatory and antimicrobial agent (9,10). The beneficial effects of RJ can be attributed to the antioxidant properties of its tri-D peptide (Tyr-Tyr, Arg-Tyr, Lys-Tyr). The antioxidant ability of these peptides is related to the properties of polyphenolic hydroxyl groups present in them. It is also due to subsequent inhibition of free radicals through the hydroxylation mechanism of these amino acids (11). Reports have shown that RJ administration reduces liver toxicity and oxidative stress caused by sumithion and malathion as organophosphate insecticides (12-14). The compounds produced by the enzymatic hydrolysis of the RJ have high antioxidant activity against a variety of active oxygen species from the superoxide anion radicals and hydroxyl radicals (15). Moreover, a novel investigation proved the existence of specific proteins in RJ targeting hepatitis. These two proteins are the major proteins of RJ 2 and its X1 isoform, which reduce liver damage and also improve hepatitis C and B by interfering with the replication of these two viruses (16,17).

Considering the antioxidant properties of RJ and the lack of adequate studies on the effects of RJ about its protective role in damage induced by pesticides like dichlorvos on body organs, in this study, we investigated the protective effect of RJ on dichlorvos-induced liver tissue damage in rats.

MATERIALS AND METHODS

Experimental design

Forty-eight male Wistar rats (230 ± 5 g) were used in the study and kept in the animal house of the institute at a temperature of 22 ± 2 °C under controlled environmental conditions with water and food *ad libitum*. The Ethical Committee of Kermanshah University of Medical Sciences approved the animal experiments based on the ethical guidelines of the WMA Declaration of Helsinki (Ethic No. IR.KUMS.REC.1397.593). The experiments were done following one-week housing for the adaptation of the animals. Then, the animals were divided into eight groups and received dichlorvos or normal saline at 10 AM and 1 h later for RJ. All treatments were given orally. The experiment was prolonged for 28 continuous days. The rats were randomly divided into 8 groups, 6 each, as follow: control group receiving normal saline; dichlorvos (18185, Sigma-Aldrich; US) group receiving dichlorvos at a dose of 4 mg/kg; groups 3-5 (RJ1-RJ3) receiving RJ (R0376. Sigma-Aldrich; US) at the doses of 50, 100, and 150 mg/kg, respectively (18); groups 6-8 (dichlorvos + RJ1-RJ3) exposed to both dichlorvos (4 mg/kg) and RJ at 50, 100, and 150 mg/kg, respectively.

Twenty-four h after the last treatments, the rats were then anesthetized with ketamine hydrochloride (K2753-5G, Sigma-Aldrich, US; 100 mg/kg) and xylazine hydrochloride (X1126-5G, Sigma-Aldrich, US; 10 mg/kg), and their livers were isolated. Blood was collected from the heart (nearly 5 mL) and 1^3 cm of the liver was isolated from the rats for further analysis.

The histological examination of liver tissues

The process of tissue preparation was based on the conventional histology method (paraffin-embedded method). Subsequently, five histological sections (5 μm thickness) with 4 sectional intervals were prepared from the paraffin-embedded block of each specimen by

tissue microtome. The sections were then stained by hematoxylin and eosin (H&E) dyes, then five pictures from each subject were captured by Olympus BX‑51T‑32E01 microscope linked to a DP12 camera with 3.34 million-pixel resolution and Olysia Bio-software (Olympus Optical; Japan). Quantitative analysis of the livers including counting the diameter of hepatocytes, and measuring the diameter of the central vein, was also performed. They were calculated by double-blinded observers on the 5 fields for each section to exclude any observing bias. The quantitative measurements were done with the help of a Motic camera with software (AE-3; Motic S.L.C. Barcelona, Spain) attached to a light microscope (×200) (AE-3; motic S.L.C. Barcelona, Spain) according to the randomized selected captured pictures.

Measuring the activity of liver enzymes in plasma

Serum aminotransferase activity including alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) was measured by special spectrophotometry in accordance with manufacturers' instruction of related kits (Pars Azmun, Tehran, Iran) (19).

Evaluating liver levels of nitric oxide

Griess reagent (sulfanilamide and N-1- naphthylethenediamine dihydrochloride; NEED) diluted in an acidic medium was adopted. Briefly, 6 mg of zinc sulfate was added to 400 µL of the homogenized liver (homogenate in a homogenizer added 0.25M sucrose solution (with 1mM EDTA) (10% W/V)) and the suspensions were transmitted to the separate wells. Subsequently, 100 µL chloride vanadium, 50 µL sulfonamide, and 50 µL NEED were added per 100 µL of the mixture. The wells were reserved in the dark environment at the temperature of 30 °C and the opacity or optical density of each sample was measured by an ELISA reader (450 ± 10 nm) (20).

Liver total antioxidant capacity measurement

Total antioxidant capacity (TAC) was evaluated using an acquisition kit (Cat No: TAC-96A) ZellBioGmbH-Germany, which was designed based on oxidation colorimetric resuscitation. In this assay, the TAC of homogenate liver was tantamount to some antioxidant in the sample that was compared with ascorbic acid as standard. Homogenizing was performed in homogenizer in accordance with 0.25 M sucrose solution (with 1mM EDTA) (10% W/V). The kit's sensitivity was 0.1 mM, and the diagnostic range was 0.125-2 mM, and the last absorbance read at 490 nm Wavelength, and unit conversion is done (21).

In situ detection of apoptosis

To detect apoptotic cells, the TUNEL assay was used according to the procedures included in the Apop Tag *in situ* apoptosis detection kit (Roche, Germany). Non-parenchymal tissues around the liver were removed, then this tissue was dissected and paraffin-embedded blocks were prepared using an automatic tissue processor. Then, 5 µm coronal histological thin sections were cut from paraffin-embedded blocks with the help of a microtome instrument (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany). Five sections of the animal were chosen. For the unification of the section selection, the first section was the 4^{th} and the last was the 24^{th} (5 sections interval).

Briefly, after routine deparaffinization and blocking of endogenous peroxidase with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature, incubation with 100 µg/mL proteinase K (Sigma, USA) was performed for 15 min at room temperature. Following prehybridization treatment, the sections were exposed to terminal deoxynucleotidyl transferase with digoxigenin-11-dUTP and dATP and incubated in a moist chamber for 60 min at 37 °C. Anti-digoxigenin-11-dUTP labeling for 30 min at room temperature was followed by exposure to 0.05% diaminobenzidine tetrahydrochloride (DAB). Counterstaining was achieved with 0.5% methyl green solution.

Photomicrographs of the apoptotic cells, methyl green-stained nuclei cells, were distinguished under a fluorescent microscope (400× magnification) and the apoptotic index

(AI) was measured using the following equation (22):

$$AI = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells counted}} \times 100$$

Statistical analysis

The data were presented as mean ± SEM and the normality of the samples was confirmed by the Kolmogorov Smirnov test. To compare the statistical differences between groups One-way ANOVA was performed followed by Tukey's test using SPSS software (version 16). $P < 0.05$ was considered statistically significant.

RESULTS

Histological characteristics of liver

The quantitative evaluations were done according to histological slides are presented in Fig. 1. The central venous diameter in the dichlorvos group was significantly increased compared to the control group. A significant increase in the central venous diameter was observed in dichlorvos + RJs groups compared to the control group. Despite the raised in central venous diameter in RJs intra-group, this difference was not statistically significant. The diameters of the central vein in the RJs and dichlorvos + RJs were decreased in comparison with the dichlorvos group (Fig. 2A). The diameter of hepatocytes in the dichlorvos and dichlorvos + RJ1 groups was significantly more than that in the control group. The rate of this parameter was significantly declined in dichlorvos + RJs compared to the dichlorvos group. There was no significant difference in this parameter by the increasing dose in dichlorvos + RJs (Fig. 2B).

Effects of RJ and dichlorvos on serum levels of liver enzymes

Figure 3A-C indicated that the serum levels of AST, ALT, and ALP were significantly increased in the dichlorvos group compared to the control group. Also, the ALT serum level in the dichlorvos + RJ1 group was significantly higher relative to the control and RJs. While there were no statistical differences for serum levels of AST and ALP in the dichlorvos + RJ groups compared to the control group. These three variables showed no significant differences in RJ groups compared to the control groups. There were no significant differences for RJs intra-groups comparison. Administration of RJ significantly decreased these three parameters compared to the dichlorvos group. Although the serum levels of AST, ALT, and ALP were reduced with increasing doses in the dichlorvos + RJ groups, the decrease was not statistically significant.

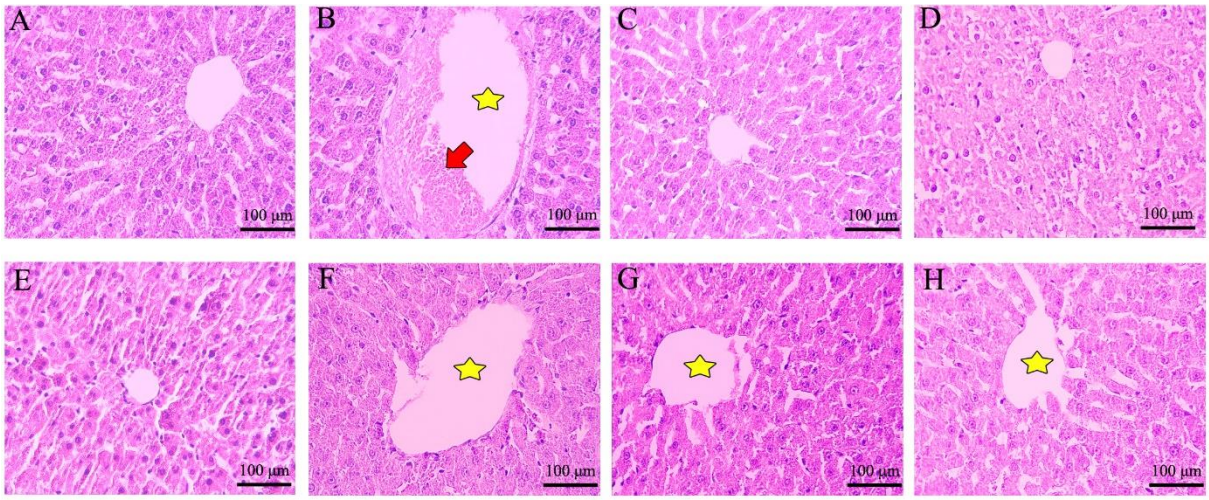

Fig. 1. Histopathologic sections of the liver tissue in control and experimental groups following administration of dichlorvos and different doses of RJ. (A) Control group, received normal saline 0.09%; (B) received dichlorvos at 4 mg/kg/day; (C-E) received royal jelly at 50, 100, and 150 mg/kg, respectively; (F-H) received dichlorvos at 4 mg/kg/day and royal gelly at 50, 100, and 150 mg/kg, respectively. The red arrow referred to the intra-venous red blood cells accumulation and the yellow star pointed to the venous dilatation. H & E, ×400.

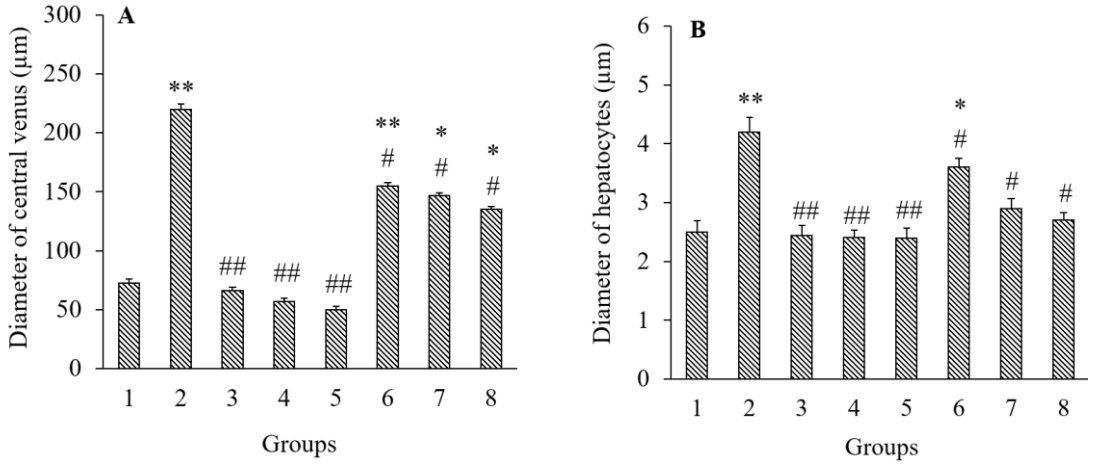

Fig. 2. The effect of dichlorvos and royal jelly on the liver tissue-related parameters in rats. Group 1 received normal saline 0.09% as a control; group 2 received dichlorvos at 4 mg/kg/day; groups 3-5 received royal jelly 50, 100, and 150 mg/kg, respectively; and groups 6-8 received dichlorvos at 4 mg/kg/day and royal jelly at 50, 100, 150 mg/kg, respectively. Data represent mean ± SEM. **P* < 0.05 and ***P* < 00.1indicate significant differences compared to the control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ relative to dichlorvos group.

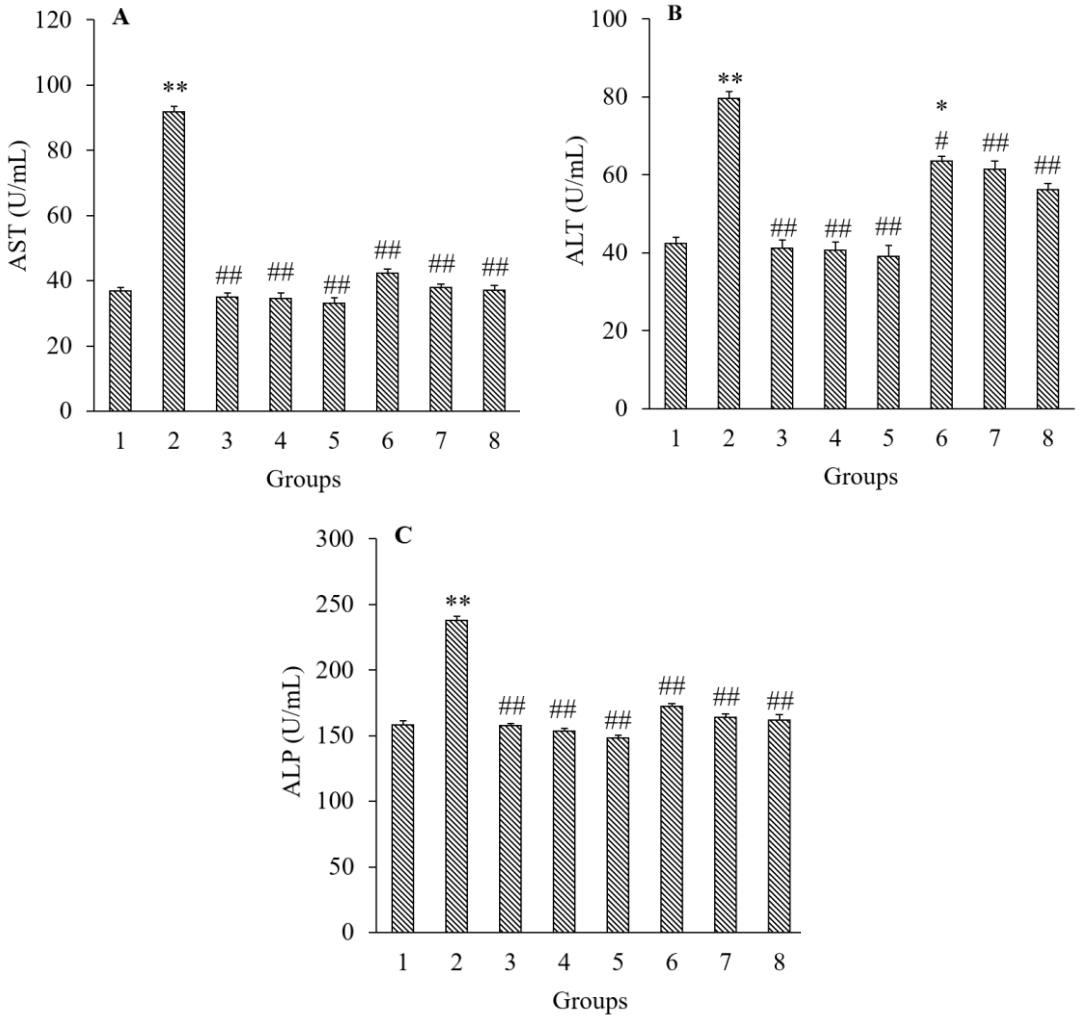

Fig. 3. The effects of dichlorvos and royal jelly on the liver enzymes in rats. Group 1 received normal saline 0.09% as a control; group 2 received dichlorvos at 4 mg/kg/day; groups 3-5 received royal jelly at 50, 100, and 150 mg/kg, respectively; and groups 6-8 received dichlorvos at 4 mg/kg/day and royal jelly at 50, 100, 150 mg/kg, respectively. Data represent mean ± SEM. **P* < 0.05 and ***P* < 00.1indicate significant differences compared to the control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ relative to dichlorvos group. AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase.

Effects of RJ and dichlorvos on nitric oxide in liver tissue of rat

Measurement of nitric oxide (NO) levels of liver tissue in the dichlorvos group showed a significant increase compared to the control group. In dichlorvos + RJ groups, there was a significant increase in this parameter compared to the control group. Although in RJ groups, the NO level was reduced in a dose-dependent manner, which was not statistically significant. NO levels were significantly declined in RJ-receiving groups compared to dichlorvos groups. Intra-group analysis between the dichlorvos + RJs determined no significant differences in NO liver levels (Fig. 4A).

Effects of RJ and dichlorvos on TAC in liver tissue of rat

There was a significant decrease in TAC levels in the dichlorvos and dichlorvos + RJ1 groups compared to the control group. Intra-group analysis between the RJ groups did not show any significant changes. A significant liver TAC increase occurred in RJs and dichlorvos + RJs compared to the dichlorvos group. TAC was increased dose-dependently in the dichlorvos + RJs groups (Fig. 4B).

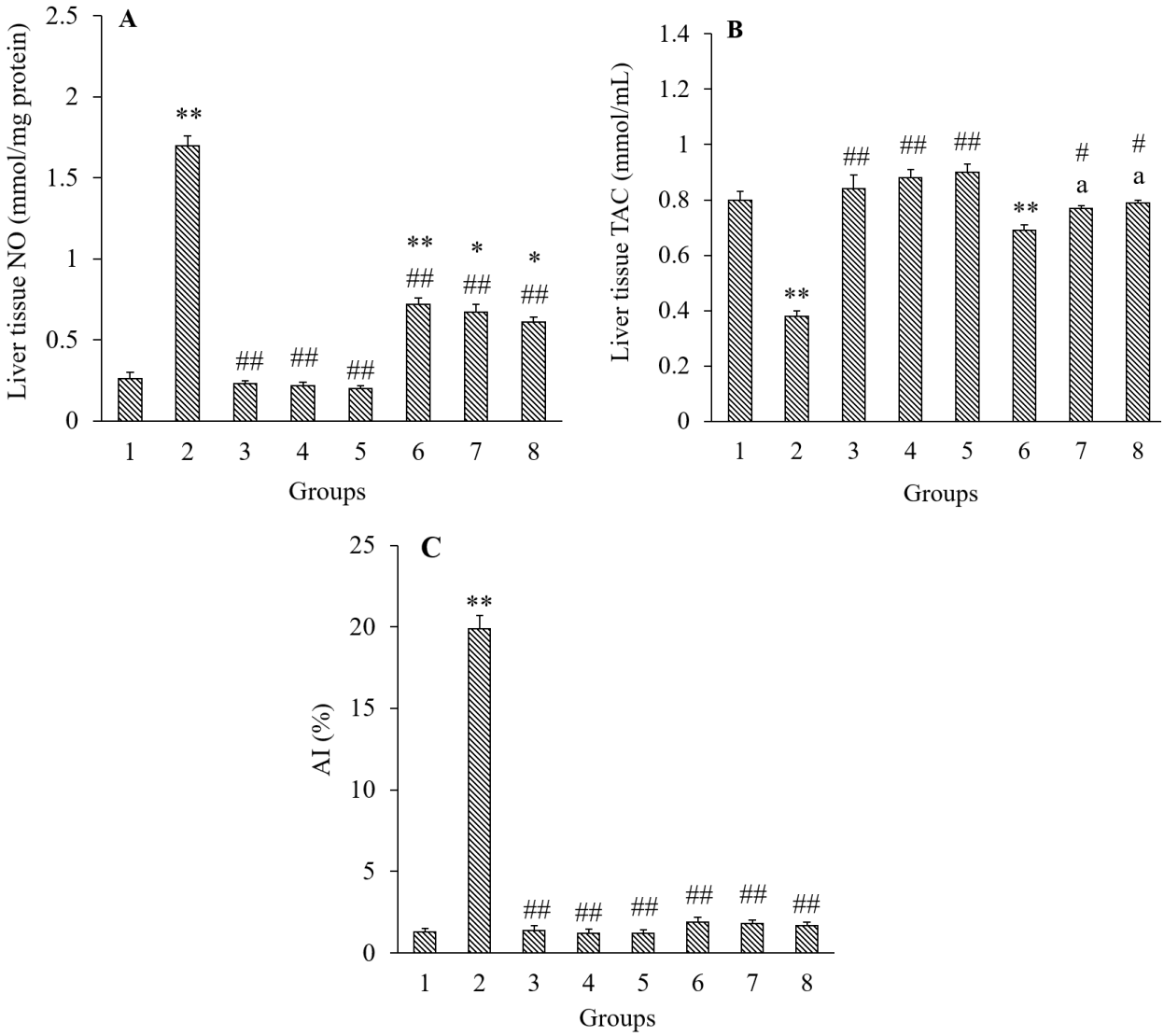

Fig. 4. The effect of dichlorvos and royal jelly on NO, TAC, and AI in rats. Group 1 received normal saline 0.09% as a control; group 2 received dichlorvos at 4 mg/kg/day; groups 3-5 received royal jelly at 50, 100, and 150 mg/kg, respectively; and groups 6-8 received dichlorvos at 4 mg/kg/day and royal jelly at 50, 100, 150 mg/kg, respectively. Data represent mean ± SEM. *$P < 0.05$ and **$P <$ 00.1indicate significant differences compared to the control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ relative to dichlorvos group; $^{a}P < 0.05$ versus dichlorvos + royal jelly at 50 mg/kg group. NO, Nitric oxide; TAC, total antioxidant capacity; AI, apoptotic index.

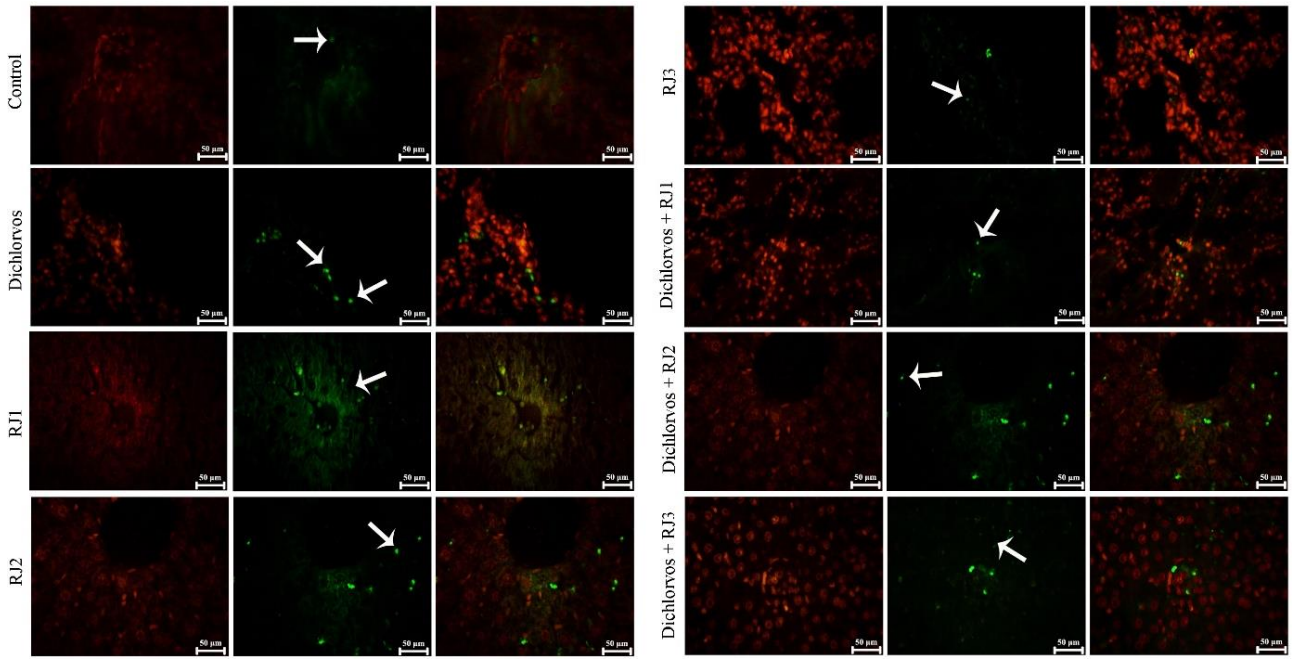

Fig. 5. Apoptosis induction in the rat liver following dichlorvos and the use of RJ (RJ1, 50 mg/kg; RJ2, 100 mg/kg, RJ3, 150 mg/kg). Left: cytoplasm staining, middle: nuclei staining, and right: merge. The white arrows referred to the shiny green nuclei of apoptotic cells. TUNEL assay was applied for staining. Magnifications, ×400. RJ, Royal jelly.

Effects of RJ and dichlorvos on the number of apoptotic cells

The AI-related quantitative data are offered in Fig. 4C and the tunnel-stained sections were conducted to measure the levels of apoptotic cells in the liver are shown in Fig. 5. The AI levels demonstrated a significant increment in the dichlorvos group than the control group, at the same time, no statistically different alterations were detected between the control and other experimental groups. AI was significantly decreased in RJs and dichlorvos + RJ groups compared to the dichlorvos group. There were no significant differences in the liver AI among dichlorvos + RJs.

DISCUSSION

Dichlorvos, an organophosphorus pesticide that produces various side effects on normal tissues, was used in the present study to induce liver damage in Wistar rats. RJ, a natural product is secreted and fed by the worker bees to larvae which are potential candidates to become queen bees in the future. It is a potent antioxidant and has many medical benefits. Thus, the present study showcased the harmful consequences of dichlorvos on the liver and the recovery of the liver from these consequences by RJ administration.

Dichlorvos treated rats showed increased the diameters of central vein in the liver, while the numbers of hepatocytes reduced. Impairments in both of these parameters were recovered by RJ. It seems the number of hepatocyte and central veins alterations result from the metabolic activity of the liver cells increment so that these cells can facilitate the excretion of toxins from the body. In line with our research, another study showed the enlargement of the central veins diameter followed by administration of malathion (23). Moreover, it has been reported that exposure to dichlorvos leads to hepatic ulcers, hepatocytes irritation, increased sinusoidal space, necrosis, and hypertrophy in the nucleus of the hepatocytes (24). Thus, here we suggest that by lowering the number of hepatocytes, a type of cell death may occur in dichlorvos-administrated rats.

The present study demonstrated that RJ decreased the levels of liver enzymes that were increased by dichlorvos. The increment in the activity of liver function index enzymes in serum demonstrated a liver injury in the present study. These enzymes can be released into the bloodstream due to the incidence of necrosis or cell membrane damage. The results are in agreement with other findings which reported that malathion administration induced the

enhancement of liver enzymes (23,25). It can be said that the abovementioned effects of RJ are due to its antioxidant properties, as well as its involvement in increasing the activity of the body's defense system against free radicals. Similar to the finding of the current study, it has been reported that honey has an antioxidant and hepatoprotective activity that minimizes liver damage in treated rats (26). Moreover, RJ by antioxidant activity diminishes liver enzyme conflicts as the criterion of liver toxicity (27,28).

In the present study, the increased liver levels of NO in the dichlorvos-treated group demonstrated the dominant role of this compound in reducing the antioxidant capacity of rats. This fact also reinforces the theory of oxidative stress induced by dichlorvos. It seems that the production of lipid peroxidation and subsequent induction of reactive oxygen species from saturated fatty acids in cell membranes results in the formation of compounds such as NO. Thus, increasing levels of NO have a decisive role in regulating oxidative stress and tissue degradation. The other organophosphates like malathion and diazinon stimulate raising levels of NO in the liver (29,30).

Based on the results of this study, administration of RJ was found to decrease the level of NO and enhance the TAC across the groups treated with it. Other researchers' reports also stated that RJ administration decreases NO (31,32). Another study showed that the administration of RJ in mice, in which cardiac toxicity was induced by paclitaxel, significantly reduced NO production and increased levels of TAC (18). However, measurement of other criteria like malondialdehyde, superoxide dismutase, glutathione peroxidase, catalase, high- and low-density lipoproteins, etc are useful for better proofing this statement in line with TAC and NO.

In the present study, the administration of RJ has protected against hepatocellular apoptosis due to the fact that in all RJ receiving groups, a lower AI was observed in comparison to the dichlorvos group. In fact, free radicals infiltrate into liver cells and cause single point necrosis in parenchymal cells.

The necrotic cells release the pro-inflammatory mediators which can exacerbate liver damage induced by poison. Free radicals' production and subsequent oxidative stress can be one of the most critical and essential causes of liver cell death. The organophosphorus compounds stimulate oxidative and genotoxic injuries. These consequences trigger cell death by cytochrome C released from mitochondria and the resulting activation of caspases (1). It can be stated that RJ, due to its antioxidant activity and suppression of inflammatory reactions, reduces the effects of dichlorvos.

In general, the present study showed dichlorvos side effects on liver-related enzymes, liver histology, and its oxidative-antioxidant balance, and the usefulness of RJ to relieve these catastrophic outcomes. It has been proved that RJ possesses hepatoprotective potential against liver damage induced by dichlorvos. This claim is along with other studies that show that oxidative stress induced by chemical or surgical operation would diminish by medicinal plants or chemical compounds (33-35).

CONCLUSION

The present study demonstrated that dichlorvos produces oxidative stress. Oxidative stress caused by dichlorvos in the liver is noticeable by a reduction in enzymatic activity, increased NO levels, and a decrease in TAC. These catastrophic consequences of dichlorvos induced apoptosis in the liver tissue. RJ reduced the dangerous effects of dichlorvos by improving the activities of functional enzymes present in the liver that are most probably due to its antioxidant potential.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Author's contribution

C. Jalili was involved in conceptualization, data curation, validation, and funding acquisition; M.H. Farzaei performed the analysis and prepared the original draft of the manuscript; I. Rashidi contributed to investigations, writing, and editing of the manuscript. A. Mohammadnezamian performed experiments. A. Ghanbari contributed to project administration, supervision, writing, and editing of the manuscript, validation, and funding acquisition. All authors read, critically reviewed, and approved the final manuscript.

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