Protective effect of crocin on diazinon induced cardiotoxicity in rats in subchronic exposure

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ABSTRACT

This study was designed to evaluate the effectiveness of crocin, main component of Crocus sativus L. (Safron) against subchronic diazinon (DZN) induced cardiotoxicity in rats.

Methods: Rats were divided into 7 groups; control (corn oil, gavage), DZN (15 mg/kg/day, gavage, ), crocin (12.5, 25 or 50 mg/kg/day, i.p) plus DZN, vitamin E (200 IU/kg, i.p, three times per week) plus DZN and crocin (50 mg/kg/day, i.p) groups. Treatments were continued for 4 weeks. Creatine phosphokinase MB (CK-MB), malondialdehyde (MDA) and glutathione (GSH) levels were evaluated in heart tissue at the end of treatments. Levels of apoptotic proteins (Bax, Bcl2, caspase 3) and cytosolic cytochrome c were analyzed by Western blotting. Transcript levels of Bax and Bcl2 were also determined using qRT PCR.

Results: DZN induced histopathological damages and elevated the level of cardiac marker CK-MB. These effects were associated with increased MDA level, lower level of reduced GSH and induction of apoptosis through elevation of Bax/Bcl2 ratio (both protein and mRNA levels), cytochrome c release to the cytosol and activation caspase 3 in cardiac tissue. Crocin (25 and 50 mg/kg) or vitamin E improved histopathological damages, decreased MDA and CK-MB, increased GSH content and attenuated the increase of Bax/Bcl2 ratio, activation caspase 3 and release of cytochrome c to the cytosol induced by DZN. In summary, DZN induced mitochondrial-mediated apoptosis in heart tissue of rat following subchronic exposure. Crocin, as an antioxidant, showed protective effects against DZN cardiotoxicity by reducing lipid peroxidation and alleviating apoptosis.

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1. Introduction

Organophosphate (OP) pesticides, known as cholinesterase inhibitors, are widely used to control of household and agricultural pests [1]. Environmental pollution caused by pesticide residues is an important concern in long term exposure [2]. Residual amounts of OPs could be detected in various agents like soil, water, vegetables, fruits and other food products [3]. DZN (0,0-diethyl-0-[2-isopropyl-6-methyl-pyrimidin-yl] phosphorothionate) is an organophosphate insecticide generally used around the world to control insects in crops, lawns, fruit and vegetables and as a pesticide in domestic animals and agriculture [4]. Acute and chronic toxicity of DZN in different tissues have been proved in human and animals. DZN may change the level of liver enzymes and biochemical indices and cause swelling of mitochondria in hepatocytes [5]. Spleen, thymus, lymph nodes and other organs are also affected by DZN [4]. Intoxication with DZN also changes haematological parameters and induces genotoxicity [6]. Although the main mechanism of OP intoxication is the inhibition of acetylcholinesterase (AChE) and overstimulation of cholinergic receptors, however, researches have shown that cholinergic hyperexcitability is not responsible for all of toxic effects of OP poisoning. Recent studies indicate that acute and chronic toxicity of OPs like DZN induce oxidative stress leading to generation of free radicals and change in antioxidants or reactive oxygen species (ROS) scavenging enzymes in mammals and other organisms in different tissues [7].

Lipid peroxidation has been suggested as one of the molecular mechanisms involved in DZN-induced cardiac toxicity [7]. It was reported that DZN also accumulates in different tissues leading to histological and biochemical damages [4].

Increasing evidence suggests that oxidative stress is a major apoptotic stimulant in different diseases such as cardiovascular...
diseases and ROS can induce apoptosis. So that this process can be suppressed by various antioxidants [8]. Involvement of low dose exposure of DZN and other OPs in apoptotic pathways have been reported recently. However, mechanisms by which OPs modulate this process are poorly investigated [6,9–12].

Antioxidants play an important role in preventing free radical mediated damages by directly scavenging them. Vitamin E or α-tocopherol is one of the most biologically active antioxidants in the biological system. It protects cells or tissues against lipid peroxidation by its chain breaking action. Many studies show that vitamin E could protect tissues against apoptosis induced by toxic substances [10].

Crocin, a carotenoid isolated from *Crocus sativus* L. (saffron), is responsible for the red color of saffron. Crocin is a pharmacologically active component of saffron. Modern pharmacological studies have demonstrated that crocin can be used as a new therapeutic agent. It has antimutator [13,14], antioxidant, radical scavenging [15–17], hypolipidamic [18,19] and memory-improving effects [18,20].

Moreover, the cardioprotective effects of saffron and its active components such as crocetin and crocin have been reported in some studies that are related to modulation of endogenous antioxidant enzymatic activities and cardiac biomarkers [6,21].

Besides antioxidant effects, crocin inhibits apoptotic pathway besides suppression of tumor necrosis factor, modulation of Bcl-x family proteins and inhibition of DNA fragmentation. Crocin also blocks the cytochrome c-induced activation of caspase-3 [22].

Also, it was shown that crocin could be considered as a protective agent in cytotoxicity induced by acrylamide via decrease in cellular ROS production in PC12 cells. Moreover crocin reduced the increase of Bax/bcl2 ratio induced by acrylamide [23].

The protective effect of crocin against DZN toxicity have been proved previously. Crocin attenuated the DZN toxic changes in hematological and biochemical parameters in rats. Also crocin decreased the level of 8-iso-prostaglandin F2α, TNF-α, and S100β induced by DZN in rats [6,24].

Since toxic effects of sub-chronic exposure of DZN in the cardiac system has not been fully elucidated, the present study was designed to determine the possible toxic effects of DZN on specific biochemical cardiac enzyme, morphological changes of the rat heart and apoptotic pathway. Moreover the possible protective effects of crocin on DZN toxicity were evaluated.

2. Materials and methods

2.1. Chemicals

DZN (Bazodin®, Syngenta, Singapore, purity 96%) and vitamin E (OSVE Pharmaceutical Co., Tehran, Iran) were purchased. TBA (2-thiobarbituric acid), n-butanol, phosphoric acid, potassium chloride, and MDA were obtained from Merck. Reduced GSH were obtained from Sigma–Aldrich. Stigmas of *C. sativus* L. from Novin Saffron (collected from Ghain, Khorasan province, Northeast of Iran) was obtained and analyzed in accordance to the ISO/TS 3632-2. Crocin was extracted and purified as defined by Hadizadeh and colleagues [25]. Other chemicals used in this study were described in the related section.

2.2. Animals and treatment

Adult male Wistar rats (weight 200–250 g) were provided by animal center, School of Pharmacy, Mashhad University of Medical Sciences. Rats were maintained on a 12 h light/dark cycle and at a temperature of 23 ± 1 °C with free access to food and water. These conditions were maintained constant throughout the experiments. All animal experiments were carried out in accordance to Mashhad University of Medical Sciences, Ethical Committee Acts.

Rats were randomly divided into seven groups: (1) control group (corn oil); (2) DZN treated group (15 mg/kg); (3) DZN + crocin 12.5 mg/kg treated group; (4) DZN + crocin 25 mg/kg treated group; (5) DZN + crocin 50 mg/kg treated group; (6) DZN + vitamin E 200 IU/kg treated group and (7) Crocin 50 mg/kg group. All groups consisted of six rats. DZN was administrated via gavage once a day for 4 weeks. Corn oil (vehicle of DZN) was given in the same way to control rats. Crocin and vitamin E were intraperitoneally administrated for 4 weeks once a day and three days a week, respectively.

2.3. Biochemical evaluation

At the end of the study period (4 weeks), rats were killed and blood was collected. The heart tissues were removed and washed in normal saline, then samples were taken and stored at −80 °C until the analysis.

2.3.1. Measurement of malondialdehyde in the heart tissue

To measure MDA, an important marker of oxidative stress, the heart tissues from different groups were homogenized for 2 min at 4 °C (POLYTRON® PT 10–35, Kinematica, Switzerland) in 1.15% KCl in order to provide a 10% homogenate. These homogenates were centrifuged (Hettich, Germany) at 6000g for 10 min to obtain supernatants. Total protein contents and MDA were measured in supernatants. The protein contents of homogenates were determined using Bio-Rad Protein Assay Kit according to the manufacturer protocol. MDA levels were determined according to the method of Fernandez et al. [26]. This method is based on the spectrophotometric measurement of the color developed by reaction of MDA to thioarbituric acid (TBA). Briefly, 3 ml of phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 ml of supernatant in a falcon tube and the mixture was incubated for 45 min in a boiling water bath. After cooling, 4 ml of n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 3000g for 20 min. The organic layer was transferred to a fresh tube and its absorbance was measured at 532 nm.

2.3.2. Measurement of reduced GSH in the heart tissue

Cardiac GSH content was measured according to the method of Moron et al. [27]. The hearts were homogenized in ice cold phosphate buffered saline (PBS), pH 7.4, to obtain 10% homogenate (w/v). Homogenates were centrifuged at 3000g for 10 min. Protein and GSH contents were determined in supernatants. Reduced GSH contents were measured using 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) which produced a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). Briefly, equal amounts of samples and 10% trichloroacetic acid (TCA) were mixed and centrifuged at 3000g for 5 min. 0.5 mL of 0.04% DTNB reagent was added to 0.5 mL of supernatants plus 2 mL PBS (0.1 M, pH 8.0). Then, the absorbance of yellow colored TNB was measured at 412 nm. Tissue GSH contents were expressed as nmol/mg protein.

2.3.3. Measurement of creatine phosphokinase-MB (CK-MB)

The activity of CK-MB in serum was measured using commercial colorimetric kits (Biosystem, Spain) by auto analyzer (Tokyo-Boeki Prestige).

2.4. Histopathological evaluation

For histopathological examination, hearts of all animals were removed at the end of the experiment after euthanasia and fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Sections were cut at 6 μm and stained with...
sequences of different Primers used for real-time PCR reactions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Forward</td>
<td>5′-GGTTGAGAACCTCCTGAGGA-3′</td>
</tr>
<tr>
<td></td>
<td>Reversed</td>
<td>5′-GGTCTAGCTCGATCATCACA-3′</td>
</tr>
<tr>
<td>BAX</td>
<td>Forward</td>
<td>5′-TGCACTGCACTCCA-3′</td>
</tr>
<tr>
<td></td>
<td>Reversed</td>
<td>5′-ATGTGTTGTCAGTTGACTCC-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5′-GGGAATTGCCTGACATT-3′</td>
</tr>
<tr>
<td></td>
<td>Reversed</td>
<td>5′-GGGGTTGGCCCACTTCT-3′</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of DZN and crocin treatment (4 weeks) on MDA Level (A) and GSH content (B) in the heart rat tissues. Crocin and vitamin E were administered intraperitoneally once a day and three times per week, respectively. DZN (corn oil as a vehicle) was given through gavage to rats once a day. Data are shown as mean ± SEM, **P < 0.01 (A) and *P < 0.05 (B) compared to the control group, ***P < 0.001 compared to DZN group, Tukey–Kramer test, n = 6.

Western blot analysis was carried out on protein extracts from the heart tissue for Bax, Bcl2, caspase 3 and cytosolic cytochrome c. For Bax, Bcl2 and caspase 3, heart tissues were homogenized in the homogenization buffer containing Tris 50 mM pH 7.4, 2 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 10 mM β-glycerol-phosphate, 0.2% W/V sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Sigma, P8340) using polytron homogenizer (POLYTRON® PT 10–35, Kinematica, Switzerland) in ice and then were centrifuged at 4 °C for 15 min at 10,000g. Supernatants were frozen at −80 °C until further use. For cytochrome c, the cytosolic fraction was isolated by mitochondrial isolation kit for tissue according to the kit protocol (Pierce, cat. 89801). Briefly heart tissues were immediately removed, minced and homogenized by 40 strokes in a dounce homogenizer (Sigma). The homogenates were centrifuged at 700 and 3000g for 10 and 15 min at 4 °C. The resulting supernatants contain the cytosolic fractions, and the pellets contain the enriched mitochondria fractions. The cytosolic fractions were frozen and stored at −80 °C until further analysis. The total protein content in the cytosolic fraction was determined using Bradford protein assay kit (Bio-Rad). Levels of cardiac Bax, Bcl2, caspase 3 (pro and cleaved caspase 3), cytochrome c and β-actin were measured by immunoblotting analysis. Briefly, equal amounts of protein extracts (50 μg) were loaded to SDS–PAGE gel. After electrophoresis, proteins were transferred to PVDF membrane. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline tween for 3 h. The primary antibodies were rabbit monoclonal anti-serum against Bcl2 (Cell Signaling, #2870), cytochrome c (Cell Signaling, #4280) and caspase-3 (Cell Signaling, #9665) and rabbit polyclonal anti-serum against Bax (Cell Signaling, #2772) and β-actin, (Cell Signaling, #4967). All antibodies were used at a dilution of 1:1000. Anti rabbit IgG labeled with horseradish peroxidase (Cell Signaling, #7074) was used as secondary antibody. Protein bands were visualized using an enhanced chemiluminescence (Pierce ECL Western blotting substrate) and Alliance gel doc. (Alliance 4.7 Gel doc, UK). UV tec software (UK) was used to semi quantify protein bands. All protein bands were normalized against β-actin protein.
Table 2
Effect of crocin and DZN on histopathological changes in cardiac tissue of rat after 4 weeks treatment. Results are shown as the mean ± S.E.M.

<table>
<thead>
<tr>
<th>Histopathological findings</th>
<th>Control</th>
<th>Diazinon</th>
<th>DZN + Cro12.5 mg/kg</th>
<th>DZN + Cro25 mg/kg</th>
<th>DZN + Cro 50 mg/kg</th>
<th>Crocin 50 mg/kg</th>
<th>DZN + Vit E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltration of inflammatory cells</td>
<td>0.00 ± 0.00</td>
<td>2.00 ± 0.00**</td>
<td>1.33 ± 0.33</td>
<td>0.33 ± 0.33***</td>
<td>0.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
</tr>
<tr>
<td>Multifocal necrosis</td>
<td>0.00 ± 0.00</td>
<td>2.66 ± 0.31**</td>
<td>2.33 ± 0.33</td>
<td>0.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
</tr>
<tr>
<td>Cardiac cell hypertrophy</td>
<td>0.00 ± 0.00</td>
<td>3.00 ± 0.00***</td>
<td>2.33 ± 0.33</td>
<td>1.33 ± 0.33***</td>
<td>1.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
<td>1.00 ± 0.00***</td>
</tr>
<tr>
<td>Congestion</td>
<td>0.00 ± 0.00</td>
<td>3.00 ± 0.00***</td>
<td>2.33 ± 0.33</td>
<td>1.33 ± 0.33***</td>
<td>1.00 ± 0.00***</td>
<td>0.00 ± 0.00***</td>
<td>1.00 ± 0.00***</td>
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</table>

**P < 0.01 and ***P < 0.001 vs group control, *P < 0.05 and ###P < 0.001 vs group DZN.

Fig. 3. (A) Normal cardiac muscle cells of control rats. Hematoxylin and eosin, ×640. (B) DZN treated rats show coagulative necrosis of cardiac muscle cells (arrowhead) associated with hemorrhage (astrix) and infiltration of inflammatory cells (arrow) Hematoxylin and eosin, ×320. (C) Cardiac sections of rats received crocin 12.5 mg/kg plus DZN showing severe congestion (astrix) and focal necrosis with infiltration of inflammatory cells (arrow). Hematoxylin and eosin, ×320. (D) Mild infiltration of inflammatory cells was observed in rats received crocin 25 mg/kg plus DZN. Hematoxylin and eosin, ×320. (E) Heart tissue of rats received crocin 50 mg/kg plus DZN showing hypertrophy of cardiac muscle cells (arrowhead). Hematoxylin and eosin, ×640. (F) Cardiac muscle cells with almost normal appearance in rats received crocin 50 mg/kg. Hematoxylin and eosin, ×320. (G) Mild congestion (astrix) and focal hypertrophy (arrow) of cardiac muscle cells was observed in rats received DZN plus vitamin E. Hematoxylin and eosin, ×320.
2.6. Isolation of RNA and real-time PCR

Total RNAs were isolated from different samples using Tripure Isolation Reagent (Roche, cat #11667157001) according to the manufacturer instruction. Quality (260/280 and 260/230 ratios) and quantity of isolated RNAs were determined using Nanodrop (NanoDrop™ 2000, USA) and samples were stored at −80 °C until use. To measure transcript levels, step one thermal cycler (ABI) and Express one-step SYBR R Green ER™Kit (Invitrogen, cat #11780–200) were used. Primers for Bax, Bcl2 and β actin were chosen according to previous studies [28] and/or were designed using Beacon Design™ software (BioSoft) (Table 1). Melting curve analysis was performed to analyze quality of primers and products. Expression of target genes was normalized against β-actin. ΔΔCT method was used to measure fold increase of genes in compare to control group.

2.7. Statistical analysis

All results are expressed as mean ± SEM. ANOVA followed by Tukey–Kramer test were performed to compare means. P values less than 0.05 were considered as significant.

3. Results

3.1. Biochemical evaluation

A significant increase was observed in MDA level in the DZN treated group (P < 0.001). MDA levels were significantly decreased in crocin (25 and 50 mg/kg) plus DZN (P < 0.01 and P < 0.001, respectively) and vitamin E plus DZN (P < 0.001) as compared with DZN groups (Fig. 1A). In DZN treated rats, cardiac content of GSH was significantly decreased (P < 0.01). However, crocin and vitamin E plus DZN significantly increased the content of GSH compared to DZN treated rats (P < 0.05 and P < 0.01, respectively) (Fig. 1B). Serum CK-MB activity showed high cardiotoxicity in DZN treated group (P < 0.001). Coadministration of 25 and 50 mg/kg of crocin or vitamin E and DZN reduced serum CK-MB activity as compared with the DZN group (P < 0.001) (Fig. 2).

3.2. Histopathological analysis

Histopathological changes of cardiac tissues are presented in Table 2 and Fig. 3. All histological findings were normal in the control group (Fig. 3A). Histological changes were observed in DZN treated group (Table 2 and Fig. 3B). DZN induced cardiac damage included coagulative necrosis of cardiac muscle cells associated with hemorrhage, hypertrophy and infiltration of inflammatory cells. The cardiac lesions were significantly improved in the DZN plus crocin (25 and 50 mg/kg) plus DZN (P < 0.01 and P < 0.001, respectively) or vitamin E compared to DZN-treated group (Table 2 and Fig. 3C). DZN induced an increase in cytosolic levels of cytochrome c (P < 0.001). Crocin (25 and 50 mg/kg) or vitamin E could attenuate the release of cytochrome c to cytosole in DZN treated rats (Fig. 6).

3.3. Western blot analysis for apoptotic proteins (Bax, Bcl-2 and caspase 3)

Western blotting showed that myocardial protein expression of Bax/Bcl2 was up-regulated in DZN group (P < 0.001) (Fig. 4). Also myocardial protein expressions of cleaved caspase 3/pro-caspase 3 were up-regulated by DZN (Fig. 5). Cotreatment of DZN with crocin (25 and 50 mg/kg) or vitamin E significantly decreased the myocardial ratio of Bax/Bcl2 (P < 0.01 and P < 0.05, respectively) (Fig. 4). Furthermore crocin (25 and 50 mg/kg) or vitamin E plus crocin (25 and 50 mg/kg) or vitamin E significantly decreased the myocardial ratio of Bax/Bcl2 (P < 0.01 and P < 0.05, respectively) (Fig. 4). Also myocardial protein expressions of cleaved caspase 3/pro-caspase 3 were up-regulated by DZN (Fig. 5). Cotreatment of DZN with crocin (25 and 50 mg/kg) or vitamin E significantly decreased the myocardial ratio of Bax/Bcl2 (P < 0.01 and P < 0.05, respectively) (Fig. 4). Furthermore crocin (25 and 50 mg/kg) or vitamin E plus crocin (25 and 50 mg/kg) or vitamin E significantly decreased the myocardial ratio of Bax/Bcl2 (P < 0.01 and P < 0.05, respectively) (Fig. 4).

3.4. Effect of DZN and crocin on release of cytochrome c

DZN induced an increase in cytosolic levels of cytochrome c (P < 0.001). Crocin (25 and 50 mg/kg) or vitamin E could attenuate the release of cytochrome c to cytosole in DZN treated rats (Fig. 6).

3.5. Effects of DZN and crocin on mRNA level of Bax and Bcl2 in heart tissue

An induction of Bax/Bcl2 mRNA expression ratio was observed in DZN treated group (2.6-fold). mRNA expression of Bax/Bcl2 ratio was down regulated in concurrent administration of DZN and crocin (25 and 50 mg/kg) or vitamin E (Fig. 7) (P < 0.001).

4. Discussion

The present study showed that DZN (15 mg/kg) exhibits cardiac toxicity in subchronic exposure (28 days) by inducing morphological damages as well as elevated cardiac marker CK-MB. These changes were associated with increased oxidative stress and apoptosis in cardiac tissues. Co treatment with crocin (25 and 50 mg/kg) or vitamin E showed protective effects against DZN induced...
cardiac toxicity by reducing oxidative stress, apoptosis and other DZN-induced disorders.

It has been proved that chronic exposure to OPs such as DZN inhibits cholinesterase enzyme and introduces oxidative stress damages and free radicals production in different organs such as cardiovascular system [6,7,29–31]. Our data indicated that cardiac level of MDA was significantly increased, while content of GSH was significantly decreased following DZN administration. Malondialdehyde (MDA), the end product of lipid peroxidation, is routinely used to evaluate the presence of free radicals and lipid peroxidation induced cardiovascular toxicity [7,31] and the content of reduced GSH is a marker of oxidative damage in heart tissue. GSH acts as free radicals scavengers and protects heart from oxidative stress [32]. These findings were in agreement with another studies demonstrated that DZN significantly increases MDA and decreases reduced GSH in some tissues of rats [7,33–36]. The histopathological findings indicated that DZN subchronic exposure could induce congestion, infiltration of inflammatory cells and multifocal necrosis in cardiac tissue.

We also observed that serum CK-MB level, was increased by DZN. CK-MB is one of the sensitive markers existing for the evaluation of damage to the heart muscle.

According to the data, exposure to DZN may result in peroxidation of polyunsaturated fatty acids, leading to the degradation of phospholipids and finally cellular damages which was shown in this study as an increase in cardiac biomarker and histological damages. Our results supported the hypothesis that oxidative stress and free radicals play an important role in DZN cardiotoxicity [7].

Crocin is a potent antioxidant agent [37]. Cardioprotective effects of crocin (20 mg/kg/day, 21 days) against isoproterenol-induced toxicity via antioxidant effect, has been shown previously [38]. Results of the present study showed that concurrent administration of crocin (25 and 50 mg/kg) and DZN decreased the MDA and increased GSH content in the heart tissue. Also crocin could protect the heart from histological damages induced by DZN via anti-oxidative [19,40] and anti-inflammatory effects [6,39]. So, in this study the protective effect of crocin could be attributed to the inhibitory effect on lipid peroxidation which leads to stabilizing of plasma membranes and prevents the release of cardiac enzymes.

Vitamin E or $\alpha$-tocopherol is one of the most biologically active antioxidants in the biological system. Vitamin E protects tissues against lipid peroxidation by its chain breaking action. In the present study, vitamin E was used as a positive control. It has been reported that cotreatment of vitamin E and DZN reduced MDA level and pathological damages induced by DZN in rats [7]. Also, vitamin E prevented the increase of CK-MB and CK levels due to doxorubicin-induced myocardial damage [41]. Our results showed that vitamin E could protect heart tissue against the oxidative damages induced by DZN in rat.

Evidence suggests that oxidative stress is a major apoptotic stimulus in various diseases such as cardiovascular problems [42]. Several studies revealed that apoptosis could be a possible mechanism of toxicity in low-dose exposure to some OPs [10,43]. Also, it is reported that DZN induced cell death in NTerá2-D1 cell line through triggering the caspase cascade [44]. According to the results, lipid peroxidation could be considered as a mechanism of
DZN cardiotoxicity, therefore to understand whether subchronic exposure to DZN can induce apoptosis in heart tissue, some key factors involved in apoptosis pathway were evaluated in this study. The proteins of Bcl-2 family are involved in the intrinsic apoptotic pathway [45]. Bax is a pro-apoptotic protein and Bcl2 possesses antiapoptotic properties through stabilizing mitochondrial membrane and suppressing the release of cytochrome c. The balance between these proteins is important in the progression of apoptosis [46].

In this study DZN increased the ratio of Bax/Bcl2 in both mRNA and protein levels (P < 0.001). This increase in Bax/Bcl-2 ratio by DZN could be considered as a predictor of DZN induced apoptosis in heart tissue.

To confirm the induction of apoptosis by DZN, the activation of caspase 3 was also evaluated. According to the results, DZN cleaved (17 and 19 kDa) and activated the caspase 3 (Fig. 5).

It has been proved that mitochondria play an important role in apoptosis by releasing of cytochrome c into the cytosol. In the cytosol, cytochrome c activates caspase 9. Then caspase-9 directly cleaves and activates caspase-3. Moreover, the members of the Bcl-2 family proteins control cytochrome c release process [12]. In this study we have demonstrated that DZN decreased the cytosolic cytochrome c content (P < 0.001), so it was concluded that the increasing of the ratio of Bax/Bcl2 caused the disturbance of mitochondrial integrity and permeability and led to the release of cytochrome c to the cytosol and ultimately activated caspase 3 and induced apoptosis through intrinsic pathway.

Antioxidants are known to regulate gene expression and signal transduction pathways, so that can prevent the occurrence of apoptosis. Vitamin E inhibits free radical induced apoptosis by its scavenging property. Moreover it has been reported that combination of vitamins E and C protect endometrial apoptosis induced by diclorvos through free radical scavenging and diminishing the

**Fig. 6.** Effect of crocin and DZN on the cytosolic cytochrome c level in the rat heart tissue by Western blotting. (A) Representative Western blots showing specific bands for cytosolic cytochrome c and β-actin as an internal control. These bands are representative of six separate experiments. (B) Densitometric data of protein analysis. Data are expressed as the mean ± SEM. ***P < 0.001 vs group control and ###P < 0.01 vs group DZN.

**Fig. 7.** The effect of DZN and crocin on Bax/ Bcl2 mRNA expression in the rat heart tissue after 4 weeks treatment by real time PCR. The transcript level of each sample was normalized against β-actin transcript level. These reactions are representative of six separate experiments. Data are expressed as the mean ± SEM. ***P < 0.001 vs. control and ###P < 0.001 vs DZN treated group.
activation of caspases 3 and 9. Caspases are susceptible to oxidation because of cysteine residues and antioxidants such as vitamin E has been shown to inhibit oxidative stress mediated caspase activation and ultimately inhibit apoptosis [10].

Moreover, vitamin E is associated with lipid-rich membranes such as mitochondria intracellular and thus helps to maintain the membrane stability [47]. So it may be inhibit the release of cytochrome c from mitochondria to the cytosol by maintenance of integrity of mitochondria inner membrane.

Antiangiogenic effect of crocin has been shown through inhibitory effect against TNF-α induced cell death [48], modulatory properties on expression of Bcl2 family proteins and inhibition of release of cytochrome c to the cytosol [49].

Our results showed that crocin (25 and 50 mg/kg) plus DZN clearly reduced the increase of the protein level as well as the mRNA expression of the Bax/Bcl2 ratio, compared to the DZN group ($P < 0.01$ and $P < 0.01$, respectively). Also crocin reduced the cytochrome c level in cytosolic fraction ($P < 0.001$) and inhibited the activation of caspase 3 induced by DZN in heart tissue. Moreover, the effect of the crocin in the highest dose (50 mg/kg) was similar to the control group. Concurrent administration of vitamin E and DZN attenuated the alterations induced by DZN in apoptotic pathway as well. Furthermore the protective effect of crocin against apoptosis induced by DZN in cardiac tissue was as much as vitamin E or even more at doses of 25 or 50 mg/kg. It could be concluded that crocin, similar to the vitamin E, could maintain a redox balance in cardiac tissue and modulates the susceptibility of biological membranes to interact with ROS, as indicated by the marked reduction in MDA level and subsequently prevent tissue against apoptosis. Also, like vitamin E it can maintain the mitochondria membrane stability and reduce the increase of Bax/Bcl2 ratio induced by DZN. Also, it can inhibit caspase 3 activation by free radical scavenging property and inhibition of sestine residue of caspase 3 oxidation [10]. Regarding the hydrophilicity of crocin, it may be suggested that like hydrophilic antioxidant vitamin C, crocin can restore the antioxidant property of oxidized α-tocopherol by recycling the tocopheroxyl radical [10].

5. Conclusions

In summary we believe that this is the first report regarding the induction of mitochondrial dependent apoptosis induced by DZN in cardiac tissue. Our data also indicate that crocin has protective effects against DZN-induced cardiotoxicity through attenuating lipid peroxidation, increasing reduced GSH, reducing histopathological damages and alleviating apoptosis by decreasing Bax/Bcl-2 ratio, the cytosolic cytochrome c content and inhibition of caspase-3 activation.

Conflicts of interest

None.

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