Chemico-Biological Interactions 203 (2013) 547–555

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

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

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ARTICLE INFO

Article history:
Received 19 December 2012
Received in revised form 13 February 2013
Accepted 11 March 2013
Available online 21 March 2013

Keywords:
Diazinon
Crocin
Saffron
Crocus sativus
Apoptosis
Oxidative stress

ABSTRACT

This study was designed to evaluate the effectiveness of crocin, main component of *Crocus sativus* L. (Saffron) 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), malondealdehyde (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 histophatological 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 of 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.

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 poisioning. 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

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http://dx.doi.org/10.1016/j.cbi.2013.03.010

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 antitumor [13,14], antioxidant, radical scavenging [15–17], hypolipaemic [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 through 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 F2 β, TNF-a, 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 Ghaen, 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 6000*g* 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 thiobarbituric 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 3000*g* 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 3000*g* 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 3000*g* 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