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Original Article

Crocin Protects Against Atrazine-induced Toxicity in Primary Rat Hepatocytes

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Abstract

Background: Atrazine is a typical herbicide that has domestic and agricultural uses all over the world. A review of the literature, however, revealed evidence of its destructive effects on many human tissues and signaling networks. As such, this study explored the impact of crocin, a natural pigment, on reducing the liver damage caused by atrazine in primary rat hepatocytes.

Methods: As biochemical cytotoxicity indicators, cell death, lactate dehydrogenase (LDH) leakage, reactive oxygen species (ROS) generation, lipid peroxidation (LPO), glutathione (GSH) level, and mitochondrial membrane potential were assessed.

Results: In the first step, LD50 concentration of atrazine was evaluated using a methyl thiazolyl tetrazolium (MTT) test in rat hepatocytes. The findings indicated that cellular function declines at LC50 concentration (400 M). On the contrary, crocin (50 μM) substantially boosted hepatocyte viability, decreased ROS production and LPO, replenished cellular GSH pools, and improved mitochondrial function.

Conclusion: Overall, the data suggest that crocin may play a protective function in atrazine-induced liver injury in which the main mechanisms of toxicity appear to be the generation of ROS and mitochondrial damage.

Keywords: Crocin, Saffron, Oxidative Stress, Hepatotoxicity

Introduction

In agricultural settings, atrazine (ATZ) or 2-chloro-4-ethylamino-6-isopropylamino-s-triazine is one of the most widely applied herbicides.[1] This herbicide is typically applied in sprays, liquids, concentrates, or granules. ATZ exerts minimal biodegradation in soil and water, which is most often found as a pesticide in surface waterways, so it influences the environment extensively.[2]

Most body organs are affected by ATZ; furthermore, its impacts on the neurological, excretory, and reproductive systems have been thoroughly studied. ATZ is an endocrine disruptive pollutant that can cause thyroid cancer in female spouses of pesticide applicators in agricultural cohorts.[3,4] alter the reproductive system of male rats, and deplete testis and epididymis antioxidant reservoirs. The liver exhibits signs of ATZ poisoning. The submandibular salivary glands of rats given oral ATZ exhibited oxidative stress, degeneration, and apoptosis.[5] Rats given ATZ exhibited damaged hepatocytes, substantially elevated levels of total bilirubin, alanine aminotransferase, and aspartate aminotransferase, and decreased glutathione (GSH).[6] Rats treated with even low dosages of ATZ exhibited lipidosis in hepatocytes, hepatic peri-acinar necrosis, and portal lymphocytic inflammation.[6,7] Genotoxicity has also been observed in the liver after subacute exposure to ATZ.[8] Since ATZ is a commonly used herbicide, cases of poisoning are also common. As the liver is the primary organ of chemical exposure, there is no surprise that hepatotoxicity frequently occurs. Therefore, the introduction of novel therapeutic agents to prevent herbicide-induced hepatotoxicity is an urgent issue.

Saffron (*Crocus sativus* L.) is employed in complementary medicine as a natural therapy against human diseases because it has antioxidant, anti-inflammatory, and anti-carcinogenic characteristics in addition to being hypolipidemic.[9,10] Crocin, crocetin, and safranal are the three primary components of saffron. Crocin exhibits strong antioxidant activity and scavenges free radicals.[11]

One of the most effective strategies for combating hepatotoxicity in recent years has been the phytomedicine approach. The free radical scavenging functions of phytochemicals are mainly responsible for this protection. Therefore, the chief objective of the present investigation was to demonstrate the protective role of crocin against ATZ-associated liver injury.

Methods

Chemicals

Fetal bovine serum and Dulbecco's Modified Eagle medium (DMEM) were obtained from Invitrogen Gibco. Albumin bovine serum was purchased from the Roche diagnostic company (IN). Collagenase (Type II), thiobarbituric acid, and all other chemicals were acquired from Sigma-Aldrich Co. (Heidelberg, Germany).

Primary Rat Hepatocytes Culture

Collagenase perfusion was used to produce primary rat hepatocytes,[12] and the Trypan blue exclusion testing was implemented to assess the viability of the cells to obtain over 85% of viable cells for subsequent investigations. The separated hepatocytes were then incubated for 3 hours at 37 ˚C in a humidified atmosphere of 95% O2 and 5% CO2 and supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Hepatocytes were seeded in 12 well plates (2×10^5/mL) for various experiments and in 96 well plates (4.8×10^4/mL) for the viability tests. Crocin was administered one hour after the cells had been exposed to ATZ for 4 hours, and the cells were then incubated for another 3 hours at 37 ˚C. In the end, cultured hepatocytes were used to test cytotoxicity and other biochemical tests, including membrane injuries such as lactate dehydrogenase (LDH) leakiness, reactive oxygen species (ROS) production, mitochondrial membrane potential (MMP), GSH level, and lipid peroxidation (LPO).

Cell Viability

Cell viability was assessed using methyl thiazolyl tetrazolium (MTT) and LDH activity. After incubating cells in 96-well plates, 50 mL of medium from every group was exposed to freshly made -NADH solution and left for 20 minutes for LDH measurement. At the same time, an enzyme-linked immunosorbent assay microplate reader was applied to quantify the reaction mixture's absorbance at 340 nm. The leftover medium was subjected to the MTT test by being incubated by MTT (1 mg/mL) for 4 hours at 37 ˚C. After the medium was picked up, the produced formazan crystals were dissolved in dimethyl sulfoxide. Finally, using an enzyme-linked immunosorbent assay microplate reader, the absorbance was read at 570 nm, and the consequent data were represented as a percentage of the non-treated group.

Preparing of Cell Lysate

Hepatocytes were exposed to a centrifugation step (5000 g, 5 minutes) before washing with ice-cold phosphate-buffered saline (PBS) to create the cell lysate. Afterward, the cell pellet was reconstituted with PBS and the resultant was sonicated three times for 30 seconds each time using an ultrasonicator (Parasonic 30S, Iran). Furthermore, Lowry's technique was applied to estimate the amount of total protein.[13]

Reactive Oxygen Species

Using the fluorescent probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), oxidative stress was studied. This dye can penetrate the cells. Rat hepatocytes were treated and then exposed to the dye solution. Hepatocytes were then incubated for 1 hour at 37 ˚C. DCFH-DA is broken down by intracellular esterases to DCFH, which can then react with different types of ROS to produce the extremely fluorescent substance known as dichlorofluorescein (DCF). The amount of DCF was determined from a DCF standard via a spectrophotometer with an excitation and emission wavelengths of 485 and 535 nm, correspondingly.

Lipid Peroxidation

In sum, a solution of combined potassium chloride and ferric chloride was added to 0.5 mL of the cell lysate for 30 minutes at 37 ˚C. The reaction was broken off by adding 2.0 mL of an ice-cold solution of hydrochloric acid, thiobarbituric acid, trichloroacetic acid, and butylated hydroxytoluene. This solution was re-heated at 90 ˚C for a further 30 minutes. The mixes were then chilled and centrifuged at 7000 g (5 minutes). Further, the percentage of LPO was used to express the results according to the absorbance of the supernatants.

The Level of Glutathione

The level of GSH in hepatocyte cell lysates was calculated using the method developed by Popet et al.[14] To do this, cell lysate (50 µL) was exposed to DTNB reagent [(150 µL) 12 mM (NADPH 12mM, GSH reductase 50 U/mL, DTNB 0.1 mM] and left over for 45 minutes. Then, the results were reported as nmol/mg protein through a calibration curve based on the amounts of GSH.

Mitochondrial Membrane Potential

Hepatocytes were subjected to ATZ and crocin treatment before being incubated for 20 minutes at 37 ˚C in the dark with a medium encompassing 1.6 M of Rhodamine 123. The method is based on the selective lump of Rhodamine 123 by active mitochondrion. The residual Rhodamine 123 was quantified with a spectrophotometer apparatus at 490 nm excitation and 520 nm emission wavelengths, and the results were reported as nmol/mg protein.

Statistical Analysis

For three separate experiments, the results were presented as the mean ± standard error of the mean. A one-way analysis of variance was utilized for the statistical analysis, followed by Tukey's post hoc test, and the *P* value below 0.05 was regarded as statistically significant.

Results

The viability of rat hepatocytes was treated with ATZ in escalating doses to perform the MTT test. Concentration-dependent toxicity was noted, with the ATZ LC50 (the concentration that caused 50% of the hepatocytes to

die) being 400 µM, as shown in Figure 1A. Moreover, crocin (50 µM) could significantly reduce the ATZ-induced toxicity, as illustrated in Figure 1B. Lower crocin concentrations, however, did not exhibit any significant protective effect. This study looked into LDH leakage, a crucial indicator of damaged cell membranes. When crocin was added, LDH leakage significantly decreased, indicating protection against ATZ-related membrane damage. Nevertheless, the treatment of hepatocytes with ATZ markedly accelerated LDH leakage (Figure 2).

Hepatocytes exposed to ATZ exhibited a surge in cellular ROS generation. Moreover, crocin at a 50 µM concentration could significantly reduce DCF fluorescence (Figure 3). Oxidative danger frequently occurs after the breakdown of cellular lipids. Furthermore, the LC50 concentration of ATZ boosted ROS production while also causing the membrane lipids to degrade, and crocin (50 µM) significantly reduced the quantity of LPO (Figure 4).

The major endogenous antioxidant protection against xenobiotic-induced toxicities in cells is GSH. ATZ (400 µM) substantially plummeted cellular GSH levels in comparison with the control group, while crocin (50 µM) therapy of hepatocytes effectively refilled cellular GSH reservoirs (Figure 5). Figure 6 depicts the effect of ATZ on the activation of mitochondrial membrane breakdown. ATZ significantly decreased MMP in the culture media when applied to hepatocytes, but crocin (50 µM) exhibited observable effects on reversing mitochondrial activity (Figure 6).

Discussion

Exogenous substances such as xenobiotics and poisons are extensively metabolized in hepatic tissue, producing a variety of metabolites that can either be less harmful or more toxic depending on their structure.[15] Moreover, the risk of hepatotoxicity increases because the liver is the primary site of xenobiotic exposure.[16,17] The investigation of potential toxicological pathways and the introduction of protective chemicals become crucial correspondingly. The occurrence of oxidative hazard is regarded as one of the main mechanisms of liver damage. ATZ-mediated toxicity has been linked to oxidative damage.[18] After intoxication, ROS formation can disrupt the cell membrane.[19] Furthermore, ROS-induced changes in cellular constituents might result in the denaturation of proteins, peroxidation of unsaturated fatty acids, and depletion of the cellular antioxidant pool.[20] In the present study, LPO production as a sign of oxidative stress following ATZ administration is consistent with earlier research.[21] Hence, using antioxidants to combat such toxins may be a beneficial strategy. Natural antioxidants have drawn interest in this regard due to their safer performance. According to a study by Hosseinzadeh et al, crocin effectively reduced ischemia-reperfusion-related oxidative injury *in vivo*.[22] Likewise, according to Mehri and colleagues' study, crocin reduced the acrylamide-associated neurotoxicity in rats by preventing

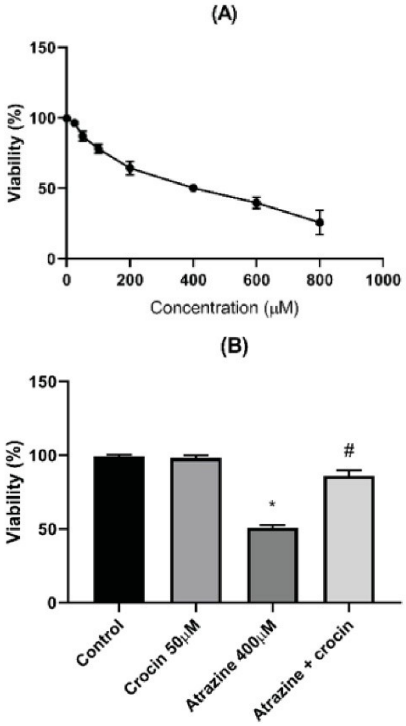

Figure 1. Viability of Rat Hepatocytes Tested with MTT Assay. (A). Effect of crocin (20 µM) on the viability of ATZ-treated hepatocytes (B). Results are represented as mean ± SD of 3 independent experiments. *Note.* MTT: Methyl thiazolyl tetrazolium; ATZ: Atrazine; SD: Standard deviation. * Significant compared to non-treated; # Significant in comparison with ATZ–treated hepatocytes ($P < 0.05$)

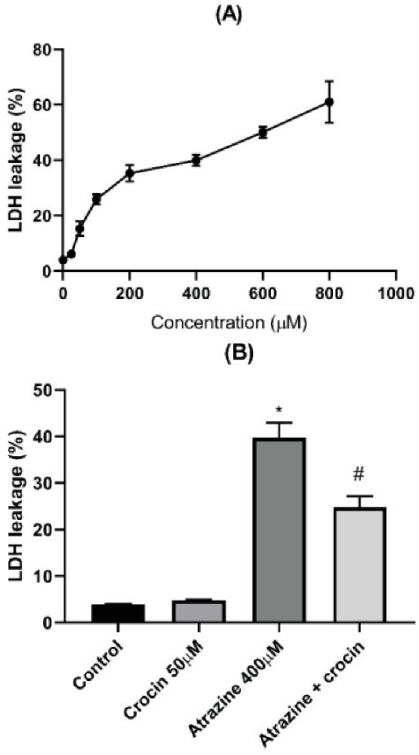

Figure 2. Membrane Leakage of Rat Hepatocytes Assessed by LDH Test (A). Effect of Crocin (50 µM) on the Membrane Integrity of ATZ-treated Hepatocytes (B). Data are represented as mean±SD of 3 independent experiments. *Note.* LDH: Lactate dehydrogenase; ATZ: Atrazine. * Significant compared to control; # Significant compared to ATZ–treated hepatocytes ($P < 0.05$)

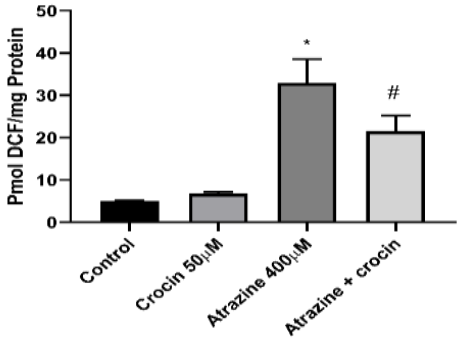

Figure 3. Effect of Crocin (50 μM) on the ROS Formation of ATZ-Treated Cells. *Note*. ROS: Reactive oxygen species; ATZ: Atrazine; SD: Standard deviation. Results are represented as mean ± SD of 3 independent experiments. * Significant in comparison with non-treated; # Significant compared ATZ–treated hepatocytes ($P < 0.05$)

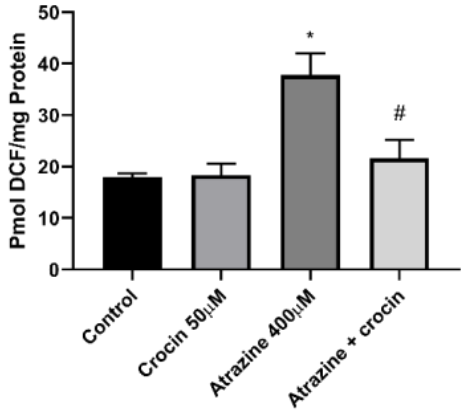

Figure 4. Effect of Crocin (50 μM) on the LPO of ATZ-Treated Cells. *Note*. LPO: Lipid peroxidation; ATZ: Atrazine; SD: Standard deviation. Results are represented as mean ± SD of 3 independent experiments. * Significant in comparison with non-treated; # Significant compared with ATZ–treated hepatocytes ($P < 0.05$)

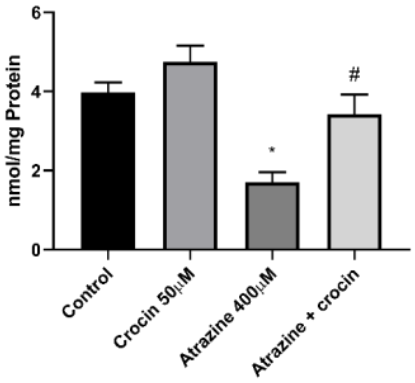

Figure 5. Effect of Crocin (50 μM) on the Glutathione Level of ATZ-Treated Cells. *Note*. ATZ: Atrazine; SD: Standard deviation. Results are represented as mean ± SD of 3 independent experiments. * Significant in comparison with non-treated; # Significant compared with ATZ–treated hepatocytes ($P < 0.05$)

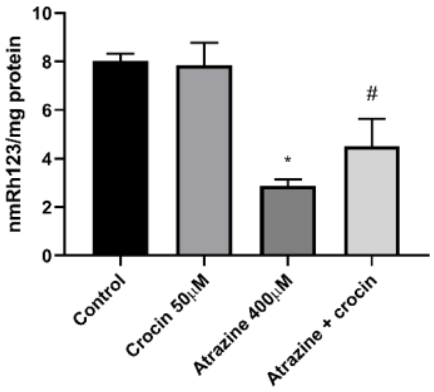

Figure 6. Effect of Crocin (50 μM) on the MMP of ATZ-Treated Cells. *Note*. MMP: Mitochondrial membrane potential; ATZ: Atrazine; SD: Standard deviation. Results are represented as mean ± SD of 3 independent experiments. * Significant in comparison with non-treated; # Significant compared with ATZ–treated hepatocytes ($P < 0.05$)

oxidative stress.[23] Moreover, it has been found that the antioxidant capabilities of crocin and saffron extract decrease malondialdehyde levels and ROS-stimulated peroxidation of membrane lipids.[24] Saffron and crocin have also been reported to regulate oxidative indicators in the hippocampal function.[25] These studies support our findings, reporting that after crocin administration, ROS formation and further LPO were plunged, GSH levels were replenished, and MMP increased. Different studies have revealed the cytotoxic effects of ATZ in which oxidative stress-related pathways play a pivotal role. For instance, ATZ induces oxidative-stress-mediated apoptosis in the testicular tissue of male offspring whose mothers were exposed to this herbicide. This can lead to a plummeted number of sperms, elevated sperm abnormality, and spermatogenesis. It has been reported that the treatment of animals with crocin during pregnancy and lactation significantly reverses ATZ-induced damages.[26] Therefore, the use of crocin as a potent antioxidant agent in reducing ATZ-induced hepatotoxicity can be explained. Furthermore, earlier research showed that crocin dramatically decreases malondialdehyde levels in animal models treated with cisplatin, cyclophosphamide, and diazinon while increasing the liver's GSH content.[27-29]

According to Lieshout et al, consuming foods high in antioxidants could hasten the binding of toxic materials with GSH and their elimination from circulation.[30] Antioxidants might then act in concert to shield crucial tissues against oxidative harm. Intrinsic anti-ROS systems in the body include antioxidant enzymes and non-enzymatic antioxidants such as GSH. Monitoring the quantity of cellular GSH can therefore provide us with information about the liver damage caused by ROS. The current study found that ATZ significantly reduces GSH levels, which is consistent with earlier research.[31] It has been demonstrated that crocin can neutralize some free radicals and raise cellular GSH.[32]

As a well-known target and the source of cellular ROS production, mitochondria are vital subcellular organelles.[33,34] Furthermore, increased ROS production may increase the sensitivity of mitochondria, which could lead to mitochondrial dysfunction, uncoupling of oxidative phosphorylation, and eventually membrane damage.[35] Cytochrome c and other pro-apoptotic

elements will therefore be liberated into the cytoplasm.[36,37] This study found that mitochondrial damage plays a role in the pathophysiology of ATZ-related hepatotoxicity. Moreover, the preventive mechanisms of crocin against ATZ-induced liver damage may involve mitochondrial pathways.

Conclusion

In summary, the findings demonstrated that crocin administration has protective effects against ATZ-induced liver damage in primary cultured isolated rat hepatocytes. The primary protective mechanisms of crocin appear to be mediated by its antioxidant and mitochondrial protecting activities, which were demonstrated as the inhibition of LPO, prevention of GSH depletion, and reduction of MMP collapse. Therefore, it might be suggested that using crocin as a potent antioxidant and mitochondrial protective agent can be beneficial against various xenobiotics that cause hepatotoxicity, particularly for ROS-related damage. However, further *in vitro* and *in vivo* experiments are required to clarify the protective mechanisms of protection, the possible association of divers signaling cascades, and validation of the doses used in animals before clinical trials, which can also be mentioned as the limitation of our study.

Ethics statement

The ethical approval for this project is IR.TBZMED.VCR.REC.1401.382 funded by Tabriz University of Medical Sciences, Tabriz, Iran.

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Conflict of interests declaration

None to be declared.

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Data availability statement

This published paper includes all data produced and/or analyzed during the current study.

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