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Crocin, the main active saffron constituent, mitigates dichlorvos-induced oxidative stress and apoptosis in HCT-116 cells

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ABSTRACT

The protective effects of Crocin (CRO), a carotenoid with wide spectrum of pharmacological effects, against the cytotoxicity and the apoptosis produced by exposure to Dichlorvos (DDVP) in HCT116 cells were investigated in this work. The cytotoxicity was monitored by cell viability, ROS generation, antioxidant enzymes activities, malondialdehyde (MDA) production and DNA fragmentation. The apoptosis was assessed through the measurement of the mitochondrial transmembrane potential ($\Delta\Psi$m) and caspases activation. The results indicated that pretreatment of HCT116 cells with CRO, 2 h prior to DDVP exposure, significantly increased the survival of cells, inhibited the ROS generation, modulated the activities of catalase (CAT) and superoxide dismutase (SOD) and reduced the MDA level. The reduction in mitochondrial membrane potential, DNA fragmentation and caspases activation were also inhibited by CRO. These findings suggest that CRO can protect HCT116 cells from DDVP-induced oxidative stress and apoptosis.

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1. Introduction

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate; DDVP) is an organophosphate pesticide and is widely used for the control of household pests, public health pests and stored product insect infestations [1]. DDVP is a direct-acting inhibitor of AChE [2], the enzyme that degrades the neurotransmitter ACh in cholinergic synapses, and disrupts nerve function that can lead to the exposed-organism death [3]. DDVP is effective against spider mites, mushroom flies, aphids, caterpillars, thrips and white flies in greenhouses, outdoor fruit, and vegetable crops [1,4]. The general public exposure to DDVP may occur via air, water, or food as this pesticide is readily absorbed through all routes of exposure [5]. DDVP exposure has been linked to substantial adverse health effects on several organ systems, including the reproductive system [6,7] and respiratory system [8].

The prevention of DDVP toxicity involves reduction of pesticide levels in foodstuffs and increasing the intake of diet components such as vitamins and antioxidants. We have previously shown that oxidative stress was involved in DDVP-induced toxicity in HCT116 cells [9]. Thus, studies on the effect of antioxidants, especially those consumed in food, appear of great interest to prevent DDVP-induced cell damages.

Crocin is a pharmacologically active compound of *Crocus sativus* L. (saffron) [10]. The antioxidant potential of Crocin has been reported *in vitro* and *in vivo* [11–15]. For example, Crocin can decrease lipid peroxidation in kidney [13] and skeletal muscle [14] during ischemia-reperfusion-induced oxidative damage in rats. In addition, this carotenoid increases cell viability in PC12 cells upon serum deprivation by inducing glutathione (GSH) synthesis, increasing Glutathione reductase (GR) and c-Glutamylcysteinyl synthase (c-GCS) activities, and decreasing ceramide formation [11,12].

Similarly, Razavi et al. [16] have demonstrated that Crocin exerted protective effects against the cardiotoxicity induced by Diazinon, an organophosphate pesticide, by reducing lipid peroxidation and alleviating apoptosis.

Abbreviations: OP, organophosphorous; DDVP, dichlorvos; CRO, crocin; ROS, reactive oxygen species; $\Delta\Psi$m, mitochondrial transmembrane potential.

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The present study was designed to determine the effect of the antioxidant molecule, Crocin against DDVP-induced toxicity in HCT116 cells.

2. Materials and methods

2.1. Chemicals

Dichlorvos, Crocin and pyrogallol were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-4,5-Dimethylthiazol-2-yl, 2,5-diphenyltetrazolium bromide (MTT), Cell culture medium (RPMI1640), foetal calf serum (FCS), phosphate buffer saline (PBS), trypsin-EDTA, penicillin and streptomycin mixture and l-glutamine (200 mM) were from GIBCO-BCL (UK). 2,7-Dichlorofluoresce diacetate (DCFH-DA) was supplied by Molecular Probes (Cergy Pontoise, France). Low melting point agarose (LMA) and normal melting point agarose (NMA) were purchased from Sigma (St. Louis, MO). All other chemicals used were of analytical grade.

2.2. Cell culture and treatment

Human colon carcinoma cells HCT116 were cultured in DMEM-F12, supplemented with 10% FBS, 1% L-glutamine (200 mM), 1% of mixture penicillin (100 IU/ml) and streptomycin (100 lg/ml), at 37 °C with 5% CO_2.

2.3. Cell toxicity assay (MTT assay)

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (a tetrazolium salt reduction assay) provides sensitive measurements of the normal metabolic status of cells, particularly that of the mitochondrion, where measurements reflect early cellular redox changes [17]. HCT-116 cells (2.5×10^5 cells/well in 96-well plates) were incubated at 37 °C for 24 h with DDVP alone or combined to CRO (50, 100 and 250 μM). A negative control containing only cells was also evaluated. After treatment, the plates were incubated in the MTT solution (final concentration of 0.5 mg/mL) for 3 h. The dark-blue formazan crystals that formed in intact cells were dissolved with DMSO, and the absorbance at 570 nm was measured with a spectrophotometer microplate reader (Bioteck, Elx 800). The results were expressed as the percentage of MTT reduction relative to the absorbances measured from negative control cells. All assays were performed in triplicate.

2.4. Reactive oxygen species determination and oxidative stress status

Reactive oxygen species (ROS) are essential intermediates in oxidative metabolism. Nevertheless, when oxidative stress occurs, ROS are generated in excess and consequently may damage cells by oxidizing lipids, disrupting DNA and proteins. The intracellular amounts of ROS were measured by a fluorometric assay with 2,7-dichlorofluorescein diacetate (DCFH-DA) used extensively to monitor oxidation in biological systems as a well-established compound to detect and quantify intracellular produced such as superoxide radical, hydroxyl radical, and hydrogen peroxide [18–20]. The conversion of the non-fluorescent (DCFH-DA) to the highly fluorescent 2,7-dichlorofluorescein product (DCF) (λ_{max} = 522 nm) happens in many steps. The fluorescent probe, after diffusing in the cell membrane, is hydrolysed by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH), which is trapped inside the cells then oxidized to fluorescent DCF through the action of peroxides in the presence of ROS [21]. HCT-116 cells were seeded on 24-well culture plates (Polylabo, France) at 10^5 cells/well for 24 h of incubation. After, the cells were incubated with DDVP alone or combined to CRO (50, 100 and 250 μM), for 24 h at 37 °C. After incubation, cells were treated with 20 μM DCFH-DA. Intracellular production of ROS was measured after 30 min incubation at 37 °C by fluorometric detection of DCF oxidation on a fluorimeter (Biotek FL 800×) with an excitation wavelength of 485 nm and emission wavelength of 522 nm. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly.

2.5. Protein extraction

Cells (10^6 cells/well) were cultured 24 h in six-well multidishes (Polylabo, France) at 37 °C, then cultures were incubated 24 h at 37 °C in the presence of DDVP with/without CRO (50, 100 and 250 μM). Cells were rinsed with ice-cold PBS, scrapped, collected in a lysis buffer (Hepes 0.5 M containing 0.5% Nonidet-P40, 1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.4) and incubated 20 min in ice before centrifugation. Protein concentrations were determined in cell lysates using Protein BioRad assay [22].

2.6. Measurement of superoxide dismutase (SOD) activity

SOD activity was determined according to the method described by Marklund and Marklund [23] by assaying the autooxidation and illumination of pyrogallol at 440 nm for 3 min. One unit of SOD activity was calculated as the amount of protein that caused 50% pyrogallol autooxidation inhibition. The SOD activity is expressed as U/mg protein.

2.7. Measurement of catalase (CAT)

CAT activity was measured according to the method described by Aebi [24] by assaying the hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25 °C. The activity of catalase was calculated using the molar extinction coefficient (0.04 mM^{-1} cm^{-1}). The results were expressed as μmol/min/mg protein.

2.8. Lipid peroxidation

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa et al. [25]. Cells were seeded on 6-well plates at 7.5×10^5 cells/well. After 24 h of incubation, they were exposed to DDVP with/without CRO (50, 100 and 250 μM), for 24 h at 37 °C. Cells were then collected and lysed by homogenization in ice-cold 1.15% KCl. Samples containing 0.1 ml of cell lysates were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4 ml with distilled water and heated to 95 °C for 120 min. After cooling to room temperature, 5 ml of mixture of *n*-butanol and pyridine (15:1, v:v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the supernatant fraction was isolated and the absorbance measured at 546 nm. The concentration of MDA was determined according to a standard curve.

2.9. Mitochondrial membrane potential (MMP) assay

The uptake of the cationic fluorescent dye rhodamine-123 has been used for the estimation of mitochondrial membrane potential [26]. In a typical experiment, the seeded cell in 96-well culture plates were treated with DDVP alone or combined to CRO for 24 h, then cells were carefully rinsed with phosphate-buffered saline (PBS), and 100 μl of rhodamine-123 (1 μM) in PBS was replaced on the plates. Cells were returned to the incubator (37 °C, 5% CO_2) for 15 min. Next, the supernatant PBS (containing unuptaked rhodamine-123) was removed and replaced by fresh PBS. The uptake rhodamine-123 was measured by fluorimetric detection. The

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