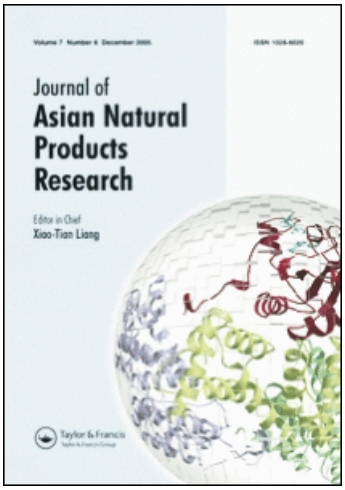

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Evidence of crocin against endothelial injury induced by hydrogen peroxide *in vitro*

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Crocin, the digentiobiosyl ester of crocetin, was investigated for its cytoprotective effect on hydrogen peroxide-induced injury in bovine aortic endothelial cells (BAECs). The morphology of BAECs was observed by inverted phase contrast and electron microscopy. The MTT assay was used to measure cell viability. Cell apoptosis was evaluated by DNA argarose gel electrophoresis. The cells treated with H_2O_2 (200 μM) showed apoptotic changes as revealed by cell shrinkage, condensation of nuclei, membrane blebbing and formation of apoptotic body. A concentration-dependent inhibition of cell injury was seen in cultures treated with crocin at dosages ranging from 1 to 10 μM. Furthermore, in the H_2O_2-treated group, agarose gel electrophoresis displayed a "DNA ladder". Whereas in the 10 μM crocin-pretreated group, cells remained intact and no "DNA ladder" was observed in agarose gel electrophoresis. Only very little DNA debris appeared on DNA-fragmentation analysis in the 1 μM crocin-pretreated group. Our data demonstrated that crocin has preventive effects on the cell apoptosis induced by H_2O_2, which may contribute to its utilisation for cardiovascular diseases (e.g., atherosclerosis and hypertension).

Keywords: Crocin; Bovine aortic endothelial cells; Hydrogen peroxide; Apoptosis

1. Introduction

Crocus sativus L. has been used in Chinese traditional medicine for anodyne, tranquid and emmenagogue [1]. Studies on the isolation and purification of its chemical components showed that crocin (crocetin di-gentiobiose) (figure 1) is one of its main bioactive ingredients, which has been shown to be effective in treating atherosclerosis [2]. However, a precise mechanism for this biological function has not yet been clearly delineated.

Accumulating evidence suggests that the endothelial cell dysfunction is associated with the pathogenesis of atherosclerosis. Endothelial cells cover the lumen of blood vessels and serve as a barrier between blood and vessel wall/tissues. Vascular endothelial

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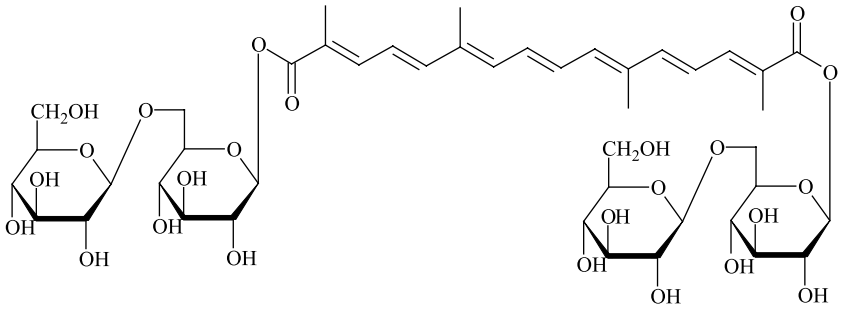

Figure 1. Chemical structure of crocin.

cells are highly vulnerable to injury caused by reactive oxygen species (ROS), because they are in close contact with the constituents of blood (e.g., leucocytes) and activated leucocytes release ROS (such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$)) for biophylaxis [3]. It is the damaged endothelial cells (endothelial dysfunction) that facilitate adhesion of platelets and other blood cells to the vascular wall and can be considered an initial cause of atherogenesis [4]. Considering that endothelial cells are subjected to ROS injury and the relationship between endothelial dysfunction and atherosclerosis, we hypothesised that crocin might function as an anti-oxidant to protect endothelial cells against ROS injury. In order to examine this issue, we used an *in vitro* model of ROS injury by exposing cultured bovine aortic endothelial cells (BAECs) to oxidative stress induced by incubation with hydrogen peroxide to investigate whether crocin has an endothelial protective effect, i.e., is capable of increasing endothelial resistance towards ROS damage.

2. Results and discussion

2.1 Crocin blocks H_2O_2-induced changes in morphology

The effect of crocin on H_2O_2-induced cell death is shown in figure 2. When cells were incubated with H_2O_2 (200 μM), they began to shrink and became round progressively (figure 2B). Some cells detached from the dish and floated in the culture medium. The condensation of nuclei, membrane blebbing and formation of apoptotic body were observed under further electron microscopy inspection (figure 3). These observations did not occur in cells pretreated with 10 μM crocin, whose morphology remained intact (figure 2C) and was in the vicinity of normal cells in RPMI-1640 medium conditions (figure 2A). Crocin alone had no effect on the morphology of BAECs (data not shown).

2.2 Effect of crocin on H_2O_2-induced cell viability inhibition

The exposure of BAECs to hydrogen peroxide for 4 h produced an obvious decrease in the cell viability, as assayed by MTT. A concentration-dependent inhibition of cell injury was seen in cultures pretreated with crocin for 6 h at dosages ranging from 0.1 to 10 μM. The cell survival was progressively ameliorated, as shown in table 1.

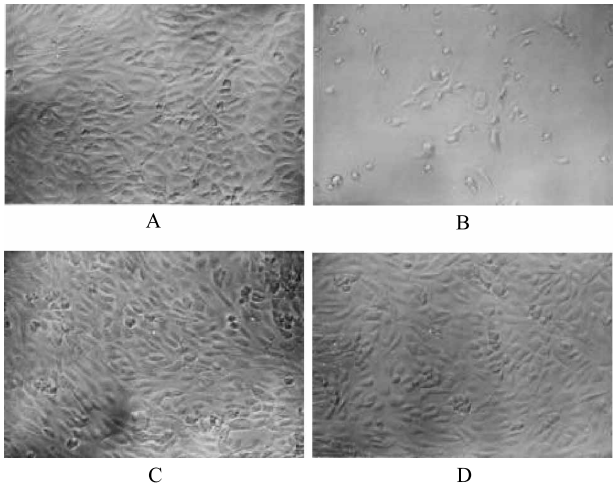

Figure 2. Morphological appearance of BAECs treated with vehicle (A), H_2O_2 (200 μM) (B), 1 μM crocin plus H_2O_2 (200 μM) (C) or 10 μM crocin plus H_2O_2 (200 μM) (D) under phase-contrast microscopy (× 100).

2.3 DNA fragmentation

Detection of internucleosomal DNA fragmentation provides a sensitive method to monitor events in apoptosis. At 200 μM concentration H_2O_2 was able to induce apoptosis of BAECs, as evident by prominent DNA laddering. When BAECs were incubated with 1 μM crocin before exposure to hydrogen peroxide, no DNA ladder and only a small amount of debris could be observed in agarose gel electrophoresis. At the higher concentration of 10 μM,

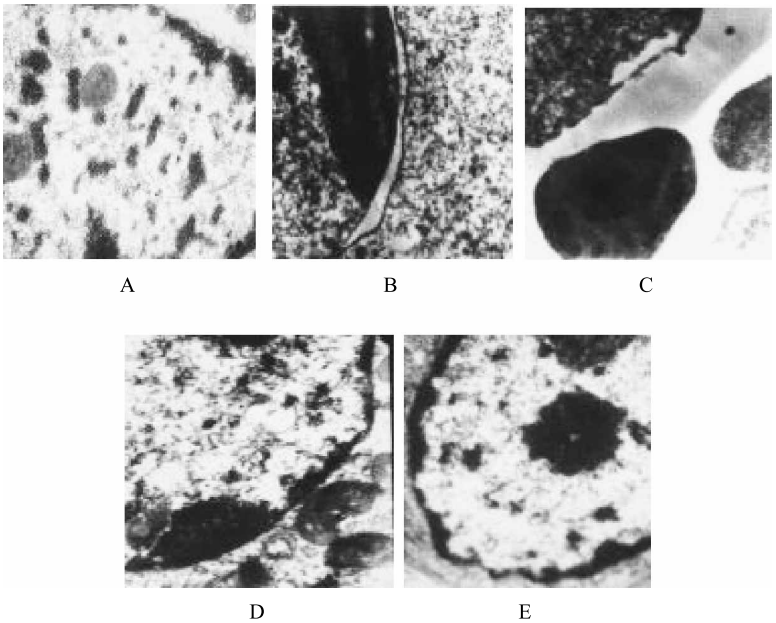

Figure 3. Ultrastructure of BAECs under electron microscopy (× 6000). (A) Normal BAECs; (B) apoptotic BAECs showing cytoplasm condensation of chromatic border; (C) apoptotic BAECs showing formation of apoptotic body; (D) BAECs pretreated with 1 μM crocin; (E) BAECs pretreated with 10 μM crocin.

Table 1. Effect of crocin on cell survibal induced by hydrogen peroxide in BAECs.

Agents (μM)	*A_{570}*	*% Survival*
Control	1.38 ± 0.17	–
H_2O_2	0.70 ± 0.10##	50.7
Crocin 0.1 + H_2O_2	0.62 ± 0.06	44.9
Crocin 1 + H_2O_2	0.91 ± 0.13*	65.9
Crocin 10 + H_2O_2	1.20 ± 0.14**	87.0

Cells were treated with or without different concentrations (0.1–10 μM) of crocin for 6 h prior to exposure to hydrogen peroxide (200 μM) for 4 h ($\bar{x} \pm s$, $n = 6$).
##$p < 0.01$ vs. control; *$p < 0.05$, **$p < 0.01$ vs. H_2O_2 group.

crocin almost blocked H_2O_2-induced apoptotic cell death completely, as the DNA ladder was not seen and DNA degradation profiles were comparable to that of the control (figure 4).

It has been recognised now that endothelial cells have many important physiological functions in the cardiovascular system [5]. Under physiological conditions, all cytokines secreted by vascular endothelial cells maintain a dynamic balance, and preserve the endothelial integrity and regular function. Under pathological conditions, the endothelial integrity is reduced (endothelial dysfunction), which initiates the process of atherogenesis.

Due to their special localisation, endothelial cells are highly vulnerable to injury caused by ROS. Evidence indicates that an exposure to high levels of H_2O_2 results in immediate cell necrosis, whereas exposure to low levels results in cell apoptosis [6]. In this study, the morphological observation showed that the 200 μM H_2O_2-treated cells did not collapse but shrunk and became round. Some cells detached from the dish and floated in the culture medium. Further electron microscopy observation demonstrated that cell injury caused by 200 μM H_2O_2 occurred by apoptosis, as revealed by condensation of nuclei, membrane blebbing and formation of apoptotic body. We then used agarose gel electrophoresis to study the effect of crocin on hydrogen peroxide-induced apoptosis. Results showed that agarose gel electrophoresis in the H_2O_2 model group displayed a "DNA ladder". The 10 μM crocin-pretreated cells remained intact without a DNA ladder. Only a little DNA debris was observed on DNA fragmentation analysis in the 1 μM crocin-pretreated group. All these results suggested that 10 μM crocin could strongly protect H_2O_2-treated cells from apoptosis, but 1 μM crocin only to a minor extent. Based on these findings and our previous report [7],

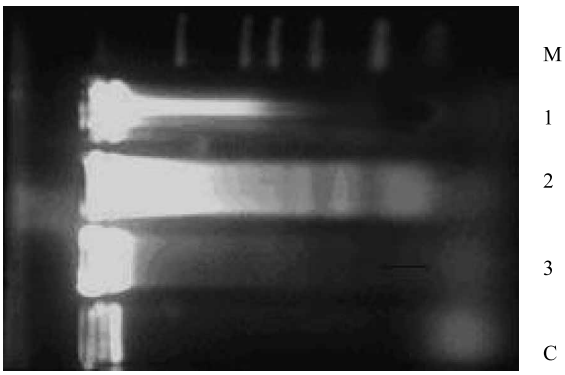

Figure 4. Gel electrophoretic analysis of BAECs DNA treated with H_2O_2 and different concentrations of crocin, respectively. Lane M: DL 2000 marker; lane C: control. Lane 2 is 200 μM H_2O_2 alone; lanes 1 and 3 are 1 μM and 10 μM crocin + 200 μM H_2O_2, respectively.

we conclude that our results are consistent with the above study and crocin might thus increase cell viability through antagonising apoptosis.

In this sense, one question arises: how can crocin protect endothelial cells from oxidative injury? It has been reported that H_2O_2, as a member of ROS, injures cell via lipid peroxidation and formation of hydroxyl radical [8,9]. Crocin is a water-soluble carotenoid with a long unsaturated carbohydrate chain, which is known to have a good anti-oxidative activity. It has been found that crocin reacts with free radicals such as lipid peroxyl radical LOO· to form an adduct (LOO-Cro˙) and lipid radical L˙ to form an adduct (L-Cro˙), and may play a role in inhibiting lipid peroxidation in cell membranes [10]. Furthermore, carotenoid may act as an anti-oxidant by scavenging not only the peroxyl radical but also the hydroxyl radical. So the anti-apoptotic effect of crocin can occur since: (1) crocin may function as a chain-breaking lipid radical scavenger to interrupt the chain reaction of lipid peroxidation; (2) it may reduce the injury caused by OH· via quenching the hydroxyl radical [11].

There is evidence to show that vessel injury resulting from endothelial cell apoptosis induced by low concentrations of H_2O_2 is more harmful than that from necrosis induced by high concentrations, because the apoptotic endothelial cell overlaying the vessel wall affects the endothelium-dependent relaxation and endothelial regeneration, and reduces the integrity of the endothelial cell lining, which is conducive to atherogenesis [6]. In the present study, we found that crocin could prevent endothelial cells from apoptosis induced by low concentrations of H_2O_2, which suggested that treatment of atherosclerosis by crocin might correlate with its anti-apoptotic effect.

It is reported that crocin inhibits neuronally differentiated PC12 cell apoptosis induced by both internal and external apoptotic stimuli [1]. The results from our study suggest that crocin has a beneficial effect on inhibiting endothelial apoptosis in the condition of H_2O_2-induced oxidative stress, which is consistent with the above-mentioned anti-apoptotic effect of crocin on PC12 cells. Although the detailed mechanism by which crocin acts remains unknown, it is suggested that the anti-oxidant and free radical scavenging effect may be involved in its cytoprotective effect. Future experiments conducted on the effects of crocin on apoptotic gene/protein modulation, e.g., the expression of Bcl-2 family proteins, release of cytochrome *c* and activation of caspase-3, would be worthwhile and might provide useful information.

In summary, crocin attenuates endothelial injury through inhibition of apoptosis caused by H_2O_2, and the treatment for atherosclerosis by crocin may correlate with its cytoprotective effect.

3. Experimental

3.1 Materials

Crocin was extracted and purified by Professor Zhi-Yu Qian (China Pharmaceutical University, HPLC $>98\%$), then dissolved in phosphate buffer. The chemical structure of purified crocin is illustrated in figure 1. Newborn bovine serum was provided by Hangzhou Si Ji Qing Co. Ltd, and other chemicals were of analytical grade.

3.2 Cell culture

Bovine aortic endothelial cells were isolated as previously described [12]. The cells were cultured on gelatin-coated plastic dishes in RPMI-1640 medium supplemented with 10%

heat-inactivated fetal bovine serum (Gibco), 100,000 U/L penicillin and 100 mg/L streptomycin (Gibco) at 37°C in a humidified CO_2 (5%) incubator.

3.3 Morphological observation

Light microscopy: Morphological characteristics of the cells were directly observed and photographed under an inverted phase contrast microscope.

Electron microscopy: Electron microscopy of BAECs was carried out on samples fixed in phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated, and embedded as described previously [13]. Thin sections were cut and stained with uranyl acetate and lead citrate, then examined under a Phillips 201 electron microscope from which transmission electron micrographs were produced.

3.4 Viability assay

The cell viability was measured by the MTT assay [14]. 20 μl of the stock solution of 3-[4,5-dimethylthiazol -2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml) (Fluka) was added to each well and incubated for 4 h. 150 μl of the solubilisation solution was added to each well. The optical density was determined by measuring light absorbance at 570 nm with an ELISA plate reader (Hua Dong Electronic Co., Nanjing, China). All MTT assays were performed in six replicated wells. Crocin-treated cells were pretreated for 6 h with crocin, then exposed for 4 h to 200 μM H_2O_2. Non-crocin-treated cells were also incubated under the same conditions as those used in the experimental protocols with crocin.

3.5 DNA fragmentation

Experiments were performed as described previously [15]. In brief, Cells (1×10^6) were incubated in a digestion buffer that contained 0.2 mg/ml proteinase K at 50°C for 5 h. The cellular DNA was extracted and digested with RNase (final concentration, 0.6 mg/ml) at 37°C for 30 min, then the sample was subjected to electrophoresis on a 2% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The gel was then stained with ethidium bromide and photographed on a UV transilluminator.

3.6 Statistical analysis

Data are expressed as mean ± standard deviation of *n* experiments. The statistical difference was analysed using Student's *t*-test.

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