RESEARCH COMMUNICATION

DNA Fragmentation and Cell Cycle Arrest: A Hallmark of Apoptosis Induced by Crocin from Kashmiri Saffron in a Human Pancreatic Cancer Cell line

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Abstract

Background: Apoptosis, a widely important mechanism that contributes to cell growth reduction, is reported to be induced by *Crocus sativus* in different cancer types. The present study was designed to elucidate apoptosis induction by crocin, a main component of *Crocus sativus* in a human pancreatic cancer cell line (BxPC-3). Methods: Cell viability was measured by MTT assay, Hoechest33258 staining was used to detect the chromatin condensation characteristic of apoptosis, and DNA fragmentation was assessed by gel electrophoresis and cell cycle analysis by flow cytometry. Results: Crocin induced apoptosis and G1-phase cell cycle arrest of BxPC-3 cells, while decreasing cell viability in a dose dependent and time dependent manner. Cells treated with 10μg/L crocin exhibited apoptotic morphology (brightly blue-fluorescent condensed nuclei on Hoechst 33258 staining) and reduction of volume. DNA analysis revealed typical ladders as early as 12 hours after treatment indicative of apoptosis. Conclusion: Our preclinical study demonstrated a pancreatic cancer cell line to be highly sensitive to crocin-mediated growth inhibition and apoptotic cell death. Although the molecular mechanisms of crocin action are not yet clearly understood, it appears to have potential as a therapeutic agent.

Asian Pacific J Cancer Prev, **11**, 675-679

Introduction

Pancreatic cancer is a leading cause of cancer-related deaths with extremely poor prognosis (Niederhuber et al., 1995, Jemal et al., 2005). The low survival rate is due to insensitivity of pancreatic cancer to most of oncologic therapies such as chemotherapy, radiotherapy and immunotherapy (Ghaneh et al., 2007, Sarkar et al., 2007). New therapeutic strategies are therefore urgently needed to combat with this deadly form of cancer.

Natural products from plant sources have extensive past and present use in treatment of diverse diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification. The importance of natural products in modern medicine has been well recognized. More than 20 new drugs, launched world over between 2000 and 2005, originate from natural products. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases infectious and non-infectious (Chin et al., 2006).

Crocus sativus is a plant of the iris family (Iridaceae) and its flower contains various chemical constituents (Abdullaev and Espinosa Aguine, 2004). Stigmas of the flower (saffron) contain crocin, anthocyanin, carotene and lycopene (Giaccio et al., 2004), and these constituents are known to have various pharmacological effects on different illness, including anti-tumor effects by inhibition of cell growth (Abdullaev et al., 1993).

Crocin, a main constituent of the CSE, exhibits a variety of pharmacological effects in mice including inhibition of skin tumor growth (Konoshima et al., 1998), improvement of learning behavior previously impaired by ethanol (Abe et al., 2000), prevention of long-term potentiation inhibition caused by ethanol (Abe et al., 2000), anti-hyperlipidemic effects (Lee et al., 2005), therapeutic efficacy for colon adenocarcinomas in rats (Garcia-Olmo et al., 1999) and anti-atherosclerotic effects (He et al., 2005) .

In recent past, some of our work on Crocin have shown potential free radical scavenging and lipid peroxidation

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inhibition (Hamid Bakshi et al., 2009), Cytotoxic and apoptogenic effect of 'CS' on different Human cancer cell lines (Bakshi et al., 2008) and *in vitro* and *in vivo* xenograft inhibition of Daltons lymphoma (Bakshi et al., 2009).

Apoptosis, or programmed cell death, is an active, gene directed form of cell death that is different from cell necrosis with respect to its morphology, biochemistry, pharmacology and biological significance. Many types of mammalian cells undergo apoptosis during normal development or in response to a variety of stimuli, including DNA damage, growth factor deprivation, and abnormal expression of oncogene or tumor suppressor genes (Raff et al., 1993; Thompson et al., 1995; Vaux and Strasser, 1996). Apoptosis is a widely accepted, important mechanism that contributes to cell growth reduction. *Crocus sativus* has been shown to induce apoptosis in different cancerous cell types (Bakshi et al., 2008).

The present study was undertaken to explore whether crocin induces apoptosis in pancreatic cancer cell line, which exhibit aggressive clinical behavior. We studied the effect of crocin on the growth in culture of Human pancreatic cancer cell line (BxPC-3).

Materials and Methods

Chemical and Reagents

RPMI-1640(GIBCO) medium, Fetal calf serum, Trypsin-EDTA solution, 3-(4,5 dimethylthiazole-2-yl)-2,5-Dimethyl tetrazolium bromide(MTT), Hoechest 33258, penicillin/streptomycin 100units, Dimethylsulfoxide(DMSO), Sodium bicarbonate, Calcium chloride, HEPES Buffer, Actionmycin D, propidium iodide (sigma), Sodium chloride and disodium hydrogen phosphate were purchased from Merck, USA. All regents were prepared using deionized (Millipore) distilled water. All other fine chemicals were obtained from Sigma, Mo, USA. All other HPLC and analytical grade solvents and silica gel required for column purification and TLC were obtained from SISCO Research Laboratories, India. The pre-coated TLC plates mesh size 60-120 was obtained from E-MERCK India.

Plant Material

Plants were collected from Pampore (in south of Kashmir province, India) in October and dried in shadow and ground. The *C. sativus* L. was identified by Department taxonomist and voucher samples were preserved for reference in the herbarium of laboratory under (BUBT-1705).

Isolation of Crocin from C. Sativus

Crocin was isolated from saffron by previously described method (Bakshi et al., 2008) with some modifications. Briefly, 500-mg saffron was washed thrice with 20-mL ethyl ether, and the residual ether was evaporated in air. It was then suspended in 15 mL of 30% methanol (v/v) in distilled water and stirred for 5 minutes at room temperature. The extract was filtered through a 0.45-μm Millipore filter. It was then diluted with 10 mmol/L phosphate-buffered saline (PBS, pH=7.4), and the concentration of crocin was adjusted to 25 μmol/L, using the coefficient e443=89,000 M^{-1} cm^{-1} reported for crocin in aqueous solution (Lussignoli et al., 1999). Crocin was stored at -20 °C in the dark for a maximum of 2 months (Tubaro et.al, 1998). The compounds were characterized on the basis of ^{1}HNMR, ^{13}CNMR mass spectral data (data not shown) and standardized by HPLC (Figure 2).

Cell culture

Human pancreatic cancer cell line BxPC-3 (ATCC, USA) (generous gift from Dr. K Menon, KARD Scientific, Boston (USA)) was grown in RPMI-1640 medium containing 10% fetal calf serum (FCS), supplemented with 100 units/mL penicillin and 100mg mL streptomycin in a humidified atmosphere in 5% CO_2 at 37°C.

Cell Viability

The viability of cells was assessed in the assay as described previously (Bakshi et al., 2008). Briefly, MTT was added to cells at a final concentration of 0.5g/L. Cells were incubated at 37°C for 5 hours , the medium was aspirated, and the formazan productwas solubilized with dimethyl sulfoxide(DMSO). Absorbance at 630nm (background) was subtracted from absorbance at 570nm.

Morphological Detection of Apoptosis

Cells were fixed for 5 minutes in 3% Para formaldehyde in phosphate buffered saline. After air dying, cells were stained for 10 minutes in Hoechst 33258 (10/L), mounted in 50% glycerol containing 20mmol/L citric acid and 50mmol/L orthophosphate, and stored at -20°C before analysis. Nuclear morphology was evaluated using a Zeiss IM 35 fluorescence microscope.

Cell cycle analysis

For cell cycle analysis, $1x10^6$ BxPC-3 cells were washed twice with phosphate buffered saline (PBS) and then incubated with PI(Propidium iodide)solution (0.5mL

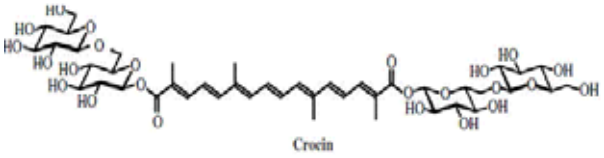

Figure 1. Chemical Structure of Crocin

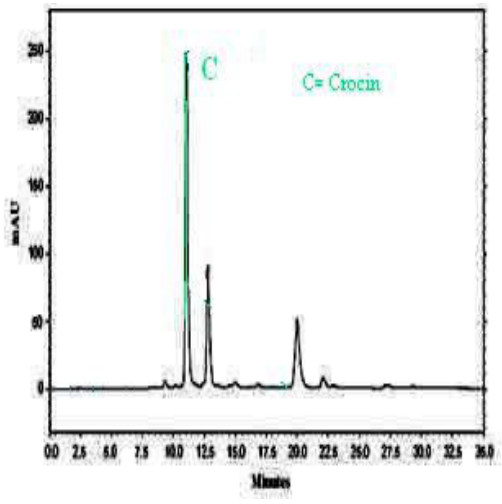

Figure 2. HPLC Profile of Crocin from Crocus

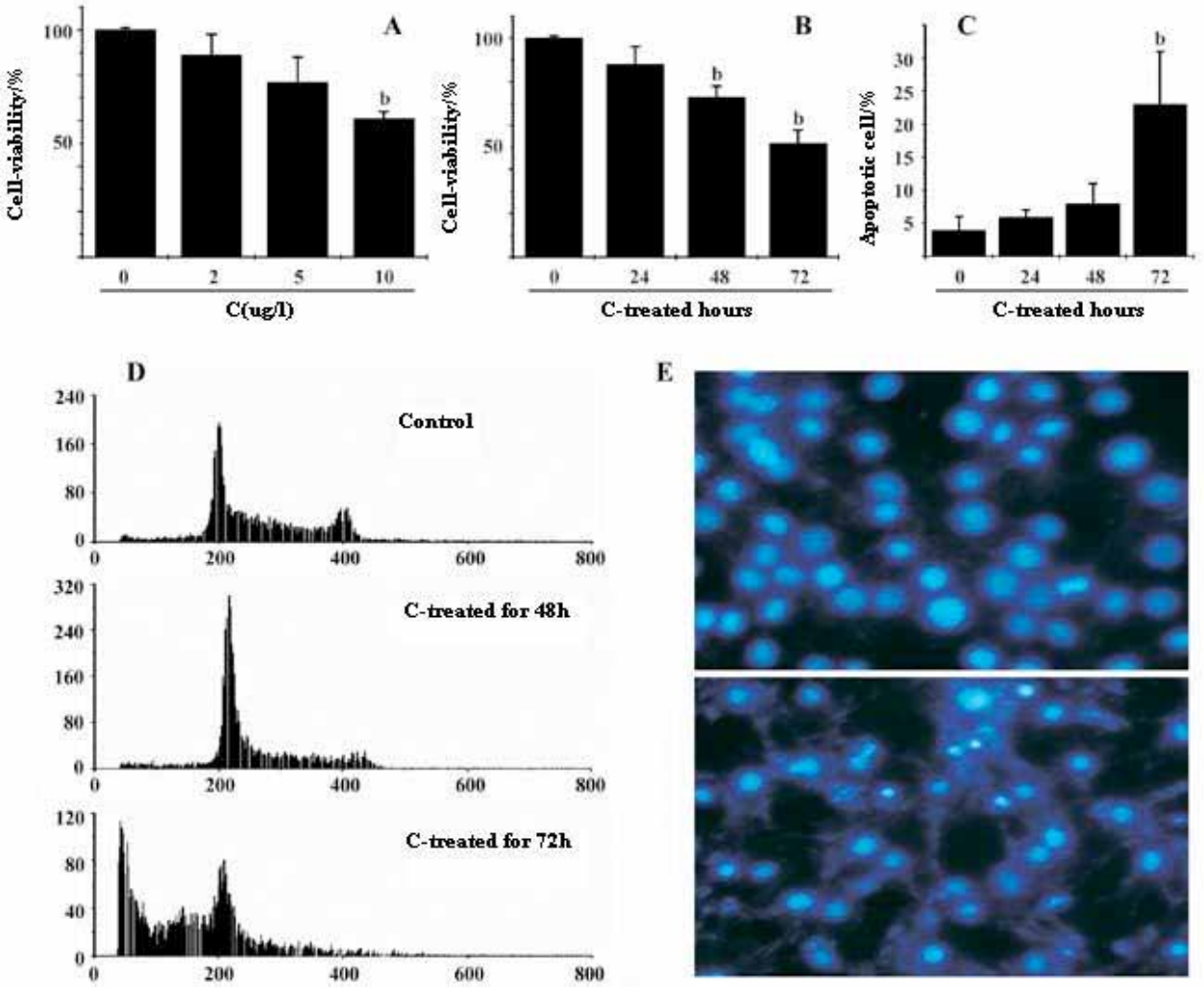

Figure 3. Induction of Apoptosis and Cell Cycle Arrest. (A) A dose-dependent effect of crocin on cell viability. (B) A time-dependent effect of crocin 10μg/L on cell viability. (C) Apoptosis assay of cells incubated with crocin by Hoechst staining, n=4. Mean±SEM, bP<0.05 vs control. (D) Cell cycle analysis using flow cytometry. Cells were incubated with crocin 10g/mL for 48 or 72 hours. (E) Morphology of apoptotic cells control vs treated. Hoechst stain x400.

of 3.4mmol/L sodium citrate, 10mmol/L CaCl, 0.1% NP-40 , and 50mg/L of PI) for 30 minutes. Fluorescence intensity was measured using flow cytometry (PAS system PARTEC, Germany). For each sample 10,000 cells were analyzed using program M cycle software (Coulter).

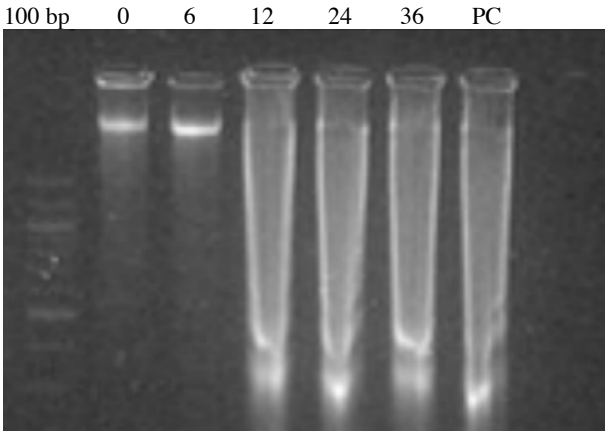

Figure 4. Agrose Gel Electrophoresis Demonstrating DNA Fragmentation. BxPC-3 cells treated with 10μg/mL crocin for 0, 6, 12, 24, 36h, induced DNA fragmentation in time dependent manner from 12 hours. Actinomycin D for 36h was a positive control (PC) at a concentration of 10μg/mL

DNA Fragmentation Analysis

Internucleosomal cleavage of DNA was analyzed as described (Kim et al., 2001). Briefly cells were seeded in 6-well plates at a concentration of 1x10^6 per ml of medium. The cells were treated with 10μg/mL of crocin. About 10μg/mL of actinomycin D was added as positive control. Cells were harvested at appropriate time points and DNA was analyzed using gel electrophoresis.

Statistical Analysis

The statistical significance of the difference were evaluated by applying analysis of variance (ANOVA) and two tailed Students t-test.

Results

Cell viability

Crocin induced apoptosis of BxPC-3 cells; Crocin decreased the cell viability in a dose-dependent manner (Figure 3A). Treated with crocin at 10g/L, Crocin

decreased the cell viability in a time-dependent manner (Figure 3B). The number of apoptotic cells was significantly increased after the treatment of crocin for 72 h. Within the incubation time (48 h), we could not detect any sign of apoptosis, although (Figure 3B) showed the cell viability was decreased 48 h after the crocin treatment. However, MTT assay has been used as an assay for proliferation like the method of thymidine incorporation. This suggests the existence of crocin also contributes to the anti-proliferative potential in Bx-PC-3 cells.

Cell cycle analysis

The treatment of crocin for 2d increased the G1 fraction of Bx-PC-3 cells. The arrest of cell cycle (G1 phase) which was obtained 48h later was less pronounced but the increase of sub G1 fraction cells became much more apparent at later time point: 72h (Figure 3D).

Morphological detection of Apoptosis

Crocin induced apoptotic pattern within 72-h incubation (Hoechst staining) (Figure 3E). Our data suggest that crocin-induced accumulation of cells in G1 stage may lead to apoptosis in later stage (eg, 72h) in Bx-PC-3 human pancreatic cancer cells.

DNA fragmentation analysis

To elucidate whether the crocin inhibits Bx-PC-3 cell proliferation through induction of apoptosis; we examined the cell death by DNA fragmentation from 0 to 36 hours and. A time and dose dependant DNA fragmentation was observed in Bx-PC-3 cells treated with crocin (Figure 4). The dose-response studies for DNA fragmentation as shown in (Figure 3) revealed that the IC50 concentration of 10μg/ml of crocin was optimum enough to induce fragmentation in Bx-PC-3 cells. Time point studies from 0 to 36 hours demonstrate typical DNA "Ladder" pattern in Bx-PC-3 cells treated with 10μg/ml of crocin from 24h onwards till 36 hours.

Discussion

Agents capable of inducing apoptosis, inhibiting cell proliferation, or modulating signal transduction are currently used for the treatment of cancer. A combination of multiple chemo-preventive agents or agents with multiple targets is considered to be more effective than a single agent (Guzman M, 2003).

Crocus sativus has been shown to induce apoptosis in human cancer cell lines (Hamid bakshi et al., 2008). Crocus sativus has been used to treat several medical conditions, such as gastrointestinal disorders, urological infections, as well as in treating malignancies (Nair et al., 1991; Rios et al., 1996; Winterhalter et al., 2000). *Crocus sativus* contains components like safranal, alpha crocin or crocin is the major component. Additionally *Crocus sativus* also contains amino acids, flavoniods and other chemical compounds (Nair et al., 1994; Winterhalter et al., 2000). Among these, crocin is the most important since it is the major component in *Crocus sativus* and has shown significant biological activities (Winterhalter et al., 2000).

The result of present study demonstrated that crocin, a principal component present in *Crocus sativus*, is strong suppressor of human pancreatic cancer Bx-PC-3 cell proliferation. It is important to point out that growth inhibitor effect of crocin were observed at concentration that may be generated through dietary intake. We also found that crocin exerts activity against proliferation of Bx-PC-3 cells by arresting cells in the G1 phase of cell cycle and causing apoptosis. However exact molecular mechanism of crocin action is not yet clearly understood. It is ongoing work in our laboratory and soon we will come up with systematic explanation of mechanism of crocin action.

In summary, data from this study suggest that crocin from CS may be efficacious in treating pancreatic cancer. However, before coming to conclusive statement more research will be needed to fully delineate the part they play in cancer and molecular mechanism of crocin action, which is not yet clearly understood. However, it appears to have potential as a therapeutic agent.

Acknowledgement

Authors are highly thankful to Director MHRT Hospital and for providing facilities and support.

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