International Journal of Natural and Engineering Sciences
E-ISSN: 2146-0086 13 (3): 83-86, 2019

Cytotoxic Effects of Crocin on Glioma Cells

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Received: 03 September 2019
Accepted: 01 December 2019

Abstract

A large number of primary brain tumors are glial tumors and glioblastoma multiforme is the most dangerous of the group. Treatment of glioma cells is very difficult because of its rapid proliferation, and resistance to chemotherapeutic drugs used for various reasons.

We investigated the morphological and antiproliferative activity of 100 to 4000 µM crocin in C6 and T98G glioma cells for 24 and 48 hr. Morphological changes in C6 cell started from the crocin concentration of 2500 **µM**. However, the crocin concentrations did not cause any morphological changes in T98G cells. We also observed that the cytotoxic effect of crocin on C6 cells at the end of 24 hr of application began at 1500 µM, while T98G cells started at 3500 µM ($p<0,05$). The IC_{50} values of crocin on C6 cells were calculated as 3360 and 2460 µM for 24 and 48 hr respectively. Although cell viability decreased in T98G cells, IC_{50} value could not be calculated for the two different time periods. The crocin was more effective in the C6 cells than the T98G cells and it was observed that the time and concentration increased the suppressive effect of the cell proliferation. The mechanisms of suppression of glioma cells proliferation by crocin should be investigated.

Keywords: Glioma, Cytotoxicity, Crocin

INTRODUCTION

Glioblastoma multiforme (GBM) is the most dangerous and difficult to treat type of brain tumors [1]. The average survival of patients with glioblastoma ranged from 12 to 24 months [2,3]we have started a human immuno-gene therapy study. The goal is to study the effects of immunisation with autologous tumour cells expressing gene sequences for human interferon-γ. For more than two decades we have sought for efficient treatment against malignant gliomas. Our most successful treatment in the animal models is immuno-gene therapy where murine genes for the cytokines IFN-γ, IL-7 and B7-1 were chosen for their ability to stimulate different stages of the pathway for cytotoxic T lymphocyte (CTL. Therefore researchers are constantly trying to research and develop more natural, less toxic and more effective new drugs.

Although cancer is treated by methods such as radiotherapy or chemotherapy, in recent years, it is thought that natural compounds can be used in the treatment of cancer because of their various advantages such as low side effects, high potential efficacy and low toxicity [4]. Natural compounds Saffron is the worldwide name of *Crocus sativus L*. plant of the genus Crocus [5]. The natural compounds derived from saffron are known to be used for relaxing, dyspnea, anti-inflammatory, analgesic purposes in many different societies from ancient China to the present day [6]. There are different bioactive compounds in the saffron plant. Crocin is one of the most active biological molecules found in the saffron. It is a carotenoids that gives color to saffron with crocetin [5]. Different studies demonstrated that crocin have antioxidant, neuroprotective and anti-inflammatory effects [7–9]we examined in vitro the antioxidant properties of extract of C. sativus stigmas and its effect on Aβ1-40 fibrillogenesis. The antioxidant properties were determined by measuring the ferric-reducing antioxidant power and Trolox-equivalent antioxidant capacity, while its effects on Aβ-aggregation and fibrillogenesis were studied by thioflavine T-based fluorescence assay and by DNA binding shift assay. The water:methanol (50:50, v/v.

Recent *in vitro* studies have shown that crocin has a potent inhibitory effect on different cancer cells including colorectal, pancreatic, breast, and different leukemias [10–12]. Furthermore, in vivo studies have shown that crocin decreases tumor growth and therefore prolongs survival time [12,13]. In addition, no toxic effects were observed in a long-term (13 weeks) period upon high-dose (400 mg/kg) administration of crocin in rats [13]. In the literature review, although the effects of crocin on the growth of crocin were studied on different cancer cell lines, there is less information about the cytotoxic effect on the GBM which is the most dangerous of brain cancers. Therefore, in this study, the cytotoxic effects of crocin on the two different GBM cell lines was investigated *in vitro* in a concentration and time dependent fashion.

MATERIALS and METHODS

Cell culturing and treatment

In the experiments, rat glioma (C6) and human glioblastoma multiforme (T98G) cell lines previously purchased from American Culture Collection were used. Cells cultured in a humidified atmosphere of 5% CO_2, at 37 ^{0}C in 25 cm^2 flasks. The culture medium consists of Dulbecco's Modified Eagle's Medium (DMEM), 10 % fetal bovine serum (FBS) and 1 % penicilin-streptomycine solution. Trypsinization has done after glioma cell confluence was achieved with trypsin-ethylene diamine tetra acetic acid (0.25%). Viability was assessed by trypan blue dye exclusion. For each group seeded in 8 wells of a 96 well plates ($2x10^4$ cells/well) and incubated for 24 or 48 hr.

Crocin was dissolved in absolute ethanol / distilled water (1:1) to prepare a 250 mM main stock solution. The stock solution was diluted with DMEM at different rates to obtain 100, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 µM concentrations. The amount of absolute ethanol contained in 4000 µM concentration was added to the medium and its having any effect on cell proliferation was investigated. The working concentrations were prepared immediately prior to assay.

Morphological changes of cells

The morphological changes that occurred after crocin

treatment on cells were evaluated by inverted microscope. Glioma cells ($1x10^6$) in 5 mL media were seeded to 25 cm^2 flasks. The cells were treated with 100 to 4000 µM crocin concentrations for 24 hr. Then the morphological effects of crocin were detected under an inverted light microscope (Nikon Eclipse, TC100).

Cell viability assay

Crocin cytotoxicity was detected by MTT assay. MTT solution (20 µL) was added to each well and incubated at 37 ºC for 4 hr. After incubation all medium was replaced with 100 µL DMSO and the formazan crystals were dissolved in each well. Then, the dye absorbance was read at 550 nm using a microplate reader (Bio-Tek Instruments) and the percentages of living cells were calculated [14]. Statistical analyses were done by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. A p value less than 0.05 was considered to be significant.

RESULTS

Morphological changes on C6 glioma cells

In this study, crocin concentrations of 500, 1000, 1500 and 2000 µM did not cause any cellular changes. Decrease in cell number was observed starting at 2500 µM, changed C6 cell morphology (Figure 1). Although there was a slight decrease in cell viability at 3500 and 4000 µM concentrations, no change was observed in T98G cell morphology.

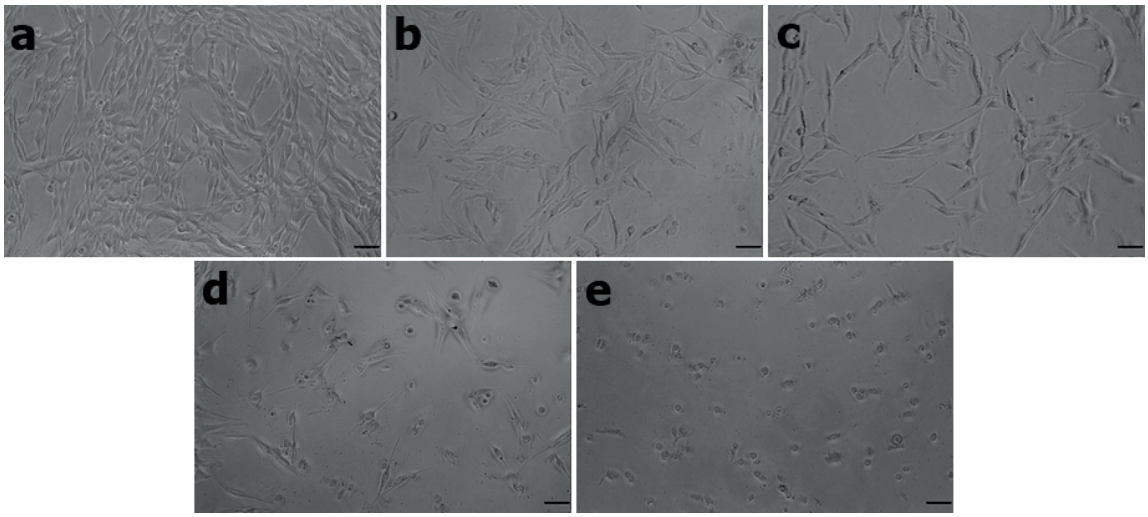

Figure 1: Morphological changes on C6 glioma cells after crocin treatment for 24 hr; a: control cells, b: 2500 µM, c: 3000 µM, d: 3500 µM, e: 4000 µM. Scale bar: 50 µm

Effects of crocin on the survival of C6 cells

It was observed that 100, 250, 500, 750 and 1000 µM crocin concentrations, did not inhibit any suppressive effect on C6 cells ($p>0.05$). However, cell survival rates for the other concentrations decreased by 11, 12, 32, 40, 53 and 68 %, respectively ($p<0.001$). The IC_{50} value of crocin on C6 cells at the end of 24 hr was calculated as 3360 µM.

The treatment of the cells with 100, 250, 500, 750 and 1000 µM crocin did not cause any change in the percentage of cell survival ($p>0.05$). However, 1500, 2000, 2500, 3000, 3500, and 4000 µM crocin after 48 hr culture period reduced the number of viable cells by 12, 21, 52, 53,72 and 82 % as compared to the control, respectively ($p<0.001$). The IC_{50} value on C6 cells was calculated as 2460 µM at the end of 48 hr.

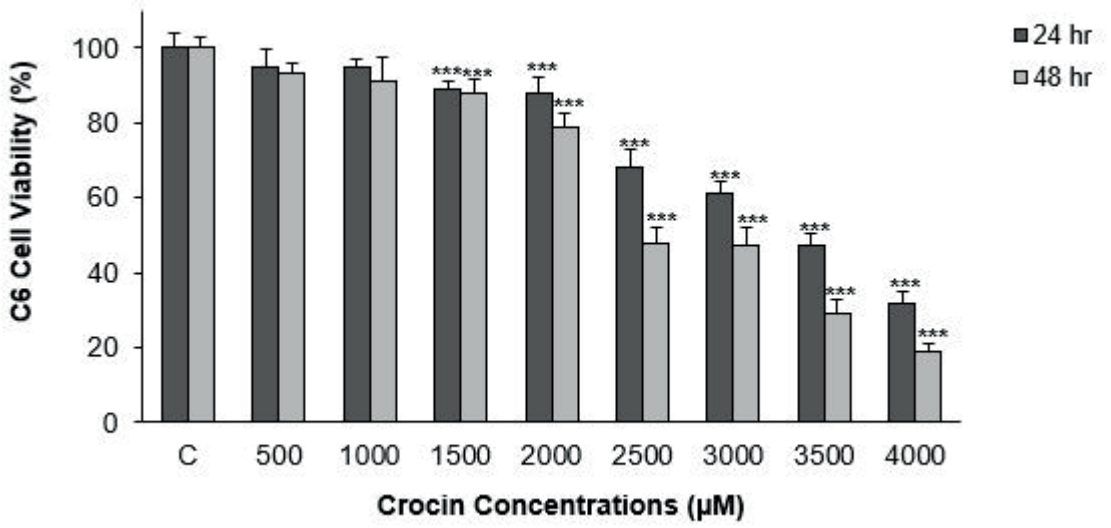

Figure 2: The effect of crocin on C6 survival for 24 and 48 hr. (C: control, ***:$p<0.001$).

Effects of crocin concentrations on the survival of T98G cells

It was determined that only 3500 and 4000 µM concentrations suppressed cell viability of T98G by 11%. ($p>0.05$) for 24 hr. Concentrations of 100 to 1000 **µM** crocin did not also affect T98G cell viability, but 1500, 2000, 2500, 3000, 3500, and 4000 µM crocin ($p<0.001$, Figure 2) decreased T98G cell viability by 15, 18, 19, 20, 23 and 38 % for 48 hr, respectively. However, The IC_{50} values for T98G cells could not be calculated for two different time periods.

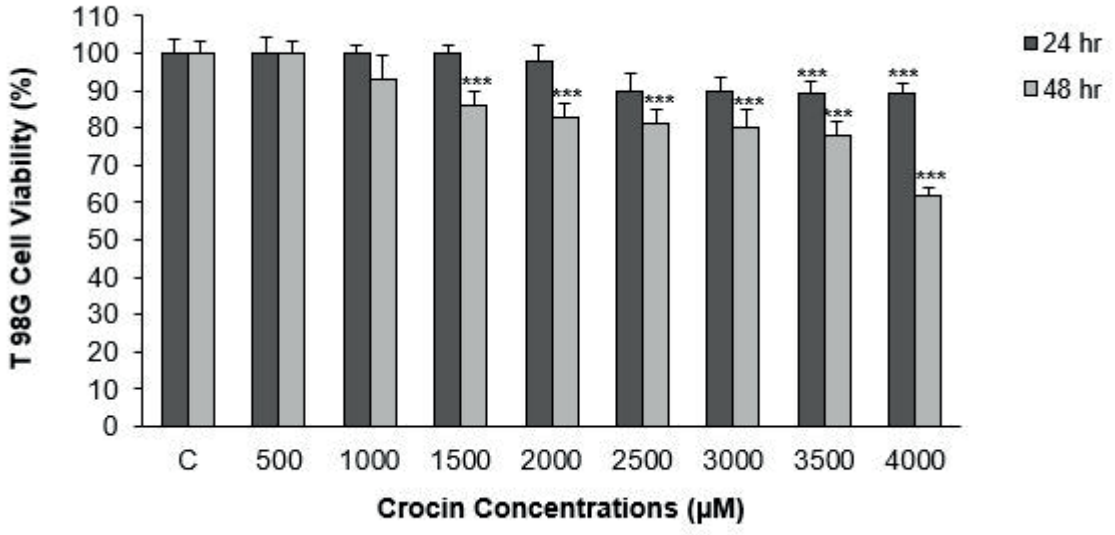

Figure 3: The effect of crocin concentrations on T98G survival for 24 and 48 hr. (C: control, ***:$p<0.001$). Ethanol had no effect on cell viability at all the concentrations tested on both C6 and T98G cells ($p>0.05$).

DISSCUSSION

Glioblastoma multiforme (GBM) is the most dangerous and difficult to treat type of brain tumors [15]. The biggest difficulty encountered in the treatment is the rapid growth of the tumor. Therefore, scientists are searching for more effective drugs for glioma treatment [16]. Crocin is a carotenoid of the saffron extract that exhibits antitumor activity against different cancer cells. However, the effects of crocin on glioma cells in vivo have not been evaluated. For this purpose, we decided to test the possible anti-proliferative effects of crocin concentrations on two different glial carcinoma cells.

In this study, morphological changes caused by the crocin treatment for 24 hr in C6 cells was visualized using an inverted light microscope. Changes in cell morphology and decrease in cell number were observed in C6 cells from a concentration of 2500 µM. However all concentrations of crocin did not have any morphological changes in T98G cells for 24 hr. Escribano et al. (1996) have been reported that crocin concentrations ranging from 0.64 to 10.2 mM for 18 hr causes morphological changes in cervical cancer cells (HeLa) such as cell shrinkage and pycnotic nuclei [17]. García-Olmo et al. (1999) examined the effect of crocin concentrations in the treatment of colon adenocarcinoma on human (HT-29) and rat (DHD/K12-PROb) cells. A significant loss of cytoplasm and cytoplasmic vacuole-like areas were observed in the tested cells. Similarly, in our study, it was observed that intracellular connections were decreased, cytoplasmic content decreased and cells were rounded, especially in C6 cells.

We also observed that the cytotoxic effect of crocin on C6 cells at the end of 24 hr of application began at 1500 µM, while that of T98G cells started at 3500 µM. The inhibitory effect on proliferation in C6 and T98G cells was observed to start at 1500 µM at the end of the 48 hr application. The study showed that crocin demonstrated cytotoxic effect in a concentrations and time dependent manner in both glioma cells. However, high crocin concentrations significantly inhibited C6 cell proliferation when compared to the T98G cells. Previous studies have studied the cytotoxic effect of various crocin concentrations in different types of cancer cell lines. Similar to our study, García-Olmo et al. (1999) examined the effect of crocin in the treatment of HT-29 and DHD / K12-PROb cells. After 24 hr of treatment, LD_{50} values were determined as 0.4 and 1.0 mM, respectively. Escribano et al. examined the inhibitory effect of HeLa cells ranging from 0.64 to 10.2 mM crocin concentrations for 18 hr and found LD 50 value at 3 mM. Aung et al. (2007) in examining the effects of crocin on three colorectal cancer cell lines (HCT-116, SW-480, and HT-29) by MTS assay for 48 hr at crocin concentration of 1000 mM observed the strongest growth inhibition in HCT-116 cells [18].

Previous studies have also reported that 15 mg/mL crocin induced apoptotic cell death via p53 activation in C6 glioma cells [19]. In another study on human hepatocellular carcinoma cells, it was determined that crocin reduced the activity of human telomerase reverse transcriptase and thus suppressed cell proliferation [20]the main pigment of Crocus sativus L., has been shown to have antiproliferative effects on cancer cells, but the involved mechanisms are only poor understood. This study focused on probable effect of crocin on the immortality of hepatic cancer cells. Cytotoxicity of crocin (IC50 3 mg/ml. Moreover, crocin significantly enhanced the chemosensitivity of human lung adenocarcinoma cells to cisplatin or pemetrexed [21]. In addition, research on crocin interaction with drugs such as temozolomide used in glioma treatment should be done.

When the results of the study were evaluated generally, crocin was more effective in the C6 cells than the T98G cells and it was observed that the suppressive effect on the cell proliferation increased in a time and concentration-dependent fashion. The mechanisms of suppression of glioma cells proliferation by crocin should be investigated further.

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