Cytogenetic behaviour of crocin on cultured lymphocytes from leukemic patients.

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ABSTRACT: Crocin is isolated from saffron, an important spice rich in carotenoids obtained from the stigmas of Crocus sativus L, commonly consumed all around the world and used as a medical drug to treat numerous diseases. In the present work a comparative study of the cytogenetic behaviour of crocin between cultured lymphocytes from chronic lymphocytic leukemic patients as well as from healthy individuals was undertaken in order to test the hypothesis that the sister chromatid exchange assay in vitro can be used for the prediction of the in vivo tumor response to the potential chemotherapeutic action of crocin. Sister chromatid exchanges (SCEs) and proliferation rate index (PRI) were evaluated in cultured lymphocytes from peripheral blood of all donors. Results showed that all tested crocin solutions didn't cause remarkable changes to the PRI values of lymphocytes neither of the leukemic patients, nor of healthy individual. Contrariwise, after crocin affection a statistically significant decrease of the SCEs frequency of lymphocytes of leukemic patients had been observed ($p<0,001$, t-test) whereas the SCEs of the healthy donor's cells presented slight, but not statistically significant increase. Our results indicate that crocin did not prove to be cytostatic in the tested concentrations, but it mainly reduced significantly the DNA damages along with being demonstrated as cytoprotective.

Key Words: Crocin, Sister chromatid exchanges, Chronic lymphocytic leukemia.

INTRODUCTION

Crocus sativus L. is a small perennial stemless herb from the iris family (Iridaceae), commonly known as saffron, that is widely cultivated in many regions of the globe[1]. Phytochemical components of saffron have been extensively reported, in which safranal, picrocrocin and crocetin glycosides, like crocin, are major components and responsible for the typical red color, bitter taste and aroma respectively[2]. C. sativus possesses a wide array of medicinally important activities, such as anticonvulsant, antihypertensive, antitussive and antigenotoxic effects[3]. Crocin is one of the major constituents of the stigma of the plant and is the diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin (Figure 1). It is characterized as an unsually soluble carotenoid, while it presents striking coloring and flavoring capabilities. It has also been shown to have antidepressant, anxiolytic, antioxidant[4,5], antithrombotic[6,7], antiinflammatory and mainly anticancer activities[8,9]. Among others, it improves memory and learning skills and presents neuroprotective properties[10].

In the present work, we conducted a comparative study of the in vitro effect of crocin on cultured human lymphocytes from peripheral blood of three leukemic patients and of one healthy individual, by estimating the frequency of sister chromatid exchanges (SCEs) and the values of proliferation rate index (PRI).

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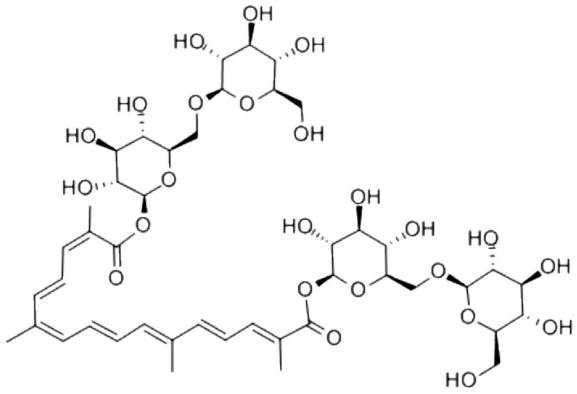

Figure. 1. Chemical structure of crocin.

The study of SCE frequency is a very sensitive, reliable and rapid method for detecting genotoxicity, which has lately been proposed also as one of the methods for evaluating chemotherapeutic efficiency in vitro and in vivo. Elevated SCE values are attributed to chromosomal instability, due to genetic or environmental factors (radiation, chemical and biological mutagens). Furthermore, PRI has been established as a sensitive indicator for the evaluation of the cytostatic activity of various enviromental hazards or therapeutic agents[11], since it reflects the effect of the different mutagenic agents in the cell cycle. The aim of our study was to test the hypothesis that the SCE assay in vitro can be used for the prediction of the in vivo tumor response to the potential chemotherapeutic action of crocin.

MATERIALS AND METHODS

In vitro SCE and PRI assays

To conduct the study, blood was taken from three women with chronic lymphocytic leukemia, 62-69 years old, that haven't undergone any treatment prior to blood collection and from one healthy donor, same gender and age. All blood donors were nonsmokers and no alcohol consumers. Human lymphocyte cultures were prepared by adding in 5mL of the chromosome medium (RPMI-1640; Biochrome, supplemented with 20% fetal calf serum, 0,63% L-glutamine, 0,63% penicillin/streptomycin and 2% phytohaemagglutinin) 11–12 drops of heparinized whole blood, 5 μg/mL 5-bromodeoxyuridine (BrdU) solution and aqueous solutions (100ng, 1μg and 10μg final concentration per culture) of crocin (Sigma) at the beginning of culture life. The cultures were incubated at 37°C for 72 hours in the dark to minimize photolysis of 5-bromodeoxyuridine. Colchicine (0,3 μg/mL) was added 2 hours before the collection of the cultures. The cells were then collected by centrifugation and exposed to 0,075M KCl for 10 min. The hypotonic solution spreads the chromosomes and hemolyses the red blood cells. The pellet was fixed three times with methanol: acetic acid (3:1). Drops of concentrated suspension of cells were placed on microslides and then allowed to air-dry. For SCE and PRI analysis, the slides were stained by a modification of the fluorescence plus Giemsa procedure to obtain harlequin chromosomes[12].

RESULTS

We have studied the cytogenetic behaviour of crocin by estimating the SCE frequency (Table 1) and PRI values (Table 2) in human lymphocyte cultures from three leukemic patients and one healthy individual. We have investigated the effect of three different crocin concentrations (100ng, 1μg and 10μg final concentrations). The results showed that a statistically significant decrease of the SCE frequency of lymphocytes from leukemic donors had been observed after crocin affection, though the SCEs of healthy donor cells presented a slight, non significant, increase (Figure 2). Contrariwise, all tested crocin solutions did not cause remarkable changes to the PRI values of the lymphocytes neither of the leukemic, nor of the healthy donors, after statistical analysis (Figure 3). Both the SCE's reduction of the lymphocytes from leukemic patients and the SCE frequency's increase of the cells from healthy donor were proportional to the concentrations of crocin solutions.

As far as the cultured lymphocytes of leukemic patients are concerned, we have observed in all of them a great increase in the frequency of SCEs compared to the control value of the healthy donor. Furtheromore, all tested concentrations of crocin have caused a statistically significant decrease ($p < 0,001$) of SCE frequency to the first leucemic patient lymphocytes, whereas only the higher crocin concentrations of 1μg and 10μg have brought a statistically significant de-

Table 1. Effect of crocin on sister chromatid exchanges in human lymphocyte cultures.

	SCEs±SE/cell			
Crocin concentrations	Healthy individual	First leucemic patient	Second leucemic patient	Third leucemic patient
(control)	7,82	12,18	9,81	10,42
100 ng	7,94	7,72 [a]	9,45	9,02
1 μg	7,95	6,36 [a]	7,5 [a]	8,4 [a]
10 μg	7,98	7,68 [a]	7,9 [a]	8,52 [a]

A minimum of 30 metaphases were scored for SCEs from each culture.
[a] Statistically significant ($p < 0,001$) decrease over the corresponding control (t-test).
SCEs, sister chromatid exchanges; SE, standard error.

Table 2. Effect of crocin on proliferation rate index (PRI) in human lymphocyte cultures.

Crocin concentrations	Healthy individual	First leucemic patient	Second leucemic patient	Third leucemic patient
(control)	2,18	1,97	1,99	1,95
100 ng	2,2	2,02	1,91	1,97
1 μg	2,21	1,89	1,91	1,84
10 μg	2,24	1,95	1,88	1,98

PRI= (M1+M2+M3)/100, where M1 is the percentage value of cells in the first division, M2 the percentage values of cells in the second division and M3* the percentage values of cells in the third and higher division. At least 100 metaphases are needed for the PRI calculation.*

crease ($p < 0,001$) on the cultured lymphocytes of the other two patients. On the other hand, the PRI values of lymphocytes of all leukemic donors were slightly decreased compared to the healthy individual after the crocin effect, but they were not significantly affected at any crocin concentration tested.

Statistical analysis

For SCE estimation, at least 30 properly spread metaphases of cells in second division from each culture were blindly scored. For PRI calculation, 100 metaphases in the first, second, third and higher divisions from each culture were blindly scored. The PRI was calculated according to the formula: PRI = (M1 +2M2 +3M3+….)/N, where "M1, M2, M3+… " indicate the number of metaphases corresponding to first, second, third or subsequent divisions, respectively, and "N" is the total number of metaphases scored (at least 100) for each culture. For the statistical evaluation of the experimental data, Student's t-test was performed to determine whether any SCE values differed significantly from the controls and the x^2-test was used for PRI comparisons.

DISCUSSION

Crocin, the main pigment of Crocus sativus L has been shown to exhibit antitumor activity against many human tumors. Although sufficient number of scientists investigates the antineoplastic effect of crocin lately, the involved mechanisms are only poor understood. Sun Y et al suggested that crocin induced apoptosis and cell cycle arrest and regulated Bcl-2 and Bax expression of HL-60 cells. In their experiments the results showed that crocin (0,625-5 mg/mL) inhibited human leukemia HL-60 cells proliferation and induced apoptosis and cell cycle arrest at G0/G1 phase, in a concentration and time-dependent manner[13]. These results are in agreement with the investigation of Hoshyar R. et al who have found that crocin triggered the apoptosis through increasing the Bax/Bcl-2 ratio and caspase activation in human gastric adenocarcinoma, AGS, cells[14]. Noureini SK and Wink M. proposed that the antiproliferative action of crocin is due on telomerase inhibition and hTERT (catalytic subunit of telomerase gene) down regulation. They have found that telomerase activity of hepatocarcinoma HepG2 cells treated

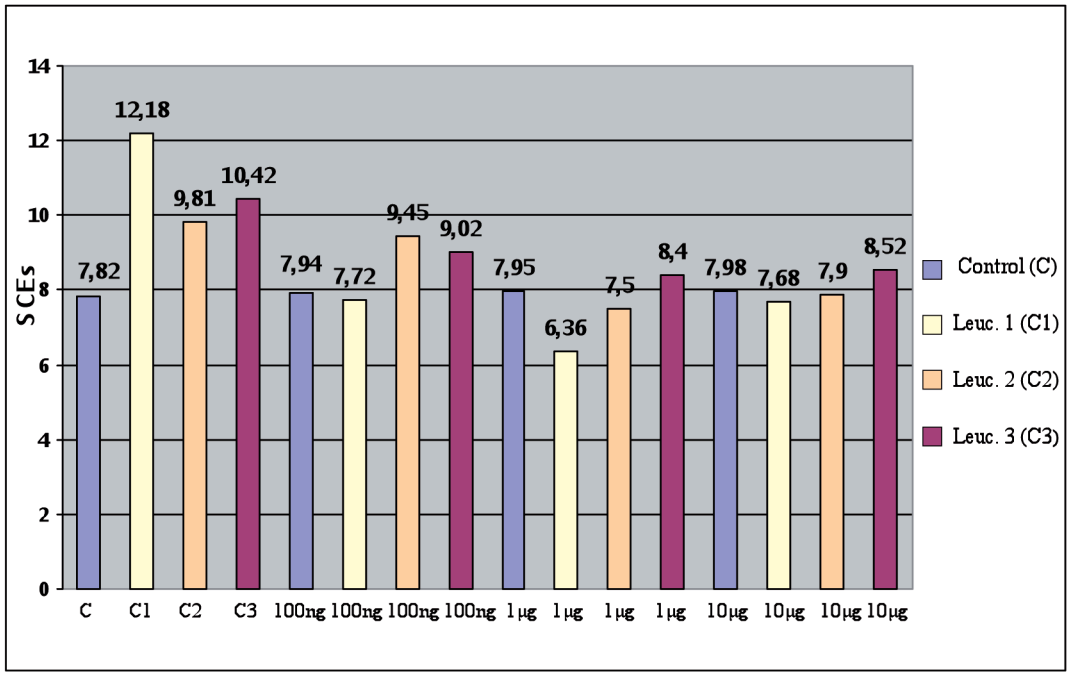

Figure 2. SCEs of cultured lymphocytes from normal (C) and leucemic (C1, C2, C3) blood donors after crocin administration (100ng, 1μg and 10μg).

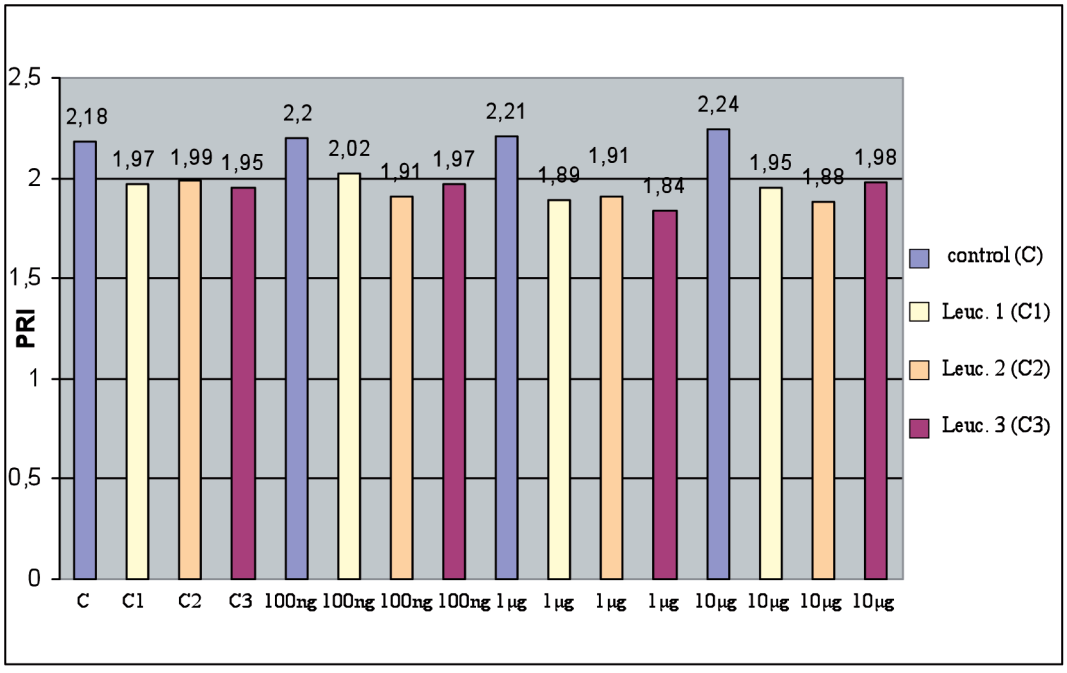

Figure 3. PRI values of cultured lymphocytes from normal (C) and leucemic (C1, C2, C3) blood donors after crocin administration (100ng, 1μg and 10μg).

with 3 mg/ml crocin has been reduced to about 51% and hTERT has showed a 60% decrease as compared to untreated control cells[15].

Increase SCE values (higher than 8 per each metaphase) of human cultured lymphocytes could be either due to a large number of DNA lesions, which could not be repaired before the cells reach S phase, and/or due to the inability of the repair mechanisms to restore damages, as usually happens in tumor cells. This is confirmed by the elevated SCE frequencies of cultured lymphocytes of the three patients with chronic lymphocytic leukemia showed in Table 1. In order to evaluate the effect of crocin on DNA consistency and/or repair enzymes efficacy of leukemic patients' cultured lymphocytes, we estimated the SCEs and PRI of these lymphocytes after the effect of crocin and we compared them with the corresponding values of healthy blood donor. The significantly diminished SCE values of lymphocytes from leukemic patients after crocin administration could be attributed not only to the lack of crocin-induced DNA damage, but also to the reinforcement of repair mechanisms by this compound. We could conclude that the DNA of leukemic patients shows increased instability, but the crocin solutions significantly reduce the instability of the DNA structure. At first glance these results seem to be contrary with the apoptotic and cytotoxic effect of crocin that found by a numerous of scientists. Crocin however, as Sun Y et al suggested[13], affects in a concentration and time-dependent manner. 0,625-5 mg/mL of crocin inhibited HL-60 cell proliferation and induced apoptosis, 6.25, 25 mg/kg of crocin inhibited the tumor weight and size[13].

3 mg/ml crocin was reduced to about 51% of telomerase activity as compared to untreated control cells[15]. The concentrations of crocin solutions in our experiment were much lower, 0,02μg/ml, 0,2μg/ml and 2μg/ml. Because of SCEs have been frequently used as a highly sensitive indicator of DNA damage and/or subsequent repair mechanisms, we chose to investigate the cytogenetic action of crocin in small concetrations.

The alteration in cell cycle kinetics as indicated by the suppression of PRI in lymphocyte cultures has been proved to be a very useful and sensitive marker of the cytostatic action of various substances, environmental hazards or therapeutic agents[9]. The cultured lymphocytes of patients with leukemia showed significant decrease of PRI values compared to the lymphocytes of healthy individuals, as expected (Table 2), while the small concentrations of crocin (0,02μg/ml, 0,2μg/ml and 2μg/ml) didn't cause antiproliferative action on them. After the effect of crocin solutions the cultured lymphocytes of patients with leukemia presented non significant change of PRI values. Crocin showed strong antiproliferative action in hepatocarcinoma HepG2 cells at the concentration of 3mg/ml[15] and it exhibited proliferative inhibition on human leukemia HL-60 cells at 5mg/ml[16].

The results of this investigation are suggested that the crocin solutions at small concentrations significantly reduce the increased instability of lymphocyte DNA from leukemic patients, while they are not affecting the rate of these cells' cycle. At least at the concentrations tested, crocin could be characterized as cytoprotective, after the significant reduction of SCEs that caused in the leukemic cells, but not as cytostatic, since we observed no significant changes in the rate of proliferation of these cells. These very interesting results of the cytogenetic study of crocin require further investigation, by expanding to in vivo experiments, since crocin could be proven to have also a potential chemotherapeutic action.

Κυτταρογενετική συμπεριφορά της κροκίνης σε καλλιεργημένα λεμφοκύτταρα ασθενών με λευχαιμία.

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ΠΕΡΙΛΗΨΗ: Η κροκίνη είναι ένα από τα κυριότερα συστατικά των στιγμάτων του φυτού Crocus sativus. Παρουσιάζει εντυπωσιακές χρωστικές και αρτυματικές ικανότητες, και χορηγείται ως φάρμακο για τη θεραπεία πολλών νοσημάτων. Στην παρούσα εργασία πραγματοποιήθηκε συγκριτική μελέτη της *in vitro* επίδρασης της κροκίνης στο γενετικό υλικό λεμφοκυττάρων καλλιεργημένων από περιφερικό αίμα τριών γυναικών με χρόνια λεμφοκυτταρική λευχαιμία, οι οποίες δεν είχαν υποβληθεί σε καμία θεραπεία προ της αιμοληψίας και φυσιολογικής μάρτυρος της ιδίας περίπου ηλικίας, με σκοπό να διαπιστωθεί αν ο προσδιορισμός της συχνότητας των χρωματιδιακών ανταλλαγών (Sister Chromatid Exchanges, SCEs) in vitro μπορεί να χρησιμοποιηθεί για την πρόβλεψη της *in vivo* ανταπόκρισης του όγκου στην πιθανή χημειοθεραπευτική δράση της κροκίνης. Οι SCEs έχουν προταθεί ως μια πολύ ευαίσθητη μέθοδος τόσο για τον προσδιορισμό της γονοτοξικότητας, όσο και για την αξιολόγηση της αποτελεσματικότητας πολλών χημειοθεραπευτικών φαρμάκων in vitro και in vivo. Παράλληλα μετρήθηκε ο δείκτης ρυθμού πολλαπλασιασμού (Proliferation Rate Index, PRI) των λεμφοκυττάρων, ο οποίος έχει καθιερωθεί ως ένας πολύτιμος δείκτης κυτταροστατικότητας. Τα αποτελέσματα αποκάλυψαν ότι κανένα από τα διαλύματα της κροκίνης δεν προκάλεσε σημαντικές αλλαγές στις τιμές του PRI ούτε των λεμφοκυττάρων των λευχαιμικών ασθενών, αλλά ούτε και των λεμφοκυττάρων της μάρτυρος. Αντίθετα μετά από την επίδραση της κροκίνης η συχνότητα των SCEs παρουσίασε στατιστικά σημαντική μείωση στα λεμφοκύτταρα και των τριών ασθενών, ενώ στα λεμφοκύτταρα της μάρτυρος μια μικρή αύξηση. Η δράση αυτή της κροκίνης, τουλάχιστον στις υπό μελέτη συγκεντρώσεις, θα μπορούσε να χαρακτηριστεί κυτταροπροστατευτική, αλλά όχι κυτταροστατική, αφού δεν προκάλεσε σημαντικές αλλαγές στο ρυθμό πολλαπλασιασμού των κυττάρων αυτών.

Λέξεις Κλειδιά: Κροκίνη, Χρωματιδιακές ανταλλαγές, Χρόνια λεμφοκυτταρική λευχαιμία.

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