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Effect of Safranal, a Constituent of *Crocus sativus* (Saffron), on Methyl Methanesulfonate (MMS)–Induced DNA Damage in Mouse Organs: An Alkaline Single-Cell Gel Electrophoresis (Comet) Assay

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The influence of safranal, a constituent of *Crocus sativus* L. stigmas, on methyl methanesulfonate (MMS)–induced DNA damage was examined using alkaline single-cell gel electrophoresis (SCGE), or comet, assay in multiple organs of mice (liver, lung, kidney, and spleen). NMRI mice were divided into five groups, each of which contained five mice. The animals in different groups were received the following chemicals: physiological saline (10 mL/kg, ip), safranal (363.75 mg/kg, ip), MMS (120 mg/kg, ip), safranal (72.75 mg/kg, ip) 45 min prior to MMS administration, and safranal (363.75 mg/kg, ip) 45 min prior to MMS administration. Mice were sacrificed about 3 h after the administration of direct mutagen MMS, safranal, or saline, and the alkaline comet assay was used to evaluate the influence of safranal on DNA damage in different mouse organs. Increase in DNA migration was varied between 9.08 times (for spleen) and 22.12 times (for liver) in nuclei of different organs of MMS-treated mice, as compared with those of saline-treated animals ($p < 0.001$). In control groups, no significant difference was found in the DNA migration between safranal- and saline-pretreated mice. The MMS-induced DNA migration in safranal-pretreated mice (363.75 mg/kg) was reduced between 4.54-fold (kidney) and 7.31-fold (liver) as compared with those of MMS-treated animals alone ($p < 0.001$). This suppression of DNA damage by safranal was found to be depended on the dose, and pretreatment with safranal (72.75 mg/kg) only reduced DNA damage by 25.29%, 21.58%, 31.32%, and 25.88% in liver, lung, kidney, and spleen, respectively ($p < 0.001$ as compared with saline-treated group). The results of the present study showed that safranal clearly repressed the genotoxic potency of MMS, as measured by the comet assay, in different mouse organs, but the mechanism of this protection needs to be more investigated using different *in vitro* system assays and different experimental designs.

Introduction

Single-cell gel electrophoresis (SCGE), or comet, assay is a rapid and very sensitive fluorescent microscopic method for quantifying DNA lesions (e.g., single-strand [SS] and double-strand breaks, oxidative-induced base damage, and DNA–DNA/DNA–protein cross-linking) in individualized cells, both *in vitro* and *in vivo* (Tice *et al.*, 1991; Fairbairn *et al.*, 1995; Tice, 1995; Gontijo *et al.*, 2001). The alkaline version of comet assay was specially developed for detection of the DNA SS breaks and alkali-labile sites (Singh *et al.*, 1988), and is also indicated to evaluate *in vivo* genotoxicity induced by carcinogen exposure (Anderson *et al.*, 1998; Tsuda *et al.*, 2000). In the standard version of the comet assay, whole cells, with or without pretreatment with a test agent, are embedded in agarose, then lysed, and subjected to an electric field. Loops of DNA-containing breaks are pulled out of the nucleus in the direction of the anode, forming a "comet tail." The relative density of DNA in the tail is related to the degree of DNA damage (Singh *et al.*, 1988).

Crocus sativus L., commonly known as saffron, is used in folk medicine as an antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac, and emmenagogue (Rios *et al.*, 1996). Further, modern pharmacological studies have demonstrated that saffron extract or its active constituents have anticonvulsant (Hosseinzadeh and Khosravan, 2002), antidepressant (Hosseinzadeh *et al.*, 2004), antiinflammatory (Hosseinzadeh and Younesi, 2002), and antitumor effects, and radical scavenger and learning- and memory-improving properties (Abdullaev, 1993; Zhang *et al.*, 1994; Escribano *et al.*, 1996; Rios *et al.*, 1996; Abe *et al.*, 1999) and promote the diffusivity of oxygen in different tissues

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(Rios *et al*., 1996). Saffron extract also has chemopreventive and genoprotective effects and protects from genotoxins-induced oxidative stress in mice (Nair *et al*., 1995; Premkumar *et al*., 2001, 2003; Abdullaev *et al*., 2002).

Safranal is a monoterpene aldehyde, formed in saffron by hydrolysis from picrocrocin during drying and storage. It is the main essential volatile oil responsible for the characteristic saffron odor and aroma (Tarantilis *et al*., 1995). Escribano *et al*. (1996) showed that saffron extract and its constituents crocin, safranal, and picrocrocin inhibit the growth of human cancer cells (Hella cells) *in vitro*. Recently, we have found that safranal could exert protective effect against cerebral ischemia–induced oxidative damage in rat hippocampus (Hosseinzadeh and Sadeghnia, 2005).

The aim of the present study was to assess the protective effects of safranal, the active constituent of *C. sativus* L. stigmas, on methyl methanesulfonate (MMS)–induced DNA damage in mouse organs.

Materials and Methods

Animals

Adult male NMRI mice weighing 25–30 g were used throughout the study. All of them were kept in the same room under a constant temperature ($22 \pm 2^{\circ}C$) and illuminated 7:00 a.m. to 7:00 p.m., with food pellets and water available *ad libitum*. The experiment was approved by the University's Ethics Committee for Animal Use.

Chemicals

Chemicals were obtained from the following sources: low melting point (LMP) agarose from Biogen, Mashhad, I.R. Iran; normal melting point (NMP) agarose from Fermentas, Glen Burnie, MD; safranal from Fluka, St. Gallen, Switzerland; sodium hydroxide (NaOH), sodium chloride (NaCl), Na_2EDTA (ethylenediaminetetraacetic acid disodium salt), Trizma base (Tris (hydroxymethyl) aminomethane), Triton X-100 (*t*-octylphenoxypoly-ethoxyethanol), dimethyl sulfoxide (DMSO), sarcosyl (sodium lauroylsarcosinate, SLS), MMS, and methanol from Merck, Darmstadt, Germany. LMP and NMP agarose were diluted in physiological saline to 0.5% and 1%, respectively.

Treatment and organ preparation

Five groups of male mice were used in this study (for each treatment group, $n = 5$). The animals in different groups received the following chemicals: (1) physiological saline (10 mL/kg, ip), (2) safranal (363.75 mg/kg, ip), (3) MMS (120 mg/kg, ip), (4) safranal (72.75 mg/kg, ip) 45 min prior to MMS administration, and (5) safranal (363.75 mg/kg, ip) 45 min prior to MMS administration. About 3 h after injection, the animals were killed by cervical dislocation, and four organs (liver, lung, spleen, and kidney) were removed. Changes in the size, color, and texture of organs were examined. After weighing the organs, they were minced, suspended at a concentration of 0.5 g/mL in chilled homogenizing buffer containing 0.075 M NaCl and 0.024 M Na_2EDTA (pH 7.5), and then homogenized gently at 500–800 rpm in ice. To obtain nuclei, the homogenate was centrifuged at 700 *g* for 10 min at $0^{\circ}C$, and the precipitate was resuspended in chilled homogenizing buffer at 0.5 g/mL and allowed to settle; precipitated clumps were then removed. Doses and times were selected based on the preliminary studies as well as literature-reported values (Sasaki *et al*., 1997a, 1997b; Tsuda *et al*., 2000; Sekihashi *et al*., 2002).

Slide preparation and alkaline SCGE assay

The *in vivo* alkaline SCGE assay was conducted based on the method described by Sasaki *et al*. (1997a) with some modifications. About 100 μL NMP agarose was quickly layered on a conventional slide and covered with a cover slip, and then the slides were placed on ice to allow agarose to gel. Five μL of the nucleus suspension, prepared as above, was mixed with 75 μL LMP agarose, and the mixture was quickly layered over the NMP agarose layer after removal of the cover slip. Finally, another layer of agarose GP-42 was added on top. The slides were immersed immediately in a chilled lysing solution (pH 10) made up of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, and kept at $0^{\circ}C$ in the dark for overnight. Then the slides were placed on a horizontal gel-electrophoresis platform and covered with a chilled alkaline solution made up of 300 mM NaOH and 1 mM Na_2EDTA (pH 13). They were left in the solution in the dark at $0^{\circ}C$ for 40 min, and then electrophoresed at $0^{\circ}C$ in the dark for 30 min at 25 V and approximately 300 mA. The slides were rinsed gently three times with 400 mM Trizma solution (adjusted to pH 7.5 by hydrochloric acid) to neutralize the excess alkali, stained with 50 μL of 20 μg/mL ethidium bromide, and covered with a cover slip.

Examination of the nuclei and statistical analysis

One hundred nuclei per organ from each animal (50 nuclei on one slide) were examined and photographed through a fluorescence microscope (Nikon, Kyoto, Japan, at 400× magnification) equipped with an excitation filter of 520–550 nm and a barrier filter of 580 nm.

Undamaged cells resembled an intact nucleus without a tail, and damaged cells had the appearance of a comet. The length of the DNA migrated in the comet tail, which is an estimate of DNA damage, was measured using CASP software. Statistical analysis was performed using one-way ANOVA followed by Tukey-Kramer *post hoc* test for multiple comparisons. The *p*-values less than 0.05 were considered to be statistically significant.

Results

In this study, the migration of DNA was measured as an indicator of DNA damage. Increase in DNA migration was varied between 9.08 times (for spleen) and 22.12 times (for liver) in nuclei of different organs of MMS-treated mice, as compared to those of saline-treated animals ($p < 0.001$, Fig. 1A–D). In control groups, no significant difference was found in the DNA migration between safranal-pretreated and saline-pretreated mice.

MMS-induced DNA migration in safranal-pretreated mice (363.75 mg/kg) was reduced between 4.54 times (kidney) and

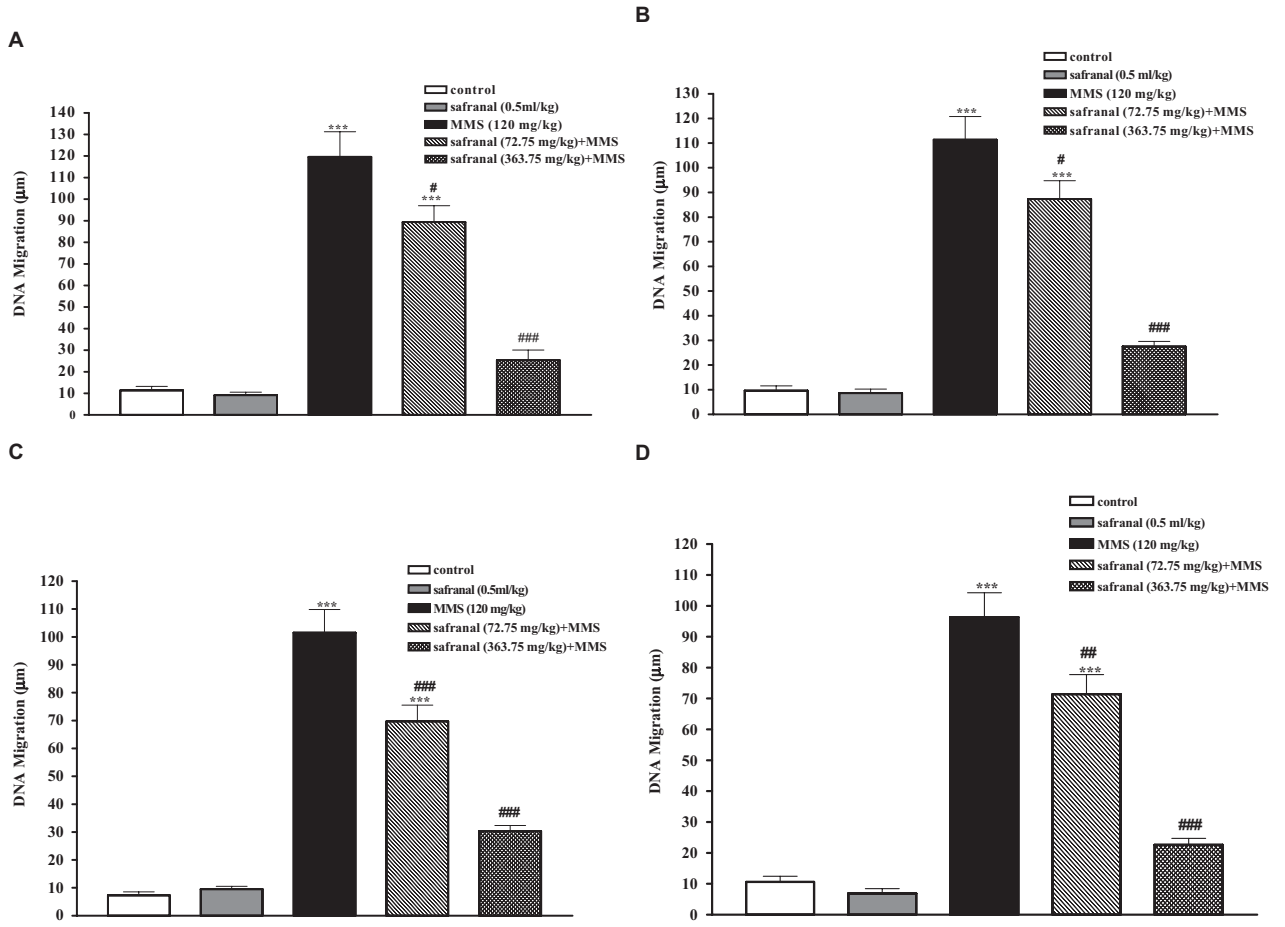

FIG. 1. Effect of safranal on DNA damage induced by methyl methanesulfonate (MMS) in mouse liver (**A**), lung (**B**), kidney (**C**), and spleen (**D**). Safranal was administered 45 min prior to MMS injection, ip. Values are mean ± SEM of five mice. $^{***}p < 0.001$ as compared with saline-treated animals; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ as compared with MMS-treated mice (one-way ANOVA followed by Tukey-Kramer test).

7.31 times (liver) as compared to those of MMS-treated animals alone ($p < 0.001$, Fig. 1A–D). This suppression of DNA damage by safranal was found to be dependent on the dose, and pretreatment with safranal (72.75 mg/kg) only reduced DNA damage by 25.29%, 21.58%, 31.32%, and 25.88% in liver, lung, kidney, and spleen, respectively ($p < 0.001$ as compared with saline-treated group, Fig. 1A–D).

Discussion

In the present study, we have shown that pretreatment with safranal suppresses MMS-induced DNA damage in multiple organs of mice.

Natural products have been traditionally accepted as remedies due to the popular belief that they produce few adverse side effects. Therefore, understanding the potential beneficial or adverse influence of natural products extensively used by human population is very important to implement public health safety measures. We did not observe adverse effects of treatment with relatively high doses of safranal, determined from DNA damage data.

Saffron has chemopreventive effects, and its extract inhibits tumor growth *in vivo* and *in vitro* (Salomi *et al*., 1990, 1991; Nair *et al*., 1991, 1994, 1995; Abdullaev 2002; Abdullaev *et al*., 2002; Das *et al*., 2004). Escribano *et al*. (1996) showed that saffron extract and its constituents crocin, safranal, and picrocrocin inhibit the growth of human cancer cells (Hella cells) *in vitro*. Abdullaev and Frenkel (1992a, 1992b) also showed that saffron could exert inhibitory effect on cellular DNA and RNA synthesis, but not on protein synthesis. Another study (El Daly *et al*., 1998) demonstrated protective effects of saffron extract against cisplatin-induced toxicity in rats. Saffron extract also has radical scavenger properties (Rios *et al*., 1996) and protects from genotoxicity as well as genotoxins-induced oxidative stress in mice (Premkumar *et al*., 2001, 2003). Using micronucleus assay, Premkumar *et al.* (2001) showed that oral pretreatment with the saffron aqueous extract (20, 40, and 80 mg/kg) for five consecutive days can significantly inhibit the genotoxicity of cyclophosphamide, mitomycin C, cisplatin, and urethane.

Among the constituents of saffron stigmas, crocins and crocetin derivatives are most abundant with established

antioxidant and antitumor effects (Abdullaev, 1993; Nair *et al*., 1995; Rios *et al*., 1996). These carotenoids scavenge free radicals, especially superoxide anions, and thereby may protect cells from oxidative stress (Bors *et al*., 1982). In rats, crocin dyes are known to exert protective effects against acute hepatic damage induced by aflatoxin B_1 and dimethylnitrosamine (Lin *et al*., 1986). It has been shown that crocetin, the deglycosylated crocin derivative, has protective effects on aflatoxin B_1–induced hepatotoxicity and protects rat primary hepatocytes against oxidative damage (Wang *et al*., 1991a, 1991b; Tseng *et al*., 1995). In another study, crocetin at nontoxic doses inhibited genotoxic effect and neoplastic transformation in C3H1OT1/2 cells induced by benzo(a)pyrene (Chang *et al*., 1996). Cancer chemopreventive as well as antitumor activities were also reported for crocins and crocetin derivatives in different assay systems (Tarantilis *et al*., 1994; Wang *et al*., 1995; Escribano, 1996; Konoshima *et al*., 1998; Garcia-Olmo *et al*., 1999).

Although the low dose of safranal significantly decreased DNA damage following MMS administration, there was also a significant difference ($p < 0.001$) between pretreatment with the low dose and high dose of safranal in different mouse organs. Moreover, while a significant difference between control groups and those treated with the low dose of safranal was seen ($p < 0.001$), there was no significant difference between control groups and those treated with the high dose of safranal. Therefore, an almost-complete protective effect of the treatment with the higher dose of safranal against MMS-induced genotoxicity can be postulated because there was a diminished tail extension of the nuclei from different organs in safranal-pretreated animals when compared to the animals treated only with the standard 120 mg/kg MMS dose.

MMS is a monofunctional alkylating agent that interacts mainly with N-7 guanosine (Dipple, 1995). The majority of induced lesions are processed via the base-excision repair pathway (Mirzayans *et al*., 1988). Horvathova *et al.* (1998) showed that most DNA lesions detected after MMS treatment in hamster V79 cells had the character of alkali-labile sites and true SS DNA breaks represented only a minor fraction, while hydrogen peroxide (H_2O_2) produced mainly true SS DNA breaks. Pretreatment with vitamin E significantly reduced the number of breaks induced by H_2O_2, but has no effect on the level of breaks induced by MMS. They suggested that MMS does not induce significant oxidative damage of DNA while most of breaks induced by H_2O_2 have the nature of oxidative lesions of DNA (Horvathova *et al*., 1998). It may be assumed that the reduced level of DNA damage in safranal-pretreated samples could be due to inactivation of the alkylating agent by safranal, which was classified as a desmutagenicity effect according to Kada and coworkers (Morita *et al*., 1978; Kada, 1983), but further investigations need to be done. Recently, Kanakis *et al*. (2007a, 2007b) have shown that safranal has antioxidative properties. This compound interacts with DNA via external binding or intercalative modes, and protects it from harmful damages.

There are several reports about the antigenotoxic and chemopreventive effects of saffron and its constituent, as mentioned above, but this is the first report of the antigenotoxic potential of safranal. According to De Flora, it is essential not only to assess the efficacy and safety of putative inhibitors by using a variety of test systems, but also to understand the mechanisms involved, for a rational implementation of chemoprevention strategies (De Flora, 1998). Therefore, this effect of safranal needs to be more investigated using different *in vitro* system assays as well as different experimental designs.

In conclusion, the results of the present study showed that safranal clearly repressed the genotoxic potency of MMS, as measured by the comet assay, in different mouse organs.

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