Safranal, a constituent of Crocus sativus (saffron), attenuated cerebral ischemia induced oxidative damage in rat hippocampus

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Abstract Increased oxidative stress has been implicated in the mechanisms of delayed neuronal cell death following cerebral ischemic insult. In this study, we investigated whether safranal, an active constituent of *Crocus sativus* L. stigmas, may ameliorate ischemia-reperfusion injury (IRI)-induced oxidative damage in rat hippocampus. Male NMRI rats were divided into six groups, namely, sham, control, ischemia and ischemia treated with safranal (four groups). The transient global cerebral ischemia was induced using four-vessel-occlusion method for 20 min. Safranal was injected intraperitoneally (727.5 mg/kg, 363.75 mg/kg, 145.5 mg/kg, and 72.75 mg/kg body weight) 5 min. prior to reperfusion and the administration was continued every 24 hours for 72 hours after induction of ischemia. The markers of oxidative stress including thiobarbituric acid reactive substances (TBARS), total sulfhydryl (SH) groups and antioxidant capacity of hippocampus (using FRAP assay) were measured. The transient global cerebral ischemia induced a significant increase in TBARS levels ($p < 0.001$), decrement in both antioxidant power (FRAP value) ($p < 0.05$) and total sulfhydryl (SH) concentrations ($p < 0.001$) in comparison with sham-operated animals. Following safranal administration the total SH contents (3.2 vs. 0.7 µmol/g, $p < 0.001$, safranal 727.5 mg/kg) and antioxidant capacity (4.12 vs. 1.16 µmol/g, $p < 0.001$; 727.5 mg/kg) were elevated in hippocampus in comparison with ischemic group. The MDA level was declined significantly in hippocampus (52.31 vs. 159.70 nmol/g, $p < 0.001$;

727.5 mg/kg). It is concluded that safranal have some protective effects on different markers of oxidative damage in hippocampal tissue from ischemic rats.

INTRODUCTION

As the brain's high oxygen consumption, high lipid contents, especially polyunsaturated fatty acids, high concentrations of transition metals, and low antioxidants activity, the oxidative stress has been implicated as a potential contributor to the pathogenesis of acute central nervous system (CNS) injury. After brain injury, for example by ischemia, the production of reactive oxygen species (ROS) may increase, leading to tissue damage via several different cellular molecular pathways. Radicals can cause damage to cardinal cellular components such as lipids, proteins, and nucleic acids (e.g., DNA), leading to subsequent cell death by modes of necrosis or apoptosis. Therefore, the use of antioxidants, free radical scavengers or trapping agents may be rational in acute and chronic CNS injuries, especially cerebral ischemia-reperfusion injury (IRI) [1-3]. Recently, there is overwhelming attention to plant products and natural agents that can limit free radical-mediated injuries, for better therapeutic management of IRI.

Crocus sativus L., commonly known as saffron, is used in folk medicine as an antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoteric, expectorant, stimulant, stomachic, aphrodisiac and emmenagogue [4]. Furthermore, modern pharmacological studies have demonstrated that saffron extract or its active constituents have anticonvulsant [5], antidepressant [6], anti-inflammatory [7] and antitumour effects, radical scavenger as well as learning and memory improving properties [4, 8-11] and promote the diffusivity of oxygen in different tissues [4]. Saffron extract also has chemopreventive and genoprotective effects and protects from genotoxins-induced oxidative stress in mice [12-15].

The aim of present study was to assess the protective effects of safranal, the active constituent of *Crocus sativus* L. stigmas, on ischemia-reperfusion injury (IRI)-induced oxidative damage in rat hippocampus.

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MATERIALS AND METHODS

Animals

Adult male NMRI rats weighing 200-300 g were used throughout the study. All of them were kept in the same room under a constant temperature (22 ± 2 °C) and illuminated 7:00 a.m. to 7:00 p.m., with food pellets and water available *ad libitum*.

The animals were divided into six groups, each of which contained 8 rats. Group 1 was the sham group in which only surgery was done without induction of ischemia; group 2 was the control group in which saline solution was given intraperitoneally. In groups 3-6, safranal (727.5 mg/kg, 363.75 mg/kg, 145.5 mg/kg and 72.75 mg/kg, i.p., [6]) was administrated 5 min prior to reperfusion and the administration was continued every 24 hours for 72 hours after the induction of ischemia.

Chemicals

DTNB (2, 2′-dinitro-5, 5′-dithiodibenzoic acid), TPTZ (2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine), TBA (2-thiobarbituric acid), n-butanol, tris, Na_2EDTA, sodium acetate, glacial acetic acid, phosphoric acid, potassium chloride, tetramethoxypropane (TMP), ferric chloride ($FeCl_3.6H_2O$), ferrous sulfate and hydrochloric acid was obtained from Merck. Safranal was purchased from Fluka.

Induction of global cerebral ischemia

Global cerebral ischemia was induced using the four-vessel occlusion method (4-VO), described by Pulsinelli *et al* [16]. Briefly, under intraperitoneal ketamine/xylazine anesthesia (60 mg/kg and 6 mg/kg, respectively), the alar foramina of first cervical vertebrae were exposed and the vertebral arteries were electrocauterized permanently. On the next day and under brief anesthesia, the common carotid arteries (CCAs) were dissected from surrounding tissues and temporarily ligated using the microvascular clamps for 20 min. At the end of the ischemic period, the ligature was removed and reperfusion was supplied. After maintaining of animals in suitable situation for 72 hours, the animals were decapitated and the hippocampus portion was homogenized in 1.5% cold KCl solution to give a 10% homogeny suspension and used for biochemical assays.

Tiobarbituric acid reactive species (TBARS) measurement

The lipid peroxidation level of the hippocampus portion was measured as malondialdehyde (MDA) which is the end product of lipid peroxidation, and reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm [17].

3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) was added to 0.5 ml of homogenate in a centrifuge tube and the mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml of n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 20000 rpm for 20 min. The organic layer was transferred to a fresh tube and its absorbance was measured at 532 nm. The standard curve of MDA was constructed over the concentration range of 0-40 µM [18].

Ferric Reducing / Antioxidant Power (FRAP) assay

The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{II}-tripyridyltriazine compound from the colorless oxidized Fe^{III} form by the action of electron donating antioxidants [19].

The FRAP reagent consist of 300 mM acetate buffer (3.1 g sodium acetate + 16 ml glacial acetic acid, made up to 1 liter with distilled water; pH=3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1.

Briefly, 50 µl of homogenate was added to 1.5 ml freshly prepared and prewarmed (37 ºC) FRAP reagent in a test tube and incubated at 37 ºC for 10 min. The absorbance of the blue colored complex was read against reagent blank (1.5 ml FRAP reagent + 50 µl distilled water) at 593 nm. Standard solutions of Fe^{II} in the range of 100 to 1000 mM were prepared from ferrous sulphate ($FeSO_4.7H_2O$) in distilled water. The data was expressed as mmol ferric ions reduced to ferrous form per liter (FRAP value) [20].

Total sulfhydryl (SH) groups assay

Total SH groups were measured using DTNB (2, 2′-dinitro-5, 5′-dithiodibenzoic acid) as the reagent. This reagent reacts with the SH groups to produce a yellow colored complex which has peak absorbance at 412 nm [21].

Briefly, 1 ml Tris-EDTA buffer (pH=8.6) was added to 50 µl homogenate in 2 ml cuvettes and sample absorbance was read at 412 nm against Tris-EDTA buffer alone (A_1). Then 20 µl DTNB reagent (10 mM in methanol) was added to the mixture and after 15 min (stored in laboratory temperature), the sample absorbance was read again (A_2). The absorbance of DTNB reagent was also read as a

blank (B). Total thiol concentration (mM) was calculated from the following equation:

$$\text{Total thiol concentration (mM)} = (A_2 - A_1 - B) \times 1.07/0.05 \times 13.6$$

Statistical analysis

Data are expressed as mean ± SEM. 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 statistically significant.

RESULTS

Change of MDA levels by safranal

The degree of free radical damage following IRI was assessed using lipid peroxidation, which was measured as MDA levels. There was an increase (64.2 %) in the MDA levels following IRI as compared with sham-operated animals (159.70 ± 15.90 vs. 97.25 ± 5.18 nmol/g tissue, p<0.001) (Figure 1). Safranal pretreatment resulted in a significant and dose-dependently reduction in the free radical-mediated lipid peroxidation as indicated by a decrease in the MDA levels, at various dose levels. In safranal-pretreated groups with doses 145.5 mg/kg, 363.75 mg/kg and 727.5 mg/kg, TBARS levels were 52.31, 76.85 and 98.74 nmol/g tissue, respectively (Figure 1).
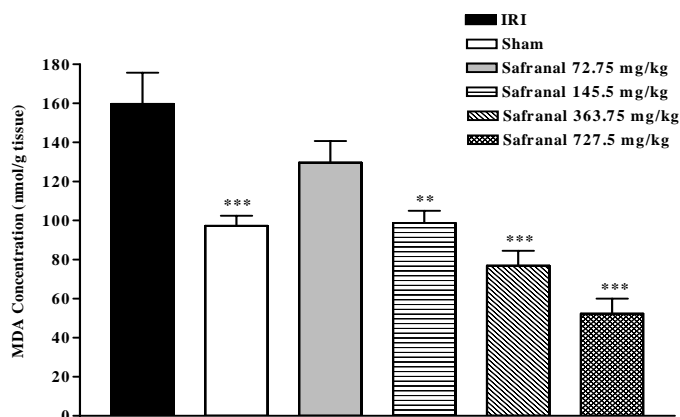

Figure 1: Effect of safranal on lipid peroxidation following global cerebral ischemia. MDA levels were measured in 10% homogenates of hippocampus portion from rats subjected to 20 min of ischemia. All drugs were administrated intraperitonealy 5 min prior to reperfusion. Values are mean±SEM (n=8). ***p<0.001 as compared with vehicle (normal saline) treated animals (One-way ANOVA followed by Tukey-Kramer test)

Change of FRAP value by safranal

IRI caused a significant reduction in FRAP value (53.2 %) of homogenate samples as compared with sham-operated animals (1.16 ± 0.2 vs. 2.48 ± 0.16 µmol/g tissue, p<0.001) (Figure 2). Safranal pretreatment increased antioxidant power (FRAP value) of brain homogenate samples, in non-dose dependent manner (from 1.16 ± 0.2 to 4.12 ± 0.33 µmol/g tissue, p<0.001; 727.5 mg/kg) (Figure 2).
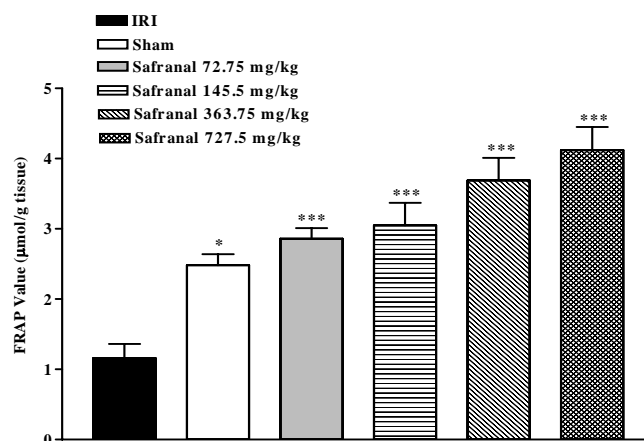

Figure 2: Effect of safranal on antioxidant power of hippocampus homogenate samples following global cerebral ischemia. FRAP values were measured in 10% homogenate samples from rats subjected to 20 min of ischemia. All drugs were administrated intraperitonealy 5 min prior toreperfusion. Values are mean ± SEM (n=8). *p<0.05, ***p<0.001 as compared with vehicle (normal saline) treated animals (One-way ANOVA followed by Tukey-Kramer test)

Effect of safranal on total thiol concentration

Following ischemia-reperfusion injury a significant reduction (77.6 %) in total SH groups (0.710 ± 0.068 vs. 3.170 ± 0.140 µmol/g tissue, p<0.001) in homogenate samples were observed (Figure 3). Safranal pretreatment induced a significant and dose dependently elevation in total thiol concentration, as compared with control group (from 0.710 ± 0.068 to 3.180 ± 0.075 µmol/g tissue, p<0.001; 727.5 mg/kg) (Figure 3).

DISCUSSION

A great deal of effort has been directed toward searching for new compounds that can be used for protection of cerebral ischemia–reperfusion injury. The results obtained in the present investigation suggest that safranal, one of the constituents of saffron stigmas with monoterpenoid structure, has an

overall protective effect against cerebral ischemia-reperfusion injury-induced oxidative stress in a rat model.
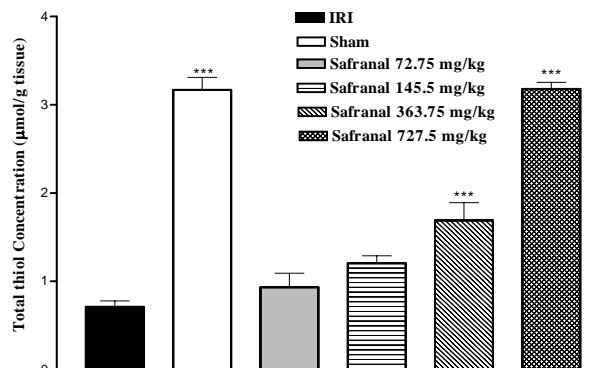

Figure 3: Effect of safranal on total thiol concentrations following global cerebral ischemia. Total sulfhydryl (SH) groups were measured in 10% hippocampus homogenate samples from rats subjected to 20 min of ischemia. All drugs were administrated intraperitonealy 5 min prior to reperfusion. Values are mean ± SEM (n=8). ***p<0.001 as compared with vehicle (normal saline) treated animals (One-way ANOVA followed by Tukey-Kramer test)

A number of processes have been implicated in the pathogenesis of oxygen deprivation–induced cell injury. These include the disturbances of cell calcium homeostasis, depletion of adenine nucleotides, activation of enzymes like phospholipases with production of toxic lipid metabolites, proteases and endonucleases and generation of free radicals (ROS) that can cause oxidative damage to cellular macromolecules [22]. It is well documented that oxidative stress is a major common pathway of cellular injury following neurological and neurodegenerative disorders such as ischemia-reperfusion, seizure, Parkinson and Alzheimer's disease and antioxidant therapy have been well documented to protect against CNS injuries [3, 23, 24].

The large numbers of polyunsaturated fatty acids (PUFAs) make cell membranes particularly vulnerable to lipid peroxidation. The oxidation of PUFAs causes them to be more hydrophilic, thereby altering the structure of the membrane with resultant changes in fluidity and permeability. Lipid peroxidation can also inhibit the function of membrane bound receptors and enzymes [22, 23]. We assessed the effect of safranal on lipid peroxidation, which was measured in terms of MDA, a stable metabolite of the free radical-mediated lipid peroxidation cascade. The MDA levels increased

significantly (p<0.001) following cerebral IRI. Safranal reversed the increase of MDA levels to a considerable extent, thereby confirming its antioxidant role in IRI.

Sulfhydryl (SH) groups are highly-reactive constituents of protein molecules, and they participate in important biochemical and metabolic process such as cell division, blood coagulation, maintance of protein systems and enzymatic activation including antioxidant enzymes (catalase, superoxide dismutase, etc.) [25]. There are also important scavengers of oxygen-derived free radicals [26]. SH groups known to be sensitive to oxidative damage and depleted following ischemic insult [27], therefore we studied the effect of this agent on the total thiol concentration during IRI. Similarly, in our studies, total sulfhydryl groups were decreased following ischemic-reperfusion injury. Safranal pretreated rats exhibited higher SH contents than their respective controls in the dose related pattern, indicating that safranal helped in replenishing the total thiol pool.

Under acute and chronic pathologic conditions such as ischemia, the balance between oxidant and antioxidant systems has been interrupted [2, 3, 28]. Therefore, we evaluate the antioxidant or reducing potential of hippocampus homogenate samples following IRI, using FRAP assay. As expected following IRI, a significant reduction in antioxidant power, as indicated by FRAP value, was observed. Safranal increased the antioxidant power of homogenate samples of hippocampus.

Saffron has chemopreventive effects and its extract inhibits tumor growth in vivo and in vitro [12, 13, 29-34]. Escribano *et al* showed that saffron extract and its constituents; crocin, safranal and picrocrocin inhibit the growth of human cancer cells (Hella cells) in vitro [9]. Abdullaev and Frenkel also showed saffron affect intracellular nucleic acid and protein synthesis [35, 36]. Another study (El Daly) demonstrated protective effects of saffron extract against cisplatin induced toxicity in rats [37]. Saffron extract also has radical scavenger properties [4] and protects from genotoxicity as well as genotoxins-induced oxidative stress in mice [14, 15]. Premkumar *et al* showed oral pretreatment with the saffron aqueous extract (40 and 80 mg/kg) for five consecutive days inhibit genotoxins-induced oxidative stress in mice liver. In this study, an increase in the levels of glutathione (GSH) concentration as well as the activities of glutathione S-transferase (GST), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) were

observed, however, normal levels of GSH could not be attained [15].

Among the constituent of saffron stigmas, crocins and crocetin derivatives are most abundant with established antioxidant and antitumor effects [4, 8, 13]. These carotenoids scavenge free radicals, especially superoxide anions and thereby may protect cells from oxidative stress [38]. In rats, crocin dyes are known to exert protective effects against acute hepatic damage induced by aflatoxin B_1 and dimethylnitrosamine [39]. 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 [40-42]. Cancer chemopreventive as well as antitumor activities were also reported for crocins and crocetin derivatives in different assay systems [9, 43-46].

There are several reports about the antioxidant activity and anti-inflammatory effects of some monoterpenoids such as α-pinene. Moreover, there have been shown monoterpenoids such as terpineol and linalool have depressant effects on central nervous system, in vivo [47] and linalool competitively inhibits glutamate receptors [48]. There are no reports about clinical uses of safranal. Much more basic pharmacological and toxicological studies need for clinical trials to evaluate the safety, tolerability and efficacy of safranal.

Our previous studies showed that safranal has a potent depressant effect on CNS and clearly suppress pentylenetetrazole-induced seizures (unpublished data) [49]. Its may be concluded that protective effect of safranal on ischemia-reperfusion injury, at least partly, due to these mechanisms, but it needs to be further investigated.

It is concluded that safranal have some protective effects on different markers of oxidative damage in hippocampal tissue from ischemic rats.

ACKNOWLEDGEMENTS

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