Original Article

Preventive Effects of Crocin, a Key Carotenoid Component in Saffron, Against Nicotine-Triggered Neurodegeneration in Rat Hippocampus: Possible Role of Autophagy and Apoptosis

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Abstract

Background: Nicotine is a behavioral stimulant that in high doses, through the neuro-inflammatory and oxidative stress pathway, can induce apoptosis and autophagy leading to cell death. Previous data indicate that crocin has neuroprotective properties. The aim of the current study is to investigate crocin's neuroprotective effects against nicotine-triggered neuro-inflammation, apoptosis, and autophagy in rat hippocampus. **Methods:** Seventy adult male Wistar rats were divided into the following seven groups: Group one received normal saline (0.2 ml/rat), group two was treated with nicotine 10 mg/kg intraperitoneally, groups 3 to 6 were treated simultaneously with nicotine and crocin (10, 20, 40, and 80 mg/kg, intraperitoneally), group 7 was treated with crocin-alone (80 mg/kg, intraperitoneally). The period of the mentioned agent administration was 21 days. On the 22nd day, an open field test (OFT) was used for evaluation of anxiety and motor activity changes. Inflammatory and oxidative stress factors and also apoptosis and autophagy biomarkers were evaluated. **Results:** All mentioned doses of crocin could decrease the nicotine-induced OFT behavioral changes. Crocin also could decrease levels of hippocampal TNF/TNF-α (tumor necrosis factor), IL1B/IL-1β (interleukin 1 beta), oxidized glutathione (GSSG), unphosphorylated and phosphorylated forms of JNK, BECN1 (beclin 1), BAX (BCL2 associated X, apoptosis regulator), and phosphorylated/inactive forms of BCL2 (BCL2 apoptosis regulator) in nicotine-dependent rats. Crocin treatments also caused increases in the reduced form of glutathione (GSH) content and activity of CAT (catalase) and mitochondrial complex enzymes in nicotine-addicted subjects. **Conclusions:** Crocin can modulate JNK-BCL2-BECN1 or JNK-BCL2-BAX signaling pathways and reduce neuronal oxidative stress, neuro-inflammation, and mitochondrial respiratory chain enzymes and exert neuroprotective effects against nicotine-induced neurodegeneration.

Keywords: *Apoptosis, autophagy, crocin, nicotine*

Introduction

Nicotine is known as a nerve stimulant and is the main bioactive ingredient in cigarettes.[1,2] The addiction to smoking is due to the properties of nicotine, in particular, stimulating the nervous system and elevating the smoker's mood.[2,3] The abuse of nicotine in the form of cigarettes has increased significantly in recent years, and this phenomenon is the basis for the occurrence of associated behavioral and biochemical consequences.[3,4] Several preclinical and clinical studies show that nicotine abuse can cause mood disorders such as anxiety and depression, as well as the consequences of cognitive impairment.[5,6] Furthermore, in the molecular dimension, nicotine abuse has harmful effects on brain function, including neurodegeneration.[7-11] Among the main causes of neurodegeneration caused by nicotine are events such as oxidative stress, neuro-inflammation, mitochondrial disorders, reduction of antioxidant defense, and signaling pathways related to cell death.[12,13] Despite all these data, the role of such pathways, including apoptosis and autophagy, in nicotine-induced neurodegeneration is still unclear and needs to be investigated.[13,14] It seems that nicotine in high doses can cause apoptosis and autophagy but a precise mechanism and linkage of the mentioned inflammatory and oxidative stress pathways in the induction and expansion of nicotine-induced apoptosis and/or autophagy remain unclear.[15-19] Apoptosis is a form of programmed cell death, which is responsible for occurrences of neurodegenerative events.[20] Autophagy

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is an important cytoprotective cellular process that removes damaged or superfluous molecules and subcellular components; autophagic dysfunction can also be responsible for some types of neurodegeneration; and autophagy is another mechanism for regulated cell death.[21]

Due to many factors in recent decades, including the increasing age of the global population, one of the important issues and challenges of medical and health systems is the introduction of neuroprotective agents for the management of the effects of neurotoxicity and neurodegeneration due to harmful compounds such as nicotine and other similar agents.[22,23] Herbal-derived flavonoids have been considered therapeutic agents against some neurotoxic agents.[23] One of these herbal agents with medicinal properties is saffron.[24-26] Saffron has important metabolites such as crocin, crocetin, safranal, and picrocrocin. Crocin is responsible for the red color of saffron, picrocrocin for the bitter taste, and safranal for the taste and smell; crocetin is a precursor of crocin.[24,25,27,28] The two compounds that are the most biologically important are crocin and crocetin. Crocin is the main glycosylated carotenoid of saffron, whereas crocetin is a hydrolyzed form of crocin with a carboxylic end group. Crocetin is particularly unique for its lipophilic properties, distinguishing it from other carotenoids. Conversely, Crocin is a hydrophilic diester of crocetin[29,30] [Figure 1].

There are substantial differences between the pharmacokinetic and pharmacodynamic properties of crocetin and crocin.[30,31] Multiple studies have shown that crocin plays an important role in the management of oxidative stress, neuro-inflammation, and cell death in neurodegenerative events.[24,26,32] Also, the role of crocin in controlling apoptosis and autophagy in neuronal cells, during destructive processes, has been previously confirmed.[24,26,29,32] Crocin also plays an important role in activating the function of mitochondria,[33,34] and this compound is additionally known as a mood and cognitive enhancer.[34-36] Despite all this information, the role of crocin in inhibiting the behavioral and molecular consequences caused by nicotine remains unknown and needs to be investigated. Thus, due to the importance of autophagy and apoptosis, and also the prominent role of mitochondrial dysfunction, neuro-inflammation, and oxidative stress, in occurrences or exacerbation of neurodegeneration, the current study was designed in part to assess the role of these pathways in conferring the neuroprotective effects of crocin against nicotine-promoted autophagy and apoptosis and alterations in mitochondrial function, oxidative stress and inflammation. This study also provides new insight and a more comprehensive understanding of the mechanisms and signaling pathways involved in nicotine-induced neurodegeneration and crocin-induced neuroprotection.

Methods

Drugs

Nicotine and crocin were purchased from the DNA Biotech Co. (Tehran, Iran). The purity of the drugs according to high-performance liquid chromatography/HPLC grade was 95%. Crocin was dissolved in normal saline for administration (0.4 mL/rat). Both agents were freshly prepared just before use.

Animals

In the present study, 70 male Wistar rats, weighing 205 grams each, were purchased from the experimental medicine center of Iran University of Medical Sciences (Tehran, Iran) and, after being transferred to the experimental studies laboratory at Masih Daneshvari Hospital (Tehran, Iran) affiliated with the Shahid Beheshti University of Medical Sciences (Tehran, Iran), were kept there for 14 days to adapt to the lab conditions. All the animals were maintained in normal laboratory conditions with 22 ± 0.5°C temperature and 30%–70% humidity throughout the project period. Cycles of light and dark extended for 12 h. All experimental procedures were accepted and approved by the Institutional Animal Use and Care Committee of the Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences (Research Protocol and Ethical Code Number = IR.SBMU.NRITLD.REC.1402.57).

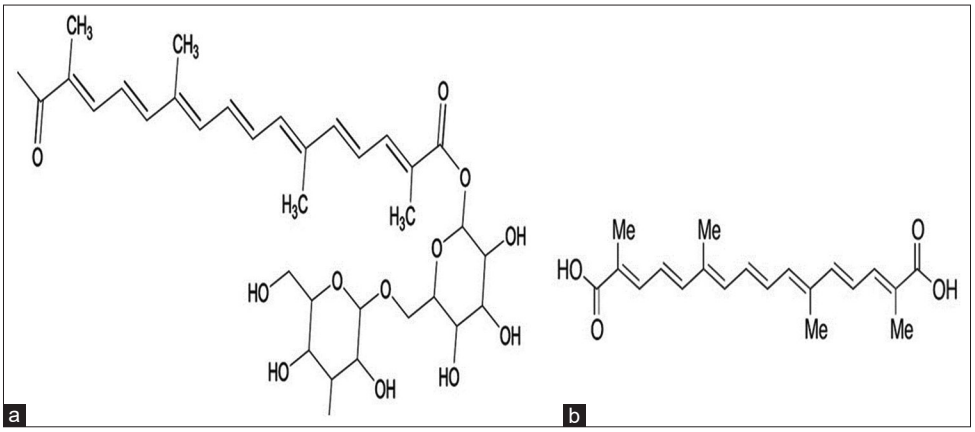

Figure 1: Chemical structures of (a) crocin and (b) crocetin

Experimental design

Experimental groupings and the timeline for drug administration and behavioral and molecular assessment are indicated in Figure 2.

It should be noted that doses of nicotine for induction of neurodegeneration and doses of Crocin for exerting neuroprotective effects were selected according to previous similar work.[7,8,24,37-43] After the animals' treatment with these agents, on the 22nd day of the procedure, all animals were evaluated by an open field test (OFT) according to the protocol below, and the level of motor activity and mood (anxiety and depression) were evaluated. In the last phases of the procedure, all animals were euthanized and their hippocampi were removed for evaluation of oxidative stress and inflammation. In addition, the JNK-BCL2-BECN1 or JNK-BCL2-BAX proteins, as markers of autophagy and apoptosis, respectively, were assessed as described below.

Behavior tests

OFT

An OFT is a standard behavioral test for the evaluation of motor activity and anxiety levels in rodents. In the present study, all animals were subjected to this experiment using standard protocols and according to previous standard studies.[44] In this test, non-anxious animals should demonstrate a strong desire to be in the center of the box and will have normal motor activity. In the OFT, the following four behaviors are evaluated in animals:

1. Rearing number: The number of rearings (searching), which is a standard behavior in rodents.
2. Line crossing (ambulation) distance: The distance traveled as determined by crossing of the grid lines.
3. Center square entries: The frequency with which the rat crossed one of the red lines demarcating the center square and with all four paws present in this square.
4. Center square duration: The duration of time that the rat spent in the central square.[44,45]

Molecular and biochemical measurements

Total protein extraction and determination of protein concentration and mitochondrial isolation

After treatment of the animals according to the protocols mentioned above, all rats were anesthetized using thiopental sodium (50 mg/kg, intraperitoneal) and euthanized. In brief, the process of inducing anesthesia in animals was based on previous standard protocols, and all of the required steps utilized including acclimation, fasting, eye protection, monitoring, heat support, fluid support, and euthanasia were based on previous studies.[46,47] After the process of anesthesia and euthanasia, the hippocampi of all animals were removed. The corresponding protocol was based on previous similar work and was as follows: All parts of the vascular system of the animals during the necropsy process were perfused with the use of 4% paraformaldehyde. In the following step, the isolated hippocampal tissues were completely isolated and were used for the determination of all targeted biomarkers; samples were prepared according to previous similar studies.[48-50] Subsequently, the mitochondria of the hippocampal cells were isolated and prepared by standard protocols and according to previous studies.[51,52] Also, the exact amounts of total protein of the prepared

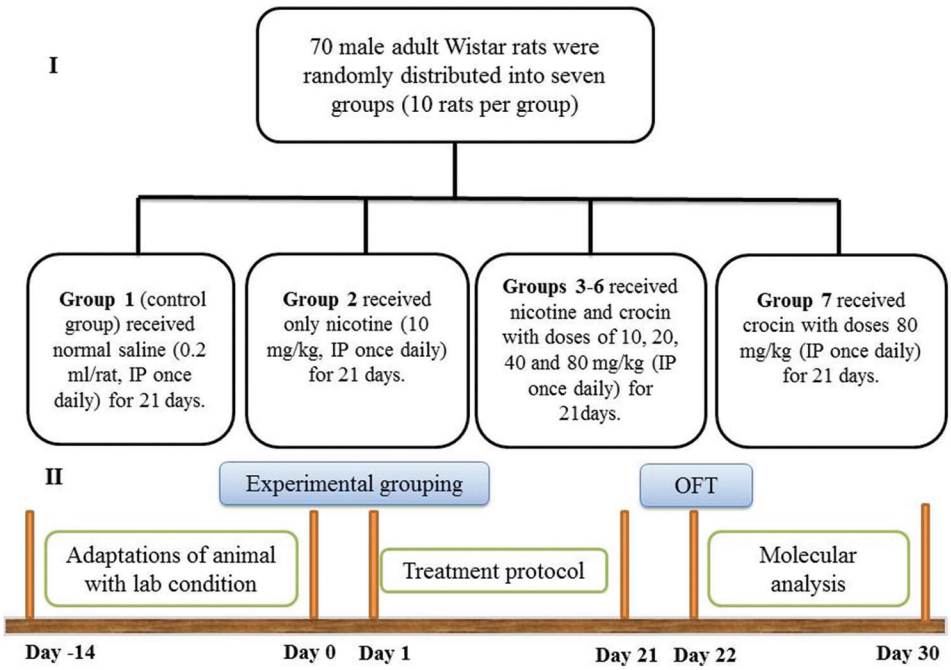

Figure 2: Schematic illustration of experimental grouping (I) and Timeline for experimental procedure and molecular evaluation (II)

hippocampus tissue were calculated by using the Bradford method as described previously.[53,54]

Evaluation of oxidative stress parameters

The level of lipid peroxidation, malondialdehyde (MDA) production, CAT activity, and also the amounts of glutathione cycle components such as GSH and GSSG were measured according to standard protocols and procedures and all of the details of the evaluation and measurements as well as the uses of necessary different reagents and chemical compounds were conducted based on previously published standard protocols and similar work.[55-59]

Evaluation of proteins involved in inflammation, apoptosis, and autophagy

The expression level and/or concentration of proteins involved in the processes of inflammation (TNF and IL1B), apoptosis, and autophagy (JNK [phosphorylated and unphosphorylated forms], BCL2 [phosphorylated and unphosphorylated], BECN1, and BAX) were determined using special ELISA kits (Abcam, Co, Boston, MA, USA) in the aforementioned hippocampal lysate cells. All protocols were carried out based on the instructions of these kits with slight changes in some steps and based on past studies, and the results are expressed as ng/ml for all of the mentioned proteins.[24,60-62]

Evaluation of mitochondrial complex enzymes chain activity

Activities of mitochondrial complexes I, II, III, and IV were assessed by using commercial kits (Abcam, Co, Boston, MA, USA). All measurements were conducted according to previous standards and similar studies and each of the values is reported as activity/mg of protein/min.[63,64]

Statistical analysis

All data were collected for each of the behavioral and molecular measured parameters and the mean ± standard error (SEM) was calculated for each data point in each group of experiments. In addition, multiple-test corrections were implemented in the analysis. The Kolmogorov-Smirnov test was used to evaluate the normality of the existing variables and the normality of all continuous variables was confirmed. Also, to evaluate the homogeneity of variances between two or more groups, Levene's or Bartlett's tests were used, and based on the results of this test, the variance between the studied groups (the homogeneity of variance) was reported. Assumptions of parametric data were estimated and carried out. The significant differences between the control and treated groups were assessed by one-way analysis of variance/ANOVA and *Tukey's post*-test. $P < 0.001$ or $P < 0.05$ was considered significant. Also, because we have considered $P < 0.05$ and $P < 0.001$ as significant levels and based on the statistical principles our P values were derived from one-tailed tests. Also, the Pearson correlation analysis was used for TNF or IL1B expression and the mitochondrial quadruple complex and a significant level ($P < 0.001$) was considered for remarkable correlation. Also, in the case of correlation analysis, the data of eight rats in each group (we had ten rats in each group) were selected, and for better evaluation of the correlation, the out-of-range data in each group were removed. In other words, to evaluate the level of correlation, we removed two out-of-range sets of data in each group. Also, for all parameters in all experiments in this study an F-test with[6,60] degrees of freedom was reported. In the results section, the number in parentheses after each experimental parameter is the F ratio followed by the P value.

Results

Crocin effects on OFT behavior in nicotine-treated animals

We first examined the effect of nicotine based on the OFT. Nicotine administration with a dose of 10 mg/kg caused decreases in the rate of central square entries (3.517; $P < 0.05$), time spent in the central region (3.654; $P < 0.05$), frequency of rearing (0.1560; $P < 0.05$), and ambulation distance (7.832; $P < 0.05$) in comparison to the control group [Table 1]. In contrast, crocin at the administered doses (10, 20, 40, and 80 mg/kg) inhibited this effect of nicotine in a dose-dependent manner and increased the rate of central square entries (3.517; $P < 0.05$), time spent in the central region (3.654; $P < 0.05$), frequency of rearing (0.1560; $P < 0.05$), and ambulation distances (7.832; $P < 0.05$) in comparison to the nicotine-alone treated group [Table 1]. Crocin alone (80 mg/kg) increased the OFT behavior, but this change was not significant in comparison to the control group [Table 1].

Crocin effects on oxidative stress biomarkers in nicotine-treated animals

Considering the effect of crocin on OFT behavior in conjunction with nicotine treatment, we next examined stress biomarkers in the treated rats. The treatment of animals with nicotine (10 mg/kg) meaningfully increased the MDA level (20.90; $P < 0.001$), CAT activity (15.01; $P < 0.001$), and GSSG level (24.20; $P < 0.001$) and also decreased the GSH level (17.65; $P < 0.001$) when compared to the control group [Figure 3]. Conversely, crocin (10, 20, 40, and 80 mg/kg) reduced the nicotine-induced rise in MDA level (20.90; $P < 0.001$), CAT activity (15.01; $P < 0.001$) and GSSG level (24.20; $P < 0.001$) and inhibited the nicotine-induced decrease in GSH level (17.65; $P < 0.001$) when compared to the nicotine-alone group [Figure 3]. Crocin treatment alone (80 mg/kg) increased the GSH content and decreased MDA, CAT, and GSSG but these changes were not statistically significant in comparison to the control group [Figure 3].

Table 1: The effects of various doses of crocin on open-field exploratory and depressive-like behavior in rats treated with 10 mg/kg of nicotine

Group	Ambulation distance (cm)	Central square entries (number)	Time spent in the central square (sec)	Number of rearing
Control	351±10	20±3	171±10	11±2
Nicotine (10 mg/kg)	232±11[a]	10±1	125±11[a]	6±2[a]
Nicotine (10 mg/kg) + Crocin (10 mg/kg)	251±12[b]	15±2	142±10[b]	11±1[b]
Nicotine (10 mg/kg) + Crocin (20 mg/kg)	281±10[b]	16±3[b]	145±8[b]	11±2[b]
Nicotine (10 mg/kg) + Crocin (40 mg/kg)	292±16[b]	17±2[b]	160±7[b]	13±1[b]
Nicotine (10 mg/kg) + Crocin (80 mg/kg)	314±14[b]	17±1[b]	169±6[b]	15±1.3[b]
Crocin (80 mg/kg)	353±18	22±1	176±14	12±4

[a]Showed significant level with $P<0.05$ vs. control group. [b]Showed a significant level with $P<0.05$ vs 10 mg/kg of nicotine. All data are expressed as Mean±SEM ($n=10$)

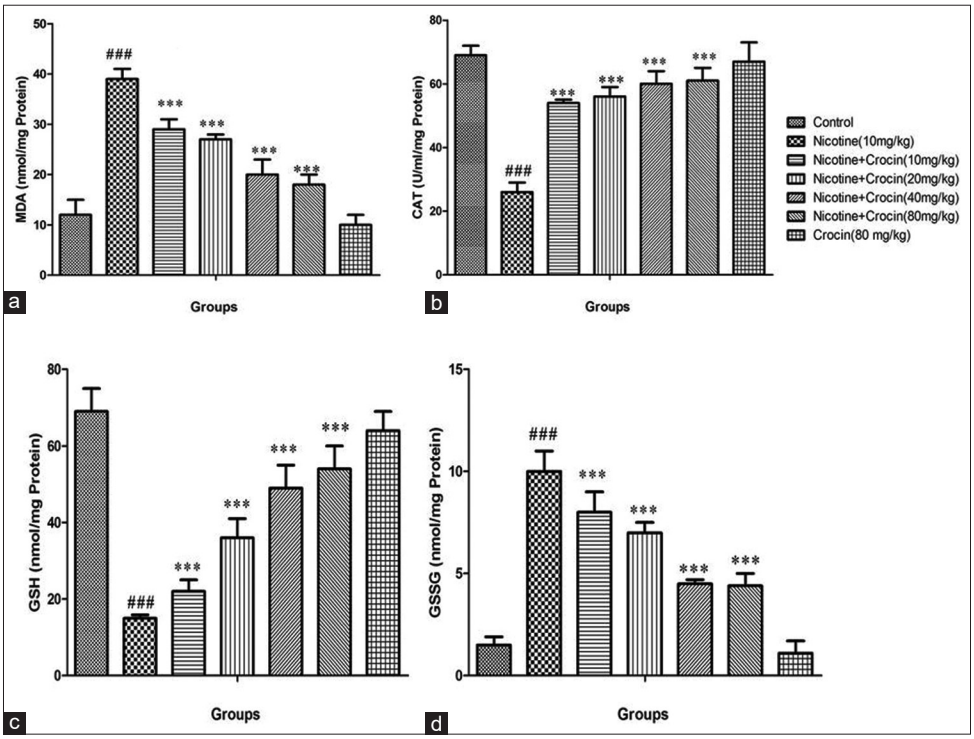

Figure 3: Oxidative stress biomarkers. The levels of (a) MDA, (b) CAT, (c) GSH, and (d) GSSG in the control group, and groups treated with 10 mg/kg of nicotine and 10, 20, 40, and 80 mg/kg of crocin in combination with nicotine. All data are expressed as mean ± SEM (n = 10). ###*P* < 0.001 vs. control group. ****P* < 0.001 vs 10 mg/kg of nicotine

Crocin effects on inflammatory biomarkers in nicotine-treated animals

To follow up on our analysis of stress biomarkers, we monitored inflammatory biomarkers in rats subjected to the treatment protocol. A dose of 10 mg/kg of nicotine remarkably augmented IL1B (32.41; $P < 0.001$) and TNF (59.22; $P < 0.001$) levels in comparison to the control group [Figure 4]. By contrast, crocin in all administered doses attenuated IL1B (32.41; $P < 0.001$) and TNF (59.22; $P < 0.001$) levels when compared to the nicotine-addicted group [Figure 4]. In the crocin-alone treated group, although IL1B and TNF levels were reduced, these differences were not significant when compared to the control group [Figure 4].

Crocin effects on the expression of total and phosphorylated forms of JNK in nicotine-treated animals

The JNK proteins are key stress-responsive kinases that can act upstream to increase the levels of the IL1B and TNF cytokines. Accordingly, we next determined the effects of nicotine and crocin on the levels and phosphorylation status of JNK; phosphorylation serves to activate JNK. Nicotine therapy significantly increased the hippocampal level/expression of both (unphosphorylated [13.25; $P < 0.001$] and phosphorylated [27.23; $P < 0.001$]) forms of JNK in comparison to the control group [Figure 5]. In contrast, crocin at the administered doses decreased the level/expression of both (total [13.25; $P < 0.001$] and phosphorylated [27.23; $P < 0.001$]) forms of

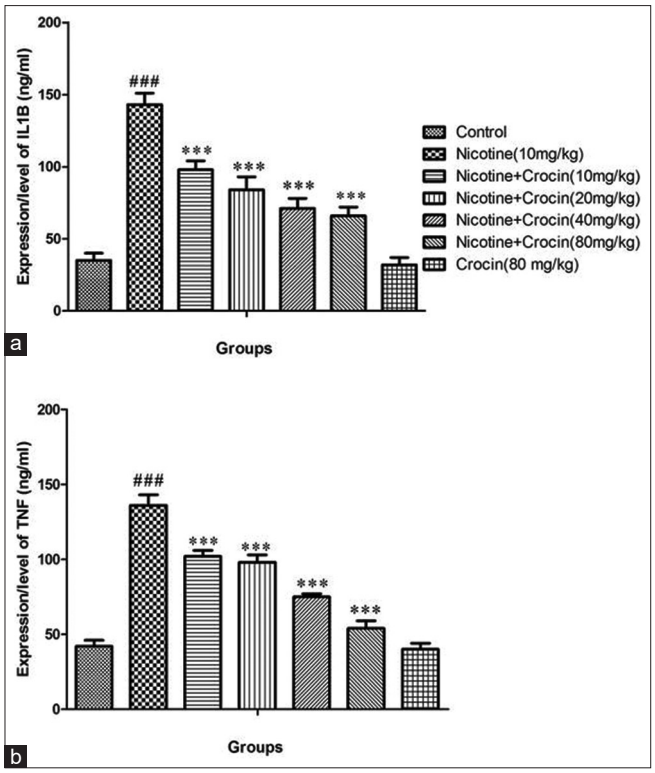

Figure 4: Inflammatory biomarkers. Alterations of the expression/level (ELISA) of (a) IL1B and (b) TNF in hippocampi in the control group and groups under treatment with 10 mg/kg nicotine in combination with crocin (10, 20, 40, and 80 mg/kg). All data are expressed as mean ± SEM (n = 10). ###*P* < 0.001 vs. control group. ***P* < 0.001 vs 10 mg/kg of nicotine

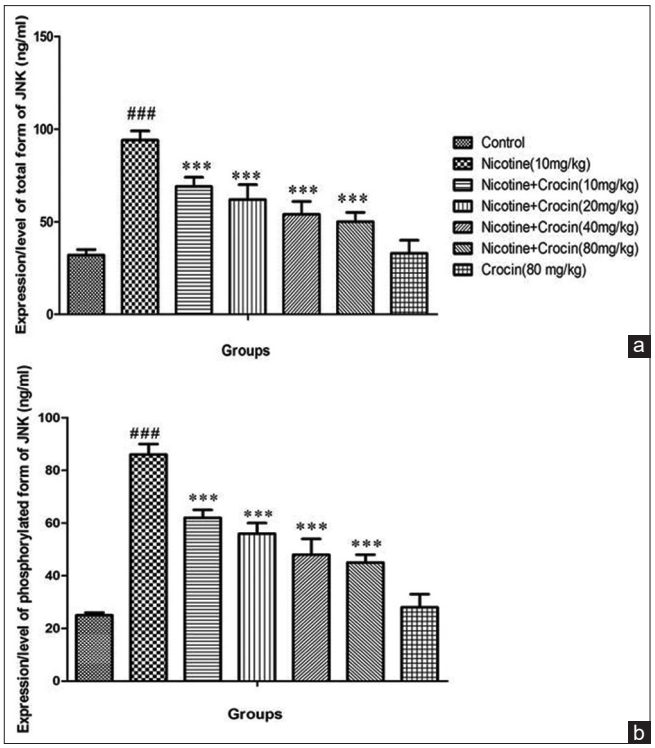

Figure 5: Total and phosphorylated JNK. Alterations of the expression/level (ELISA) of the (a) total form of JNK and (b) phosphorylated form of JNK in the hippocampi in the control group and groups under treatment with 10 mg/kg nicotine in combination with crocin (10, 20, 40, and 80 mg/kg). All data are expressed as mean ± SEM (n = 10). ###*P* < 0.001 vs. control group. ***P* < 0.001 vs 10 mg/kg of nicotine

JNK in comparison to the nicotine-alone administered group [Figure 5]. Crocin alone did not show significant effects on the JNK level [Figure 5].

Crocin effects on expression of BCL2, BECN1, and BAX in nicotine-treated animals

As mentioned above, apoptosis and autophagy are two principal mechanisms of regulated cell death. BAX and BECN1 are key components that participate in these two pathways, respectively. Furthermore, BCL2 functions as a negative regulator of both pathways by directly binding BAX and BECN1. Nicotine (10 mg/kg) injection meaningfully augmented the level of inactive BCL2 (phosphorylated form; 30.24; $P < 0.001$), decreased the level of active BCL2 (unphosphorylated form; 50.61; $P < 0.001$) and increased the BAX (16.88; $P < 0.001$) and BECN1 (22.00; $P < 0.001$) levels in rat hippocampi when compared to the control group [Figure 6]. In contrast, crocin at the administered doses administered along with nicotine decreased the inactive BCL2 level (phosphorylated form; 30.24; $P < 0.001$) and amplified the level of active BCL2 (unphosphorylated form; 50.61; $P < 0.001$) while it decreased the level/expression of BAX (16.88; $P < 0.001$) and BECN1 (22.00; $P < 0.001$) when compared to the nicotine-alone administrated group [Figure 6]. Crocin alone did not change the level/expression of these biomarkers [Figure 6].

Results of mitochondrial chain enzyme activity changes

Nicotine at the indicated dose significantly reduced quadruple mitochondrial complex enzyme activities (I [13.45; $P < 0.001$], II [17.21; $P < 0.001$], III [13.15; $P < 0.001$], and IV [16.23; $P < 0.001$]) in comparison to the control group ($P \leq 0.001$) [Figure 7]. In contrast, administration of the doses above of crocin significantly increased mitochondrial complex I (13.45; $P < 0.001$), II (17.21; $P < 0.001$), III (13.15; $P < 0.001$), and IV (16.23; $P < 0.001$) enzyme activities, in comparison to the nicotine-alone treated group [Figure 7]. Crocin alone (80 mg/kg) could not significantly alter, compared to the control group, the quadruple mitochondrial complex enzyme activities [Figure 7].

The correlation between TNF and IL1B expression and mitochondrial quadruple complex enzymes

One effect of TNF and IL1B is to decrease the mitochondrial membrane potential, causing mitochondrial dysfunction and an increase in damaging reactive oxygen species. To further understand the effects of the nicotine cascade that increases stress and inflammation, activates JNK, and induces cell death mechanisms, we monitored the effects on the mitochondrial complexes involved in

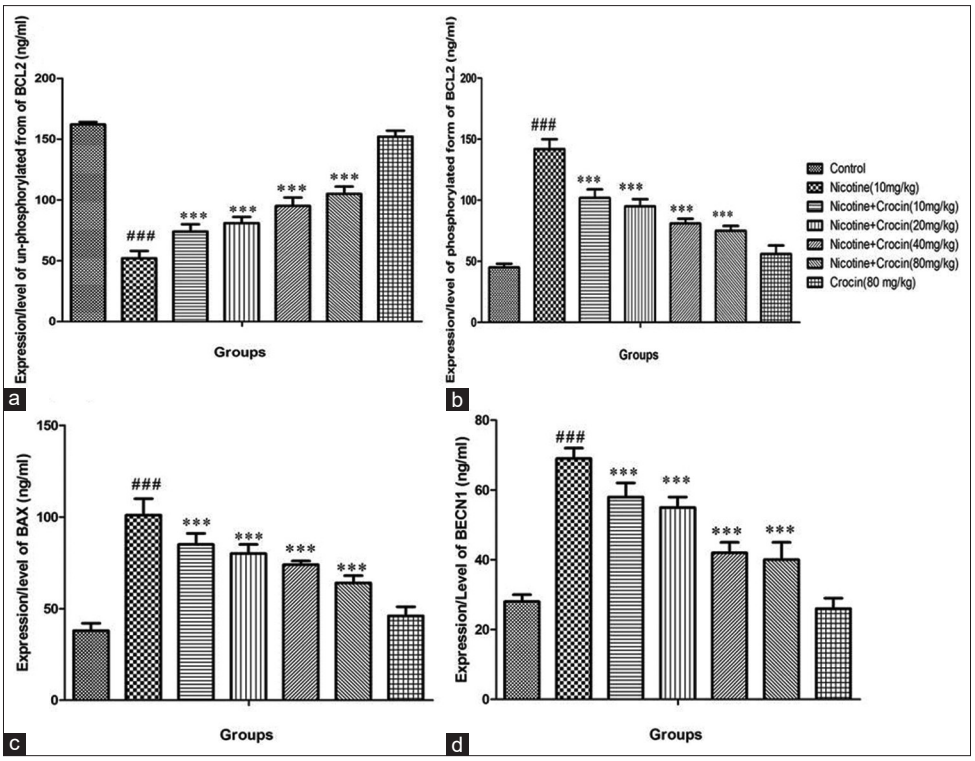

Figure 6: Apoptotic and autophagic markers. Alterations of the expression/level (ELISA) of (a) BCL2 (unphosphorylated/active form), (b) BCL2 (phosphorylated/inactive form), (c) BAX and (d) BECN1 in the hippocampi in the control group and groups under treatment with 10 mg/kg nicotine in combination with crocin (10, 20, 40, and 80 mg/kg). All data are expressed as mean ± SEM (n = 10). ###*P* < 0.001 vs. control group. ***P* < 0.001 vs 10 mg/kg of nicotine

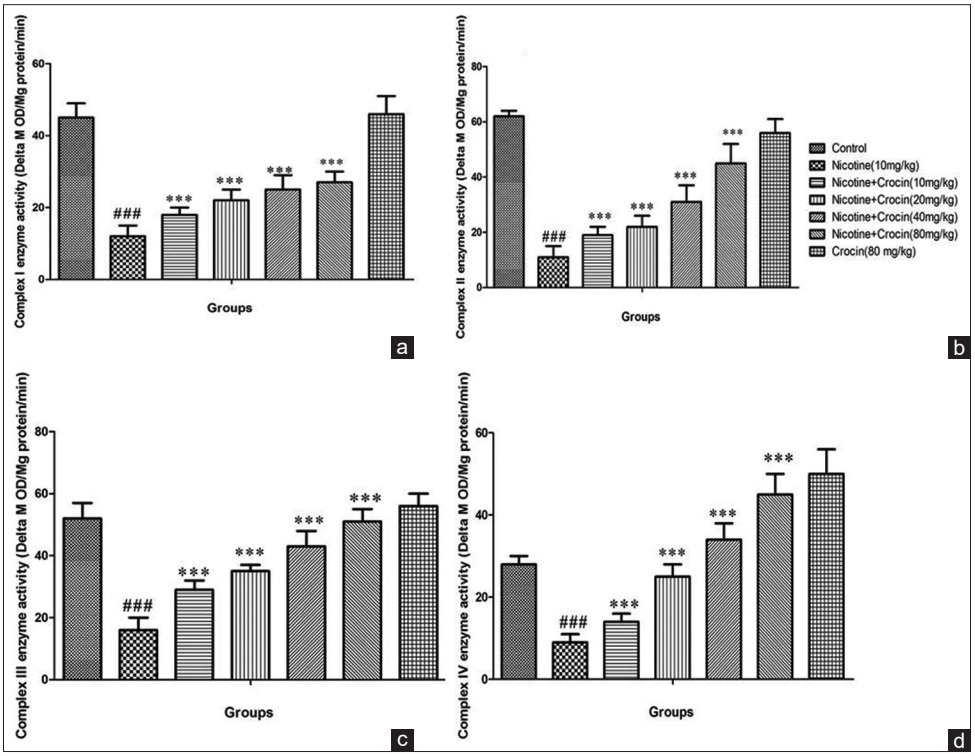

Figure 7: Mitochondrial complex enzyme activities. Alterations of (a) complex I, (b) complex II, (c) complex III, and (d) complex IV in rat-isolated hippocampi in the control group and groups under treatment with 10 mg/kg nicotine in combination with crocin (10, 20, 40, and 80 mg/kg). All data are expressed as mean ± SEM (n = 10). ###*P* < 0.001 vs. control group. ***P* < 0.001 vs 10 mg/kg of nicotine

oxidative phosphorylation. The Pearson correlation analysis demonstrated a significant correlation (with $P < 0.001$) between TNF expression and the decreased activities of mitochondrial complex I (with r = 0.8452), mitochondrial

complex II (with r = 0.9295), mitochondrial complex III (with r = 0.9524), and mitochondrial complex IV (with r = 0.8227) ($P < 0.001$) [Figure 8]. A similar result was seen between IL1B expression and the activities of mitochondrial complex I (with r = 0.8766), mitochondrial complex II (with r = 0.9187), mitochondrial complex III (with r = 0.9592), and mitochondrial complex IV (with r = 0.8132) ($P < 0.001$) [Figure 9].

Discussion

The results of the current study provide the first indication that crocin can inhibit anxiety-like behaviors and movement disorders caused by a toxic dose of nicotine in a mouse model. The present study also shows that crocin prevents the occurrence of events related to oxidative stress, inflammation, and mitochondrial disorders as well as autophagy and apoptosis in nicotine-dependent mice. The positive effects of crocin were mediated via modulation of JNK-BCL2-BECN1 or JNK-BCL2-BAX signaling pathways, which may indicate that they act as an intermediate step in the neuroprotective role of this agent.

As a psycho-stimulant agent, nicotine has a high potential for abuse and addiction.[65,66] In the first part of our study, the data show that nicotine at a dose of 10 mg/kg leads to a reduction of all of the examined OFT behaviors. According to a literature review, this dose of nicotine is considered high and can induce neurobehavioral toxicity, leading to motor activity dysfunction and anxiety.[67-69] Conversely, the result of our treatment protocol with crocin showed that this compound can reduce the behavioral disorders and anxiety-like behavior caused by nicotine in the OFT behavioral test. Treatment of animals with crocin alone caused a marginal increase in OFT behaviors but these results were not significant when compared to the control group.[70,71] This result is consistent with earlier studies about crocin's antidepressant, anti-anxiety, and motor activity modulator properties.[25,39,70-74] In addition, previous studies indicated that crocin in multiple doses can modulate drug abuse-induced neurobehavioral disturbances.[25,39,74] Thus, in the current study, crocin can exert some parts of its neuroprotective effects via modulation of nicotine-promoted anxiety and motor activity disturbances.

Molecular assessment of our work indicates that nicotine administration can cause occurrences of oxidative stress which can be seen with a rise in hippocampal MDA and GSSG levels and a reduction of CAT activity and GSH levels. These consequences and nicotine toxic dose effects on oxidative stress parameters are in agreement with previous studies showing that high doses of nicotine can lead to occurrences of lipid peroxidation and disturbances in the glutathione cycle.[18,19,75] Furthermore, nicotine abuse or chronic administration in both human and animal subjects can disturb the function of antioxidant enzymes such as SOD, CAT, GPX, and GSR.[75-78] Previous studies suggested

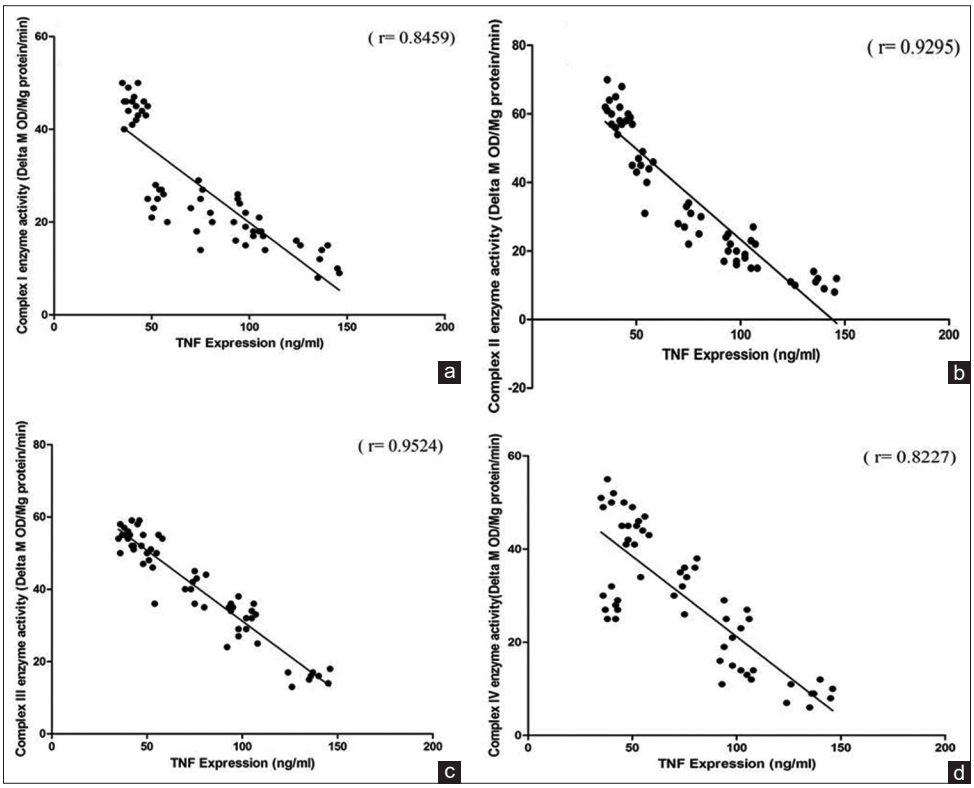

Figure 8: Analysis of TNF and mitochondrial complex enzymes. The Pearson correlation analysis between TNF expression and (a) mitochondrial complex I (with r = 0.8452), (b) mitochondrial complex II (with r = 0.9295), (c) mitochondrial complex III (with r = 0.9524) and (d) mitochondrial complex IV (with r = 0.8227) (d) ($P < 0.001$)

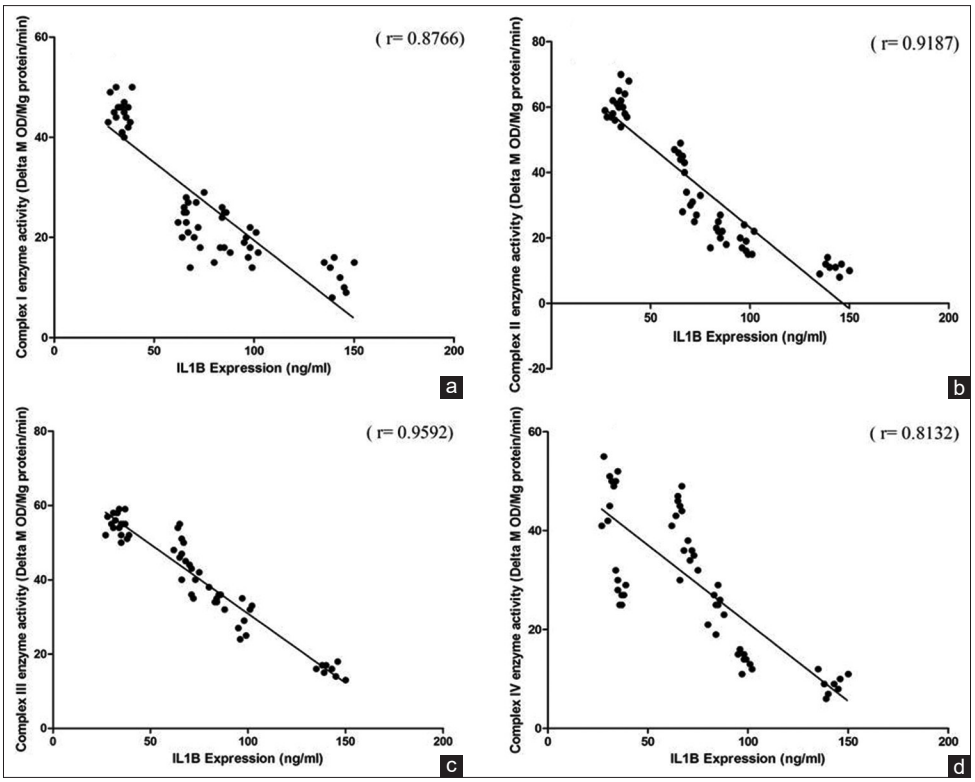

Figure 9: Analysis of IL1B and mitochondrial complex enzymes. The Pearson correlation analysis between IL1B expression and (a) mitochondrial complex I (with r = 0.8766), (b) mitochondrial complex II (with r = 0.9187), (c) mitochondrial complex III (with r = 0.9592) and (d) mitochondrial complex IV (with r = 0.8132) (d) (*P* < 0.001)

that some parts of nicotine-triggered neurodegeneration and neurotoxicity are mediated via the activation of free radicals, oxidative molecules, or reduction of cellular antioxidant defenses.[79,80] However, the precise mechanism of action of nicotine in this regard was not clarified.[18,19,75] We found that crocin with doses of 10, 20, 40, and 80 mg/kg inhibited oxidative stress and reduced the MDA and GSSG levels while it also triggered activation of CAT and elevated the GSH level. Crocin alone modestly increased these oxidative stress parameters but again the results were not significant when compared to the control group. Previous results showed the role of crocin against oxidative stress in neurodegenerative events or diseases.[24,26,33] These studies demonstrated that crocin can inhibit lipid peroxidation and also restore the glutathione level and antioxidant functions[71,73,81] as well as inhibit the creation of free radicals in neurodegenerative disorders.[81,82] Based on these properties it is expected that crocin can modulate nicotine-promoted neurodegeneration and neurotoxicity by stimulation of antioxidant enzymes or inhibition of free radicals, and lipid and protein peroxidation, and also through the improvement of glutathione effects.

Consistent with the results of changes in oxidative stress our data indicated that in animals treated with nicotine the level of hippocampal TNF and IL1B, as pro-inflammatory parameters, were increased. This finding was consistent with the previous data, which show that nicotine has a high potential for activation of neuro-inflammation and can trigger or initiate degenerative and malicious effects in neuronal cells.[83,84] Conversely, our data showed that crocin treatment (in all doses used) inhibited the nicotine-triggered elevation of TNF and IL1B levels, whereas administration of crocin alone caused insignificant reductions in these inflammatory biomarkers.[85,86] Thus, at least some of the neuroprotective roles of crocin appear to be due to the inhibition of inflammatory pathways.[25,24,39,74,87,88] Our data also indicated that nicotine administration can disturb mitochondrial function and cause a reduction of the activity of mitochondrial quadruple enzymes. Thus, we can speculate that chronic use of nicotine can lead to oxidative stress, inflammation, apoptosis, and autophagy during neurodegenerative events.[79,89-91] We found that crocin could block these nicotine-triggered effects and restore mitochondrial function, whereas the administration of crocin alone had no effect. Although the exact role of crocin in mitochondrial function in neuronal cells has not yet been determined,[33,34,92] our results suggest that the crocin-dependent enhancement of mitochondrial function can reduce occurrences of oxidative stress and inflammation, thus exerting preventive or neuroprotective effects on neurotoxicity caused by nicotine and other related substances.

Despite all the available information about the role of nicotine in the induction of inflammation and oxidative stress, and the

protective role of crocin, there is still insufficient information about the effects on cell death pathways. Thus, we examined the role of apoptosis and autophagy in mediating nicotine-triggered neurodegeneration and crocin-induced neuroprotective effects. Unphosphorylated BCL2 forms an inhibitory complex with BECN1 or BAX, biomarkers of autophagy and apoptosis, respectively.[21,93-96] Activation of JNK due to mitochondrial dysfunction or activation of TNF or IL1B receptors leads to BCL2 phosphorylation and dissociation from BECN1 or BAX, resulting in the induction of autophagy or apoptosis.[97-99] We found that nicotine increased the expression of both phosphorylated and unphosphorylated forms of JNK, whereas crocin inhibited this effect in nicotine-dependent rats. In addition, nicotine increased the levels of BAX, BECN1, and phosphorylated BCL2. In contrast, crocin caused a reduction of these effects in nicotine-addicted animals.

Therefore, the results of this study suggest that toxic doses of nicotine can cause cell death by stimulating parameters that induce apoptosis and autophagy, in agreement with the previous data.[2,13,14] In addition, nicotine-induced mitochondrial damage also causes oxidative stress and the activation of inflammatory pathways including the production of cytokines such as TNF and IL1B in the hippocampus, which in turn activates death receptors and the extrinsic apoptosis pathway.[2,13,14,19,83,100,101] The role of crocin in preventing cell death and inhibiting apoptosis and autophagy in neurodegenerative events has been mentioned in a limited number of studies,[24,26] but its role in inhibiting cell death caused by nicotine has not been clear. The present study suggests that crocin has a high potential for inhibition of inflammation and mitochondrial disorders caused by nicotine, thus preventing the occurrence of intrinsic and extrinsic pathways of apoptosis and autophagy.[24,26,39] Our data also suggest that modulation of the JNK-BCL2-BECN1 or BCL2-BAX signaling pathways in nicotine-abused subjects may be potential therapeutic targets for inhibition of neurodegeneration.[7,17,75,102-105]

Conclusions

From data obtained in the present study, we can conclude that chronic administration of nicotine in adult rats causes changes in motor activity and anxiety-like behaviors and also can cause activation of inflammatory biomarkers and dysfunction of mitochondrial dysfunction in hippocampal cells. According to our data, activation of the JNK, Beclin1, and BAX that cause activation of apoptosis and autophagy might be responsible for nicotine-induced neurodegeneration. Also, our data indicated that Crocin can interrupt nicotine-induced neurobehavioral changes and neuro-inflammation, and for the first time, the current study showed that Crocin by modulation of JNK/Bcl-2-Beclin1 or JNK/Bcl-2/BAX signaling pathways and its upstream events such as activation of neuro-inflammation, mitochondrial dysfunction and oxidative stress can be effective against nicotine-triggered apoptosis and or autophagy and consequences neurodegeneration. Based on these concepts, Crocin can be introduced as a candidate agent for the management of the behavioral and molecular sequels of smoking. Although these findings give us a new insight into mechanisms involved in nicotine-induced neurodegeneration or crocin-induced neuroprotection, further evaluation of precise molecular and cellular aspects of Crocin protective mechanisms especially against nicotine-induced neurodegeneration and neurobehavioral changes and smoking-induced sequels seems necessary.

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Ethics statement

All experimental procedures were accepted and approved by the Institutional Animal Use and Care Committee of the Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Research Protocol and Ethical Code Number = IR.SBMU.NRITLD.REC.1402.57).

Consent to participate and for publication

Current work is an experimental procedure in animal models and there is no consent for participation.

Abbreviations (alphabetical)

CAT: catalase; **GSH:** reduced form of glutathione; **GSSG:** glutathione in its oxidized form; **IL1B/IL-1β:** interleukin 1 beta; **JNK:** c-Jun-terminal Kinase; **MDA:** malondialdehyde; **OFT:** open field test; **SEM:** standard error of the mean; **TNF/TNF-α:** tumor necrosis factor.

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Conflicts of interest

There are no conflicts of interest.

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