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ORIGINAL PAPER

The Neuroprotective Effect of Curcumin Against Nicotine-Induced Neurotoxicity is Mediated by CREB–BDNF Signaling Pathway

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Abstract Nicotine abuse adversely affects brain and causes apoptotic neurodegeneration. Curcumin- a bright yellow chemical compound found in turmeric is associated with neuroprotective properties. The current study was designed to evaluate the role of CREB–BDNF signaling in mediating the neuroprotective effects of curcumin against nicotine-induced apoptosis, oxidative stress and inflammation in rats. Sixty adult male rats were divided randomly into six groups. Group 1 received 0.7 ml/rat normal saline, group 2 received 6 mg/kg nicotine. Groups 3, 4, 5 and 6 were treated concurrently with nicotine (6 mg/kg) and curcumin (10, 20, 40 and 60 mg/kg i.p. respectively) for 21 days. Open Field Test (OFT) was used to evaluate the motor activity. Hippocampal oxidative, antioxidant, inflammatory and apoptotic factors were evaluated. Furthermore, phosphorylated brain cyclic adenosine monophosphate (cAMP) response element binding protein (P-CREB) and brain derived neurotrophic factor (BDNF) levels were studied at gene and protein levels. We found that nicotine disturbed the motor activity in OFT and simultaneous treatment with curcumin (40 and 60 mg/kg) reduced the nicotine-induced motor activity disturbances. In addition, nicotine treatment increased lipid peroxidation and the levels of GSH, IL-1β, TNF-α and Bax, while reducing Bcl-2, P-CREB and BDNF levels in the hippocampus. Nicotine also reduced the activity of superoxide dismutase, glutathione peroxidase and glutathione reductase in hippocampus. In contrast, various doses of curcumin attenuated nicotine-induced apoptosis, oxidative stress and inflammation; while elevating P-CREB and BDNF levels. Thus, curcumin via activation of P-CREB/BDNF signaling pathway, confers neuroprotection against nicotine-induced inflammation, apoptosis and oxidative stress.

Keywords Nicotine · Curcumin · Neurodegeneration · CREB · BDNF

Introduction

In recent years, herbal/natural compounds with medicinal values have gained a striking attention. Natural flavonoids and their derivatives are being widely considered as supplementary therapeutics against neurodegenerative diseases [1–3].

Nicotine is the psycho-stimulant component of tobacco which carries parasympathomimetic properties [4, 5]. Its pharmacological similarity to amphetamine like stimulants makes it more prone to abuse and addiction [4, 6]. Nicotine abuse induces oxidative stress, apoptosis and inflammation in brain cells [7–9]. In-vitro studies have shown that nicotine exposure augments the production of the apoptotic proteins like caspase-3, 8 and 9 and causes DNA fragmentation in brain cells [10, 11]. Nicotine and other recreational drugs increase the release of cytochrome c and decrease mitochondrial viability and Bcl-2 production [11, 12]. Interestingly, nicotine-induced neurotoxicity appears to be more pronounced in some brain regions like hippocampus and amygdala [8, 13, 14]. Nicotine dose-dependently increases hippocampal neuronal degeneration in CA1, CA2, CA3 and DG regions [15, 16].

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Curcumin (diferuloylmethane)- the most abundant component of turmeric, is extracted from rhizomes of the plant *Curcuma longa* [17–19]. This non-nutritive yellow pigment is an established nutraceutical dietary phenol and has a significant medicinal and pharmacological value [3, 20, 21]. Curcumin exerts biological effects through its antioxidant, anti-inflammatory, antiapoptotic, immunomodulatory activity [22]. Recent studies performed in both vertebrate and invertebrate models have revealed that this natural polyphenol carries therapeutic potential for neurodegenerative diseases like alzheimer's disease and parkinson's disease [20, 23–25]. Curcumin treatment has shown to counteract oxidative stress by reducing lipid peroxidation and improving the activity of antioxidant enzymes like superoxide dismutase (SOD) and catalase [26, 27]. Furthermore, chronic treatment with curcumin reduces alcohol-induced rise in TNF-α, IL-1β, and TGF-β1 levels [28–30].

Cyclic AMP response element binding protein (CREB) is a major transcription factor involved in regulation of genes associated with synaptic and neural plasticity. Brain-derived neurotrophic factor (BDNF) is an important neurotrophic factor which primarily supports growth and survival of neurons. It is highly expressed in brain areas that are known to regulate cognition, emotions and rewards [31, 32]. It is suggested that curcumin may protect hippocampal and frontal neurons against stress-induced damage via up regulation of CREB and BDNF [33, 34]. Thus, we designed this study to assess that does curcumin confers neuroprotection against nicotine-induced hippocampal damage? And what is the role of P-CREB–BDNF signaling pathway in this protection.

Materials and Methods

Animals

Sixty adult male rats (8 weeks old, weighing 200 ± 8.0 g) were obtained from Pasteur Institute of Iran (Tehran, Iran) and were transferred to laboratory. Animals were acclimated to experimental conditions (12 h light dark cycle, 24 °C) for 2 weeks and had free access to standard food and tap water. The present study was performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The experimental protocol was approved by the Research Council of Iran University of Medical Sciences, Tehran, Iran.

Drugs

Curcumin and Nicotine were purchased from Sigma-Aldrich (USA) and dissolved freshly in normal saline just before administration. The volume of injection was adjusted to 0.7 ml/rat.

Experimental Design

Sixty adult male rats were assigned to one of the following groups:

Group 1 (negative control) was treated with normal saline (0.7 ml/rat, i.p.) for 21 days.

Group 2 (positive control) was treated with nicotine (6 mg/kg/day, s.c.) for 21 days.

Groups 3, 4, 5 and 6 were treated concurrently with nicotine (6 mg/kg/day, s.c) and Curcumin (10, 20, 40 and 60 mg/kg, i.p., respectively) for 21 days.

On day 22, Open Field Test (OFT)- a standard behavioral method for studying hippocampal degeneration was performed to evaluate the motor activity and depression in experimental animals. In addition, oxidative stress, inflammation and apoptosis were also evaluated in hippocampal tissues. Keeping in view the importance of CREB signaling and its product, BDNF, the effect of curcumin on nicotine-induced disturbances in CREB signaling pathway was studied. Furthermore, cresyl violet staining was also performed in dentate gyrus and CA1 area of hippocampus to study cells' density and neurodegeneration.

Open Field Test (OFT)

This assay was used to evaluate anxiety and locomotor activity in rodents [2, 35, 36]. Four typical behaviors in OFT were assessed and scored;

1. Line crossing (ambulation) distance: Total distance of the grid lines crossed by each rat
2. Center square entries: Number of times each rat entered the central red square with all four paws.
3. Center square duration: The time spent by each rat in the central square.
4. Rearing: Frequency with which each rat stands on its hind legs in the maze.

Mitochondrial Preparations

Animals were anesthetized using sodium thiopental (50 mg/kg, i.p) and the hippocampus was isolated from each rat. The isolated tissues were homogenized in cold homogenization buffer (25 mmol/l 4-morpholinepropanesulfonic acid, 400 mmol/l sucrose, 4 mmol/l magnesium chloride ($MgCl_2$), 0.05 mmol/l ethylene glycol tetra acetic acid (EGTA), pH 7.3) and the homogenized tissues were centrifuged at 450×*g* for 10 min. The supernatants obtained were re-centrifuged at 12,000×*g* for 10 min. Finally, the

sediments were re-suspended in homogenization buffer and stored at 0 °C. Total mitochondrial proteins in tissues were determined using Dc protein assay kit (Bio-Rad). Briefly; Bradford reagent (one part Bradford: four parts dH_2O) was added to serial dilution series (0.1–1.0 mg/ml) of a known protein sample concentration; e.g., bovine serum albumin (BSA), dissolved in homogenization buffer. These serial dilution series were prepared and used for providing a standard curve. On the other hands 10, 15, 20, 25 and 30 µl of the protein extract (homogenized cell solutions) were added to multiple wells. Bradford reagent was also added to each well. Density of colors of all wells was read by plate reader at 630 nm. Finally, by using the standard curve, protein quantity in extracts were obtained. These homogenized cell solutions, containing mitochondria of hippocampal cells, were analyzed for the measurement of oxidative stress and inflammatory markers [1, 35, 37].

Measurement of Oxidative Stress Parameters

Determination of Lipid Peroxidation

For assessment of lipid peroxidation, malondialdehyde (MDA)—a natural bi-product, was assessed. Briefly, 100 µl of SDS lysis solution was added to wells containing (100 µl) of sample solution or MDA standard. After shaking and incubation of these wells, 250 µl of thiobarbituric acid reagent was added to each well and incubated at 95 °C for 45–60 min. Next, tubes were centrifuged at 1000×*g* for 15 min and 300 µl of n-Butanol was added to 300 µl of supernatant. Then, the tubes were centrifuged for 5 min at 10,000×*g*. Finally, the absorbance was read at 532 nm and the results obtained were expressed as nmol/mg of protein [1, 38].

Determination of GSH and GSSG

For measuring GSH and GSSG levels, 25 µl of the IX glutathione reductase solution and 25 µl of the IX NADPH solution were added to a 96-well plate containing standard solution of glutathione or sample of homogenized solution. Then, 50 µl of the IX Chromogen was added to each well and mixed vigorously. Finally, the absorbance was read at 405 nm for each GSSG/GSH standard and sample. Using the standard curve, the levels of GSSG/GSH were quantified and expressed as nmol/mg of protein [1, 39].

Determination of Manganese Superoxide Dismutase (MnSOD) Activity

The previously described method was used to assess SOD activity [1]. SOD activity was measured using the following equation:

SOD activity = SOD activity = {[(A blank 1 − A blank 3) − (A sample - A blank 2)]/(A blank 1 − A blank 3)} × 100. Data were reported as U/ml/mg protein [1].

Determination of Glutathione Peroxidase (GPx) activity

GPx activity was assessed as described previously [1]. It was measured based on change in absorbance [ΔA340/min] by the following equation:

ΔA340/min = A340nm (Start) − A340nm (Stop)/Reaction time (min), any change in the absorbance is directly proportional to GPx activity.

GPx activity: ΔA340/min × Reaction volume (ml) × Dilution factor of the original sample/Extinction coefficient for NADPH at 340 nm × Volume of the tested sample. Results were expressed as mU/mg protein [1].

Determination of Glutathione Reductase (GR) activity

GR activity was assessed as described previously [1]. It was measured based on change in absorbance [ΔA340/min] by the following equation:

ΔA340/min = A340nm (Start) − A340nm (Stop)/Reaction time (min), any change in the absorbance is directly proportional to GR activity.

GR activity: ΔA340/min × Reaction volume (ml) × Dilution factor of the original sample/Extinction coefficient for NADPH at 340 nm × Volume of the tested sample. Results were expressed as mU/mg protein [1].

Measurement of Inflammatory Parameters

Determination of IL-1β and TNF-α levels

Concentrations of TNF-α and IL-1β in supernatant which contained the mitochondria of hippocampal cells, were measured using a commercially available ELISA kit (Genzyme Diagnostics, Cambridge, U.S.A). Briefly, wells containing sheep anti-rat IL-1β and TNF-α polyclonal antibody (Sigma Chemical Co., Poole, and Dorset, UK) were washed three times with washing buffer (0.5 mol/l of Sodium chloride (NaCl), 2.5 mmol/l sodium dihydrogen phosphate (NaH_2PO_4), 7.5 mmol/l Na_2HPO_4, 0.1% Tween 20, pH 7.2). Then, 100 ml of 1% (w/v) ovalbumin (Sigma Chemical Co., Poole, Dorset, UK) solution was added to each well and incubated at 37 °C for 1 h. Following three washes, 100 ml of samples and standards were added to each well and incubated at 48 °C for 20 h. After three washes, 100 ml of the biotinylated sheep anti-rat IL-1β or TNF-α antibody (1:1000 dilutions in washing buffer containing 1% sheep serum, Sigma Chemical Co., Poole, and Dorset, UK) was added to each well. Next, after 1 h incubation and three washes, 100 ml avidin-HRP (Dako Ltd, UK)

(1:5000 dilution in wash buffer) was added to each well and the plate were incubated for 15 min. After washing three times, 100 ml of TMB substrate solution (Dako Ltd., UK) was added to each well and then incubated for 10 min at room temperature. Then, 100 ml of 1 mol/l H_2SO_4 was added and absorbance was read at 450 nm. Results were expressed as ng IL-1β/ml or TNF-α/ml [1].

Real-time Reverse Transcriptase-PCR (RT-PCR) studies

Total RNA was extracted from 200 µg of hippocampal tissues by using ONE STEP-RNA reagent (Bio Basic, Canada inc.) according to the manufacturer's instructions. Extracted RNA was assessed for quantity and quality using a nanodrop (ND-1000, Thermo Scientific Fisher, US) and gel electrophoresis respectively. To eliminate genomic contamination, RNA was treated with DNase I (Qiagen, Hilden, Germany) as described by the manufacturer. Next, complementary DNA (cDNA) was synthesized using 1 µg of total RNA. The integrity and quality of cDNA was examined using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer as housekeeping. Real-time reverse transcriptase-PCR (RT-PCR) was carried out to evaluate the differences in expression patterns for BDNF and CREB genes among samples of each group. Primers were designed using Primer 3 software version 0.4 (frodo.wi.mit.edu) and are as follows:

BDNF Forward: 5′-GGAGGCTAAGTGGAGCTG AC-3′.

Reverse: 5′-GCTTCCGAGCCTTCCTTTAG-3′.

CREB1 Forward: 5′-CAGACAACCAGCAGAGTG GA-3′.

Reverse: 5′-CTGGACTGTCTGCCCATTG-3′.

GAPDH Forward: 5′-AGACAGCCGCATCTTCTT GT-3′.

Reverse: 5′-CCGTTCACACCGACCTTCA-3′.

Real time RT-PCR was performed in 20 µl reactions containing 1 µl cDNA target, 100 nmol/l forward and reverse primers and 1 × SYBR® Premix Ex Taq™ II (Takara, Tokyo, Japan). Experiments were carried out in triplicate using a CFX96™ Real-Time System (C1000TM Thermal Cycler; Bio-Rad, Hercules, CA, USA). Amplification conditions were as follow: initial denaturation at 95 °C for 10 min, followed by 40 cycles (denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min). The relative values of the mRNA expression of CREB and BDNF genes were calculated by comparing the cycle thresholds of the target gene with that of the housekeeping gene (GAPDH) using the $2^{-\Delta\Delta ct}$ method and REST 2009 software [40]. Serial dilutions of cDNAs were used for calculation of the primer sets efficiencies in real-time PCR. In this regards, the efficiencies of various primer sets were found to be similar [40].

Western Blot

We studied the immunoreactivity of CREB, CREB-P, BDNF (ligand of TrkB receptor), Bax and Bcl-2 contents in the hippocampal tissues by western blotting. Electrotransfer of the resolved bands from gel to polyvilinydene difluoride membrane (Millipore, Bedford, USA) was performed in 90 min at 0.7 mA/cm^2 using a semi-dry transfer apparatus (PeQlab). After the transferring step, the membrane was weakly stained for 3 min with Coomassie blue G-250 (1 µg/100 ml) (Sigma Aldrich, UK). Then, the membrane was dried and cut into 2 mm wide stripes. After destaining with methanol, the stripes were washed and blocked with 2% BSA overnight at 4 °C. Next day, bovine serum (dilution 1:100) was added and were incubated at room temperature (RT) for 2 h on a shaker. The membranes were then washed with PBS-T (three washing steps) and were incubated with the following conjugated polyclonal anti-rabbit antibody: BDNF (special antibody to types of BDNF which bind to TrkB receptor) and CREB (total and phosphorylated; 1:500 dilutions in BSA, 360 min, RT; Sigma Aldrich, Germany) Bax and Bcl-2 (1:1000 dilutions in BSA, 240 min, RT; Sigma Aldrich, Germany). Next, all stripes were incubated with secondary HRP conjugated polyclonal rabbit anti-Sheep antibody (1:5000 dilution in BSA, 120 min, RT; Sina Biotech, Iran). The stripes were washed and incubated with chemiluminescent substrate luminol and hydrogen peroxide (H_2O_2) for 2 min at RT. Finally, the reactive bands were detected on X-ray film within 10–20 s under safelight condition. After this process all bands' images or pictures were analyzed by AlphaEaseFC software (Miami, USA), which according to guides of this software all bands shadows were deleted and core density of each band measured and proportion of density of bands of target gene and protein, BDNF, Bax and Bcl-2, compared to density of housekeeping gene (GAPDH) calculated as proportion and this proportion were compared and analyzed between experimental groups for target genes, it should be mentioned about the P-CREB, the changes in P-CREB expression compared with CREB expression itself and results of this proportion compared between experimental groups [41].

Statistical Analysis

All data were statistically analyzed using Graph Pad PRISM Software (Version 6). The data were expressed as mean ± standard error of the mean (SEM). Differences between control and treatment groups were evaluated using one way ANOVA. Differences between the behaviors in

groups were evaluated by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

Results

Effects of Various Doses of Curcumin on Nicotine-Induced Behavioral Disturbances

As shown in Table 1, the animals in nicotine (6 mg/kg) treated group entered the central square less frequently and spent less time in the central region of the OFT in comparison to the negative control group ($p < 0.05$). Furthermore, nicotine treated animals covered less ambulation distance in OFT as compared to negative controls ($p < 0.05$). Conversely, the groups treated with nicotine in combination with curcumin (40 and 60 mg/kg) demonstrated more frequent central square entries and time spent in the central region of the OFT when compared to nicotine only treated animals ($p < 0.05$). In addition, curcumin (40 and 60 mg/kg) treatment also increased the ambulation distance in nicotine treated animals in comparison to the positive controls ($p < 0.05$).

Effects of Various Doses of Curcumin on Nicotine-Induced Lipid Peroxidation

Nicotine administration significantly increased the lipid peroxidation as indicated by elevated mitochondrial MDA levels when compared to the negative control ($P < 0.001$). Conversely, various doses of curcumin (40 and 60 mg/kg) reduced the nicotine-induced rise in MDA levels when compared to the positive controls ($P < 0.001$) (Table 2).

Effects of Various Doses of Curcumin on Nicotine-Induced GSH/GSSG Alterations in Mitochondria

Nicotine (6 mg/kg) treatment markedly reduced the mitochondrial GSH content, while increasing the GSSG levels in comparison to the negative controls ($P < 0.001$). Conversely, various doses of curcumin (40 and 60 mg/kg) improved the GSH-content and reduced the GSSG levels in nicotine treated animals when compared to the positive controls ($P < 0.001$) (Table 3).

Table 1 Effects of various doses of curcumin on open field exploratory and anxiety-like behavior in nicotine treated rats

Group	Ambulation distance (cm)	Central square entries (number)	Time spent in central square (s)	Number of rearing
Negative control	365 ± 12	17 ± 1.1	135 ± 8	14 ± 1.5
Positive control (nicotine-6 mg/kg)	311 ± 12[a]	9 ± 1[a]	96 ± 10[a]	9 ± 2[a]
Nicotine + curcumin (10 mg/kg)	320 ± 12	11 ± 1.1	104 ± 18	10 ± 0.5
Nicotine + curcumin (20 mg/kg)	334 ± 16	13 ± 3.1	113 ± 10	11.5 ± 1
Nicotine + curcumin (40 mg/kg)	344 ± 14[b]	16 ± 1.5[b]	120 ± 7[b]	12.5 ± 2[b]
Nicotine + curcumin (60 mg/kg)	350 ± 12[b]	17 ± 2[b]	127 ± 12[b]	13 ± 2[b]

All data are presented as mean ± SEM, n = 8

[a] $p < 0.05$ vs. negative control group

[b] $p < 0.05$ vs. positive control group

Table 2 Effects of various doses of curcumin on MDA level, SOD, GPx and GR activities in nicotine treated rats

Group	MDA nmol/mg of protein	SOD U/ml/mg protein	GPx mU/mg protein	GR mU/mg protein
Negative control	4.9 ± 1.2	64 ± 6.9	125 ± 6	135 ± 7
Positive control (nicotine-6 mg/kg)	15 ± 2[a]	42 ± 4[a]	92 ± 6[a]	102 ± 8[a]
Nicotine + curcumin (10 mg/kg)	13 ± 2.1	45 ± 6	95 ± 8	105 ± 9
Nicotine + curcumin (20 mg/kg)	12 ± 1.6	50 ± 7	100 ± 8	110 ± 8
Nicotine + curcumin (40 mg/kg)	8 ± 1.4[b]	58 ± 8[b]	118 ± 7[b]	126 ± 9[b]
Nicotine + curcumin (60 mg/kg)	6 ± 1.2[b]	62 ± 8[b]	120 ± 11[b]	131 ± 9[b]

All data are presented as mean ± SEM, n = 8

[a] $p < 0.05$ vs. negative control group

[b] $p < 0.05$ vs. positive control group

Table 3 Effects of various doses of curcumin on mitochondrial GSH and GSSG content in nicotine treated rats

Group	Mean ± SEM GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG
Negative control	60.8 ± 3.1	0.78 ± 0.1	76
Positive control (nicotine-6 mg/kg)	38.6 ± 3.1[a]	3.3 ± 1.4[a]	11[a]
Nicotine + curcumin (10 mg/kg)	44.4 ± 2.4	2.9 ± 0.06	15.3
Nicotine + curcumin (20 mg/kg)	48.4 ± 4	2.84 ± 0.14	17.04
Nicotine + curcumin (40 mg/kg)	54.5 ± 4.9[b]	1.5 ± 0.12[b]	36.3[b]
Nicotine + curcumin (60 mg/kg)	55.5 ± 1.7[b]	1.3 ± 0.20[b]	42.6[b]

All data are presented as mean ± SEM, n = 8

[a] $p < 0.001$ vs. negative control group

[b] $p < 0.001$ vs. positive control group

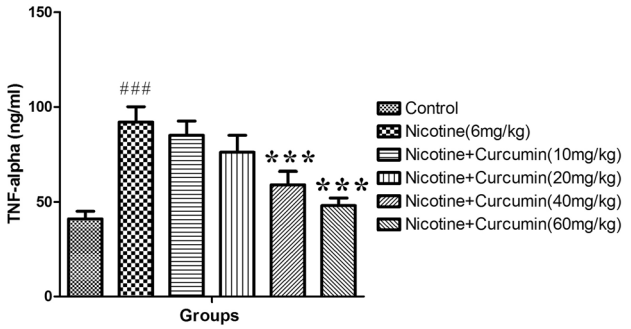

Fig. 1 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced rise in mitochondrial TNF-α level. ***$p < 0.001$ vs. positive control (nicotine treated) group, ###$p < 0.001$ vs. negative control group

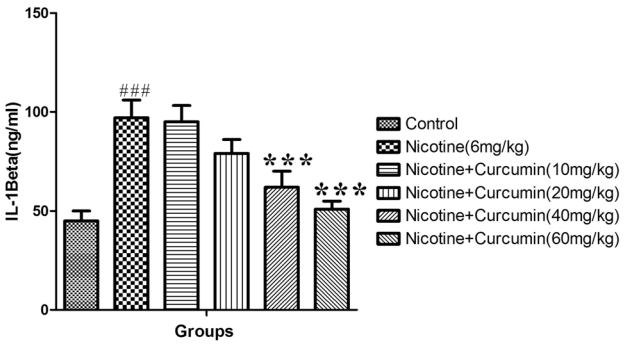

Fig. 2 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced rise in mitochondrial IL-β level. ***$p < 0.001$ vs. positive control (nicotine treated) group, ###$p < 0.001$ vs. negative control group

Effects of Various Doses of Curcumin on Nicotine-Induced Attenuation in the Mitochondrial Superoxide Dismutase (SOD), GPx and GR activity

Nicotine (6 mg/kg) treatment markedly reduced the SOD, GPx and GR activity as compared to the negative control group ($P < 0.001$). Conversely, various doses of curcumin (40 and 60 mg/kg) significantly improved the SOD, GPx and GR activities in nicotine treated animals when compared to the positive controls ($P < 0.001$) (Table 2).

Effects of Various Doses of Curcumin on Nicotine-Induced Rise in IL-1β and TNF-α Levels

The animals in nicotine treated group demonstrated a significant elevation in IL-1β and TNF-α levels as compared to the negative control group ($P < 0.001$). Conversely, curcumin (40 and 60 mg/kg) prevented the nicotine-induced rise in proinflammatory cytokines when compared to positive controls ($P < 0.001$) (Figs. 1, 2).

Effects of Various Doses of Curcumin on Nicotine-Induced Decrease in CREB Gene Expression

Nicotine (6 mg/kg) treatment markedly attenuated the gene expression of CREB in DG and CA1 regions of the hippocampus in comparison to the negative control group ($P < 0.001$). Conversely, curcumin (40 and 60 mg/kg) treatment significantly improved the CREB gene expression in nicotine treated animals when compared to the positive controls (Fig. 3a, b).

Effects of Various Doses of Curcumin on Nicotine-Induced Decrease in BDNF Gene Expression

Nicotine (6 mg/kg) treatment markedly attenuated the gene expression of BDNF in DG and CA1 regions of the hippocampus in comparison to the negative control group ($P < 0.001$). Conversely, curcumin (40 and 60 mg/kg) treatment significantly improved the BDNF gene expression

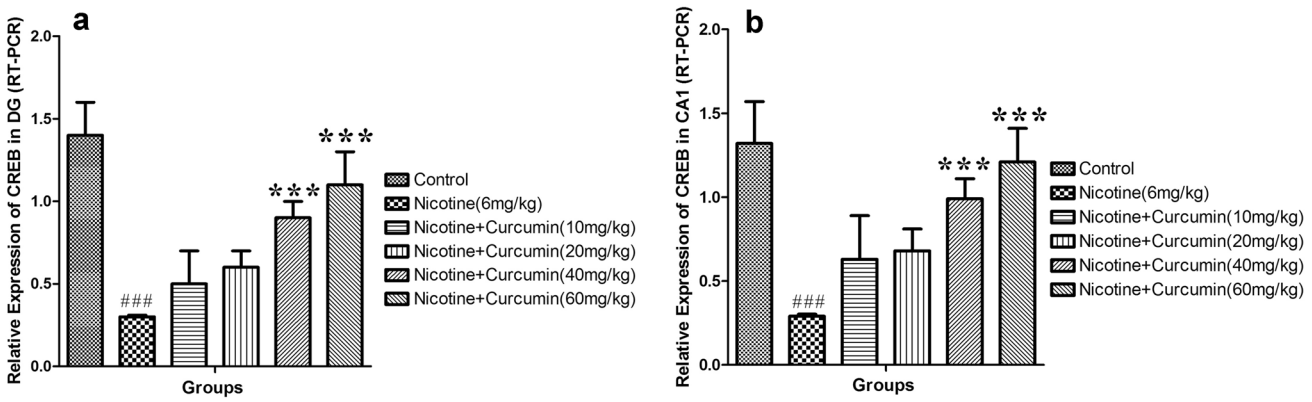

Fig. 3 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced alterations in gene expression of CREB in **a** DG, **b** CA1 region of hippocampus. ***$p < 0.001$ vs. positive control (nicotine treated) group, $^{###}p < 0.001$ vs. negative control group

in nicotine treated animals when compared to the positive controls (Fig. 4a, b).

Effects of Various Doses of Curcumin on Nicotine-Induced Alteration in Protein Expression of P-CREB/CREB

Nicotine (6 mg/kg) treatment markedly reduced the relative protein expression of phosphorylated CREB to total CREB in DG and CA1 regions of the hippocampus in comparison to the negative control group ($P < 0.001$). Conversely, curcumin (40 and 60 mg/kg) treatment significantly improved the protein expression of P-CREB/CREB in nicotine treated animals when compared to the nicotine only treated group (Fig. 5a, b).

Effects of Various Doses of Curcumin on Nicotine-Induced Reduction in the Protein Expression of BDNF

Nicotine (6 mg/kg) treatment markedly reduced the protein expression in DG and CA1 regions of the hippocampus in comparison to the negative control group ($P < 0.001$). Conversely, curcumin (40 and 60 mg/kg) treatment significantly improved the protein expression of BDNF in nicotine treated animals when compared to the positive controls (Fig. 6a, b).

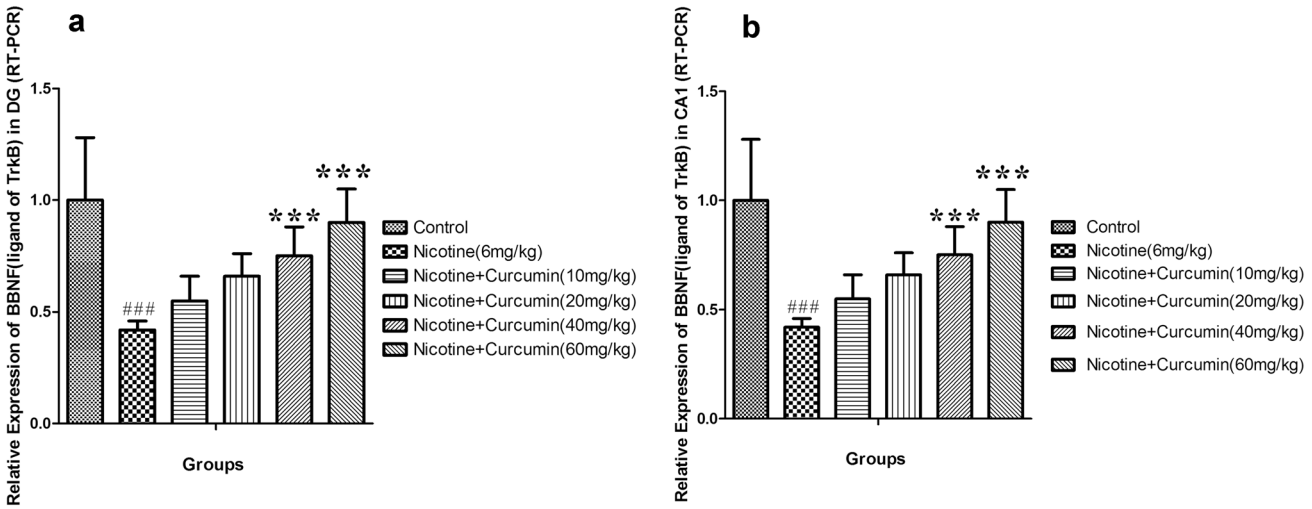

Fig. 4 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced alterations in gene expression of BDNF (ligand of **TrkB receptor**) in **a** DG, **b** CA1 region of hippocampus. ***$p < 0.001$ vs. positive control (nicotine treated) group, $^{###}p < 0.001$ vs. negative control group

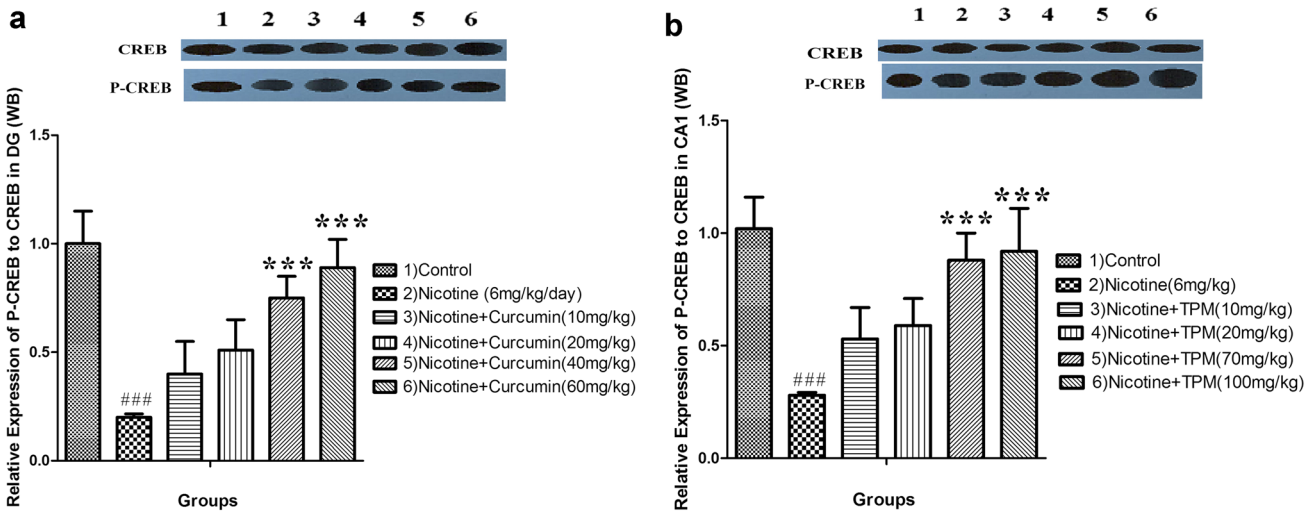

Fig. 5 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced alterations in protein expression of P-CREB in **a** DG, **b** CA1 region of hippocampus. ***$p < 0.001$ vs. positive control (nicotine treated) group, $^{###}p < 0.001$ vs. negative control group

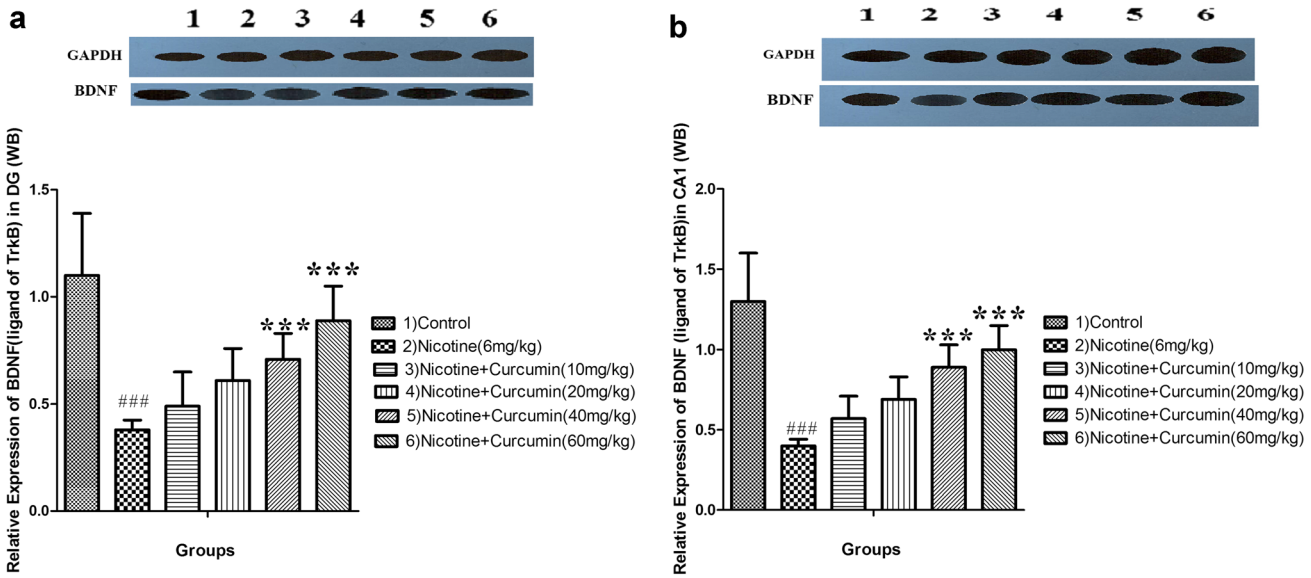

Fig. 6 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced alterations in protein expression of BDNF (ligand of **TrkB receptor**) in **a** DG, **b** CA1 region of hippocampus. ***$p < 0.001$ vs. positive control (nicotine treated) group, $^{###}p < 0.001$ vs. negative control group

Effects of Various Doses of Curcumin on Nicotine-Induced Changes in Bax and Bcl-2 Proteins Expression

Nicotine (6 mg/kg) treatment increased Bax and reduced Bcl-2 protein expression in the hippocampus as compared to the negative control group ($P < 0.001$). Conversely, curcumin (40 and 60 mg/kg) improved Bcl-2 while reducing Bax protein expression when compared to the positive controls ($P < 0.001$) (Fig. 7a, b).

Discussion

The current study shows that administration of various doses of curcumin can reduce nicotine-induced apoptosis, oxidative stress and inflammation in the hippocampus. Furthermore, our study demonstrates that the protective effects of curcumin are mediated via CREB–BDNF signaling pathway. We observed that chronic nicotine (6 mg/kg) exposure altered behavioral parameters, such as motor activity and anxiety in the OFT. Furthermore, nicotine

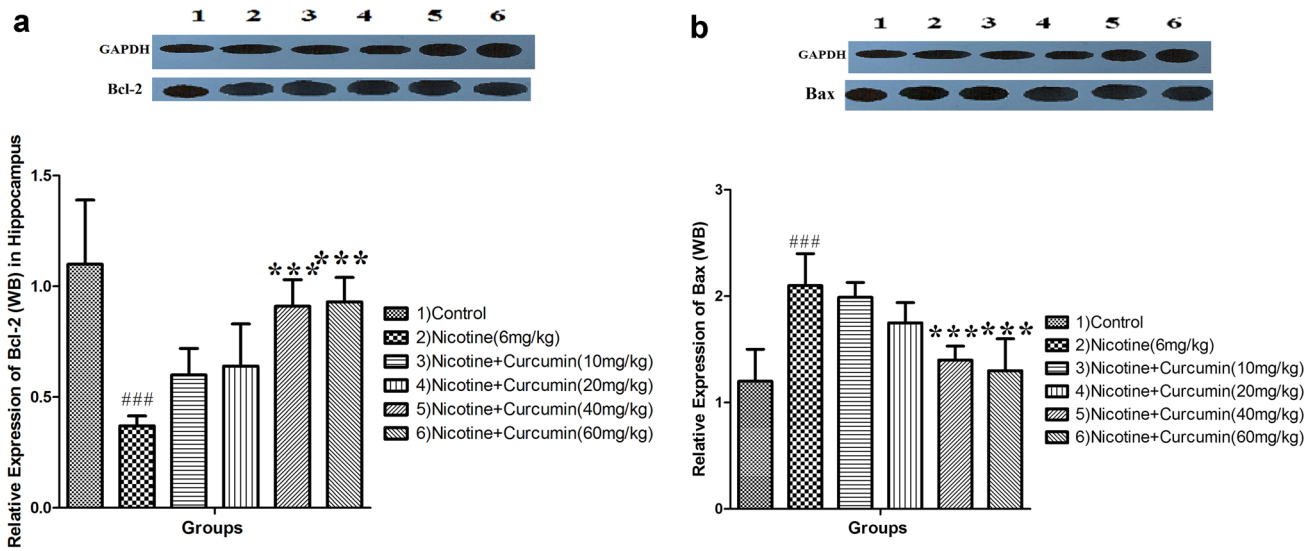

Fig. 7 Effects of various doses of curcumin (10, 20, 40 and 60 mg/kg) on nicotine-induced alterations in protein expression of **a** Bcl-2, **b** Bax in hippocampus. ***$p < 0.001$ vs. positive control (nicotine treated) group, $^{###}p < 0.001$ vs. negative control group

administration increased lipid peroxidation and GSSG content, while reducing GSH content and the activity of anti-oxidant enzymes like GPx, GR and SOD. Moreover, nicotine treatment increased the levels of IL-β and TNF-α in isolated hippocampus. Conversely, curcumin treatment attenuated oxidative stress and neuroinflammation in nicotine-dependent rats in a dose dependent manner. Nicotine also increased the protein expression of Bax, an apoptotic protein, while suppressing the protein expression of Bcl-2, an antiapoptotic protein. In contrast, curcumin inhibited nicotine -induced rise in Bax protein, and enhanced the expression of Bcl-2 in a dose dependent manner. An important finding of the current study was that nicotine exposure markedly reduced the gene and relative protein expression of total and phosphorylated CREB and protein expression of BDNF. On the other hand, curcumin administration significantly improved P-CREB and BDNF expression.

Nicotine as a psycho-stimulant compound, carries a high potential for abuse and addiction [42]. We found that nicotine (6 mg/kg) administration decreases the ambulation number and ambulation distance in the OFT. In contrast, curcumin at doses of 40 and 60 mg/kg improves ambulation number and ambulation distance in nicotine treated rats. Previous study have indicated that chronic nicotine administration tends to disturb the motor activity in rats [43]. We observed that 6 mg/kg of nicotine reduces the number of central square entries and time spent in central square in the OFT, which is indicative of anxiety induction. Previous literature has also indicated nicotine-induced rise in anxiety levels. In the current study, curcumin at doses of 40 and 60 mg/kg cause an increase in central square entry and time spent in central square in the OFT [22, 44]. Previous work from our laboratory has also revealed the anxiolytic effects of curcumin in rodents [2].

The brain is highly sensitive to oxidative damage as it contains a very limited anti-oxidant capacity [1, 45]. Chronic exposure of nicotine in adult and juvenile rats has shown to induce mitochondrial dysfunction and disturbances in respiratory enzyme activity in brain cells [16]. However, the mechanism for nicotine-induced oxidative stress is not well understood [46]. The present study shows that nicotine (6 mg/kg) enhances lipid peroxidation as indicated by a significant rise in MDA levels following the nicotine exposure. Nicotine administration also increased the mitochondrial GSSG levels, while reducing GSH content. Previous studies have shown that GSH reduces lipid peroxidation and acts as an antioxidant mediator [35]. On the other hand, administration of curcumin (10, 20, 40 and 60 mg/kg) in nicotine treated rats prevented the rise in MDA levels and thereby, reduced lipid peroxidation. Furthermore, curcumin improved GSH content and reduced GSSG levels in nicotine treated animals. Previous studies have confirmed that curcumin confers neuroprotection at least partly by scavenging free radicals [16, 47]. Curcumin has been proposed for the treatment of neurodegenerative diseases as it promotes GSH formation [48]. We observed that curcumin significantly improves the activity of major antioxidant enzymes like SOD, CAT, GR and GPx. Thus, curcumin by improving GPx and GR activities, may have enhanced the conversion of GSSG to GSH, and protected the brain against nicotine-induced oxidative stress. A pilot clinical study has reported the profound potential of curcumin in modulating oxidant-antioxidant system in drug abusers [49]. In addition, other in vivo and in vitro studies

have also confirmed antioxidative properties of curcumin [45, 50, 51].

We demonstrated that chronic nicotine exposure increases the level of pro-inflammatory cytokines like IL-β and TNF-α in the hippocampal tissue, whereas, curcumin has a strong potential for suppressing nicotine-induced neuroinflammation in a dose-dependent manner. Our results are in agreement with previous findings which have reported the rise of pro-inflammatory cytokines following nicotine abuse. It has been suggested that nicotine-induced rise in pro-inflammatory cytokines is responsible for the neurodegenerative effects of nicotine [52, 53]. In addition, curcumin has shown to block multiples sites of TNF-α and TGF-β signaling cascades, thereby protecting the brain against inflammation and injury [54, 55].

In addition to oxidative stress and inflammation, the current study confirms nicotine-induced apoptosis in the DG and CA1 areas of hippocampus. Nicotine administration increased the protein expression of an apoptotic protein, Bax, while decreasing an anti-apoptotic protein, Bcl-2. Previously it has been demonstrated that tobacco and nicotine abuse can cause brain damage via activation of multiple apoptotic pathways [56]. On the other hand, our results demonstrated the anti-apoptotic effect of curcumin against nicotine abuse, as indicated by reduced Bax and improved Bcl-2 expressions in the hippocampus. It has been shown that curcumin treatment attenuates cleaved caspase-3 and nuclear condensation resulting from brain ischemia–reperfusion injury [57].

The anti-inflammatory, anti-apoptotic and anti-oxidative effects of curcumin have been previously reported, but the putative mechanism of action remains unclear. In this regard, we evaluated the role of CREB–BDNF pathway. Our results demonstrated that nicotine administration reduces CREB and BDNF expressions in DG and CA1 areas of the hippocampus. In contrast, curcumin treatment enhanced CREB (at gene level), CREB-P proportion to CREB and BDNF gene and protein expressions in a dose dependent manner. Thus, it can be speculated that curcumin treatment restores P-CREB–BDNF signaling cascade and protects the brain against nicotine-induced injury. The transcription factor CREB regulates over hundred target genes implicated in neuronal development, survival and excitability, circadian rhythms, addiction, depression, learning and memory. In addition, dysregulation of CREB transcriptional cascade has shown to induce oxidative stress, apoptosis and neurodegeneration [58]. Many previous molecular studies demonstrated that phosphorylated form of CREB has the main role in many herbal and chemical neuroprotective properties, according to these studies proportion of P-CREB to CREB (P-CREB/CREB) in many situation such as neurodegenerative disorders were decreased while some neuroprotective agent increased the proportion [24, 59]. In consistency with these data, in current study, the relative expression of P-CREB to CREB were decreased by nicotine administration while uses of curcumin increased this proportion and relative expression of P-CREB/CREB in nicotine treated groups. BDNF is an important nerve growth factor regulated by cAMP and CREB [24, 59, 60]. According to several studies P-CREB causes production of BDNF, ligands of TrkB receptor. These studies showed that BDNF by activation of its own receptor, TrkB, can cause the production of BDNF and by mediation of this positive feedback starts the benefitial cascade in neuron which inhibits brain cell from degeneration and induces the survival of neurons [61]. In current study, it seems that decrease in P-CREB protein expression affects the mentioned cascade of BDNF/TrkB signaling pathway and activates the neurodegeneration, inflammation and oxidative stress. While curcumin administration inhibits this effect of nicotine, and cascade of P-CREB/BDNF/TrkB will be activated. Furthermore, P-CREB–BDNF signaling pathway has been implicated in regulating several functions in brain such as learning, memory, mood balances and reward mechanisms [62]. Studies have shown that chronic use of psycho-stimulants can disturb the P-CREB–BDNF cascade and cause neurodegeneration [63]. Similar to previous studies [8, 64], we observed marked neurodegeneration in the DG and CA1 areas of hippocampus caused by nicotine administration. In contrast, curcumin treatment improved cell density and preserved the tissue architecture in nicotine treated animals. Shin et al., also demonstrated curcumin-induced reduction in hippocampal cell death in animals treated with kainic acid [65].

Conclusion

Taken together, the results of the current study shows that curcumin treatment via activation of P-CREB–BDNF pathway, reduces nicotine-induced apoptosis, oxidative stress and inflammation. Thus, curcumin could be a therapeutic potential for reducing toxicities caused by nicotine abuse and other neurodegenerative diseases. However, further studies regarding human dosage and toxicity are warranted.

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