Effect of curcumin on hippocampus dentate gyrus injury induced by nicotine in rats

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ABSTRACT

Introduction: Nicotine is the most important alkaloid compound in tobacco and is a major risk factor in the development of functional disorder of several organ systems. Some plants produce Curcumin, which has antioxidant and neuroprotective properties. This study was designed to evaluate the therapeutic effects of curcumin against nicotine injury on the hippocampus CA1 region of rats.

Methods: In this study, 48 male Wistar rats were randomly assigned to eight groups: Normal control (saline) group, Nicotine control group (0.5 mg/kg); Curcumin groups (10, 30, and 60 mg/kg) and Nicotine + Curcumin groups (10, 30, and 60 mg/kg). Treatments were administered intraperitoneally daily for 28 days. Golgi staining technique investigated the number of dendritic spines. Cresyl violet staining method was used to determine the number of neurons in hippocampal region CA1. Griess technique was assessed to determine serum nitrite oxide level. Also, the Ferric reducing/antioxidant power (FRAP) method was applied to determine the total antioxidant capacity.

Results: Nicotine administration significantly increased nitrite oxide level and decreased total antioxidant capacity as well as the number of neuronal dendritic spines and neurons compared to the normal control group ($P < 0.01$). In all Curcumin and Nicotine + Curcumin groups, the number of neurons, neuronal dendritic spines, and total antioxidant capacity increased significantly compared to the nicotine control group, while nitrite oxide level decreased significantly compared to the nicotine control group ($P < 0.01$).

Conclusion: Curcumin administration can improve hippocampal CA1 region injury induced by nicotine.

Introduction

Hippocampus is a part of the limbic system and seems to be essential in the formation of different types of learning and memory (1). The dentate gyrus is a part of hippocampus and contributes in formation of new episodic memories (2). Tobacco consumption has declined dramatically in industrialized countries over the past three decades due to raising awareness of the health risks and effective control policies, while it has increased in developing countries over the same period (3). Each cigarette has an average of 10-14 mg nicotine (4). Nicotine spreads rapidly in brain tissue through pulmonary circulation within 10-

20 seconds and attaches to the nicotinic acetylcholine receptors (nAChRs) (5). Nicotine in the cigarette is an alkaloid that rapidly passes through the blood-brain barrier and stimulates the mesolimbic dopamine system (6). Nicotine can regulate brain neurotransmitters, including catecholamine, serotonin, glutamate, and gamma-aminobutyric acid (GABA). This substance also reduces the activity of superoxide dismutase, glutathione peroxidase, and glutathione reductase in the hippocampus and induces oxidative stress in some organs including the brain (7,8). Pathologic changes associated with neuronal apoptosis have been reported due to the use of nicotine

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(9). Nicotine consumption can cause hypoxia, cerebral ischemia, hemorrhage, atrophy, ataxia, cerebral infarction, and subarachnoid hemorrhage (10). Also, nicotine can induce increased oxidative stress and neuronal apoptosis, destroy deoxyribonucleic acid (DNA), produce reactive oxygen species (ROS), and increase the production of lipid peroxidation (11). It also activates the areas of the brain that play an important role in drug addiction and learning process (12). Among the brain areas where nicotine has the greatest effect is the mesocorticolimbic region that contains nucleus accumbens, ventral tegmental area, the hippocampus and the amygdala. In this region, the amygdala and hippocampus have structures that play an important role in the formation of long-term memory, and their functions are associated with a stimulation reward system (13).

In recent years, an increase is seen in the use of herbal medicines for reducing the side effects of drug use (14). For centuries, India and China have been using turmeric as an anti-inflammatory agent in treatment of colic pain, toothache, chest pain, jaundice, anorexia, and menstrual problems (15). The rhizome extract contains mainly CUR and, to a lesser extent, desmethoxy CUR, and bisdemethoxy CUR (16). CUR has anti-oxidant and anti-inflammatory properties (17). CUR could improve memory impairment induced by intravenous injection of streptozotocin (18), and increase neurogenesis and cognitive processes in elderly female rats (19). It is much more potent in breaking down free radicals than vitamin E. In addition, it can protect the brain against lipid peroxidation and break down nitrite oxide induced radicals (20). CUR seems to prevent the effects of nicotine in the brain through reduction of oxidative stress mechanism. Considering the effects of nicotine toxicity on the brain and the therapeutic properties of CUR, and considering that no study has evaluated the antioxidant effect of CUR on DG damage induced by nicotine, the present study investigates the effects of CUR on nicotine-induced toxicity in the hippocampus DG region of male rats.

Materials and Methods
Animals
Forty-eight male Wistar rats (weighing 220-250 g) were purchased from the Pasteur Institute and transferred to the animal house in medical school. During the study, the animals were kept under standard conditions for 12-h light/12-h dark and 22 ± 2℃, in special cages and on a straw bed. Water and food were available to the animals ad libitum. Standard food and tap water was used to feed the animals.

Study groups and treatment of animals
CUR powder (Sigma, USA) with a chemical formula of 4-(OH)-3-(CH₃O) was dissolved in 0.9% normal saline to obtain relevant doses. The mixture of the solute and

solvent was heated, up to 70-80°C. The heterogeneous mixture was filtered to get a homogenous solution of CUR in normal saline. Vial of nicotine (Sigma, USA) with dose of 0.5 mg/kg of body weight was dissolved in 0.9% normal saline solution. Next, 48 male rats were randomly divided into 8 groups 6 rats each. The first group (i.e., the sham group) received normal saline through intraperitoneal injection equivalent to the amount of experimental groups. In the second group (i.e., the Nicotine group), each animal received 0.5 mg/kg single dose course of nicotine in normal saline, intraperitoneally. Third to fifth groups were CUR groups and each animal received 10, 30, and 60 mg/kg of CUR intraperitoneally for 28 days at 10 AM. Sixth to eighth groups were Nicotine + CUR groups and each animal received a single dose of 0.5 mg/kg nicotine in order to induce damage. Then, they respectively received 10, 30, and 60 mg/kg of CUR intraperitoneally for 28 days at 10 AM (11,16).

Transcardiac perfusion
The transcardiac method was used for fixation. Next, 24 hours after the last injection of the drug, animals were intraperitoneally anesthetized with ketamine 70 mg/kg and diazepam 10 mg/kg. The chest was opened in the midline. Following the completion of thoracotomy, the apex of the left ventricle was pierced and a glass cannula of 1 mm diameter was inserted into it and then fixed on ascending aorta. The pericardium and the right ventricle were cut. The left ventricle pathway was cut and the ascending aorta was connected to a plastic tube by the glass cannula and descending aorta was clamped right above the diaphragm. The cannula linked to the normal saline solution was implanted into the aorta through making an incision in the left ventricle. The descending aorta was fastened and, after washing the brain, the solution was removed through the incision made in the right atrium. Formalin 5% and buffer phosphate 7% were inoculated into the brain by the cannula and the brain was fixed in 15 minutes. After perfusion, the brains were separated from the skull and stored in the same perfusion solution for three days (1).

The tissue preparing and Golgi methods
The Golgi method was used to observe neuron dendrites in DG region. The Golgi method was applied using potassium dichromate followed by silver nitrate. After brain fixation, tissue blocks were put inside 3% potassium dichromate solution for 48 hours in a dark environment. The blocks were washed in 0.75% silver nitrate solution and were put inside the solution for 72 hours. The tissues were washed in 1% silver nitrate solution. Then, paraffin embedded blocks were gotten using Automatic Tissue Processor. The steps of this process was consequently included fixation with 10% normal saline (for 72 hours), washing thoroughly under running water, dehydrating by doses of ethanol (50%, 60%, 70%, 80%, 90% and 100%, which included 3 minutes for each step and 100% ethanol

step was repeated for three times), clearing by xylene (three times and 10 minutes in each), and embedding in soft paraffin (three times and 15 minutes in each). At this stage, 5-μm coronal histological thin sections were cut from paraffin-embedded blocks, undertaken by a microtome instrument (Leica RM 2125, Leica Microsystems Nussloch GmbH; Germany), and 5 sections per animal were chosen. For unification of the section selection, the first section was the 4th and the last was the 24th (5 sections interval) and finally, the routine protocol for Golgi methods was implemented. At the end of tissue processing, the stained sections were assessed under microscope Olympus BX-51T-32E01 research microscope connected to a DP12 Camera with 3.34-million pixel resolution and Olysia Bio software (Olympus Optical Co. LTD, Tokyo, Japan) (21).

Cresyl violet method
The Cresyl violet staining method was used to determine the number of live cells in DG region. Six rat heads from each group and five slides from each mouse were selected for staining. After creating 5 μm cuts by microtome and performing tissue processing, the left hemispheres were stained using Cresyl violet staining technique. A photo was prepared and the number of cells was counted in 1 mm³. In the slides stained by means of Cresyl violet technique, the round cells without peak nose were considered as live cells (1).

Morphometrically technique
In the slides marked via Golgi staining method, the completely stained neurons with cell bodies in the central part of the tissue sections distant from the surrounding stained neurons were included in the study. The dendritic tree of pyramidal neurons was revealed through camera lucida with magnification 750 and the dendritic exclusion order from the cell body was used for counting the dendritic sections. In addition, the Sholl procedure was applied to assess the concentration of dendritic divisions. In the slips marked via Cresyl violet method, the round cells without peak nose were considered as live cells. The slides were imaged by Motic microscope and the cells were counted by Image J software 1.45 (1).

Griess technique
Griess technique uses zinc sulfate powder to eliminate the serum protein of the samples. In this study, zinc sulfate powder (6 mg) was mixed with serum samples (400 μL) and vortexed for 1 minute. The samples were centrifuged at 4°C for 10 minutes at 12000 rpm and the supernatant was used to measure the nitrite oxide. Briefly, 50 μl of sample was added to 100 μl of Griess reagent (Sigma, USA) and the reaction mixture was incubated for about 30 minutes at room temperature. Sodium nitrite (0.1 M) was used for the standard curve, and increasing concentrations of sodium nitrite (5, 10, 25, 50, 75, and 100 μM) were prepared. The Greiss solution was added to all microplate containing sodium nitrite and supernatant, and was read through an ELISA reader (stat fax100; the USA) at the wavelength of 540 nm (22).

Ferric reducing/antioxidant power (FRAP) method
FRAP method was used to measure the total antioxidant capacity of the serum. In this technique, the ability of the plasma for retaining ferric ions was measured. This process required a great quantity of Fe^{III}. A blue stain was formed when the compound of Fe^{III}-TPTZ in acidic pH returned to Fe^{II} and absorption at the maximum wavelength of 600 nm. The factor defining the speed of the Fe^{II}-TPTZ and the blue color were the only vitalizing power of the sample. Total antioxidant capacity values are strategized by means of the standard curve with diverse concentrations of iron sulfate (23).

Statistical analysis
After extracting the information, for confirming data compliance of the normal distribution, Kolmogorov-Smirnov test was conducted. For statistical analysis one-way analysis of variance (ANOVA) and Tukey post hoc test were used to determine the difference between the groups. SPSS 16 was used for data analysis; the results were expressed as mean ± standard error and $P < 0.05$ was considered statistically significant.

Results
Neurons number
The results showed a significant decrease in the number of neurons in DG region in nicotine group compared to the sham group (8.33%) ($P < 0.01$). The mean number of neurons was not significant in all CUR groups compared to the sham group ($P > 0.05$). The mean number of neurons increased significantly in CUR (dose 10 mg/kg = 18.83%, dose 30 mg/kg = 19%, and dose 60 mg/kg = 19.50%), and nicotine + CUR (dose 10 mg/kg = 14%, dose 30 mg/kg = 14.66%, and dose 60 mg/kg = 14%) in all doses compared to the nicotine group ($P < 0.01$). Also, the mean number of neurons decreased significantly in all Nicotine + CUR groups (dose 10 mg/kg = 14.12%, dose 30 mg/kg = 15.16%, and dose 60 mg/kg = 14.61%) compared to the normal control group ($P < 0.01$) (Figures 1 and 2).

Dendritic spines
The mean number of neuronal dendritic spines showed a significant decreased between the sham group and nicotine group (7.13%) ($P < 0.01$). The mean number of neuronal dendritic spines was not significant in all CUR groups compared to the sham group ($P > 0.05$). At the CUR (dose 10 mg/kg = 14.85%, dose 30 mg/kg = 14.96%, and dose 60 mg/kg = 14.93%) and nicotine + CUR groups (dose 10 mg/kg = 11.50%, dose 30 mg/kg = 11.66%, and dose 60 mg/kg = 11.83%), the mean number of neuronal dendritic spines increased significantly in all treated groups compared to the nicotine group ($P < 0.01$).
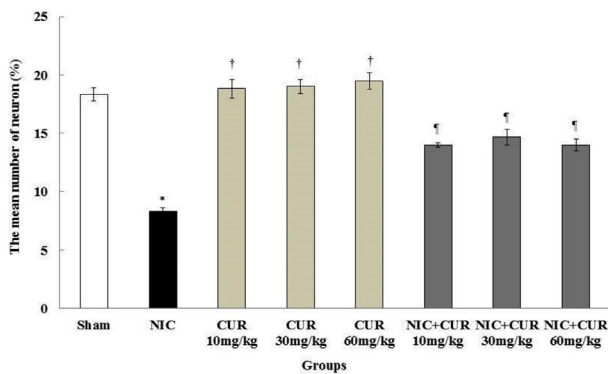
Figure 1. Effect of nicotine, CUR, and nicotine + CUR administration on the mean number of neurons in the DG region; *Significant difference compared to the sham group ($P < 0.01$); †Significant difference compared to the nicotine group ($P < 0.01$); ¶Significant difference compared to the nicotine and sham groups ($P < 0.01$). CUR: Curcumin, Nic: Nicotine, DG: Dentate gyrus.
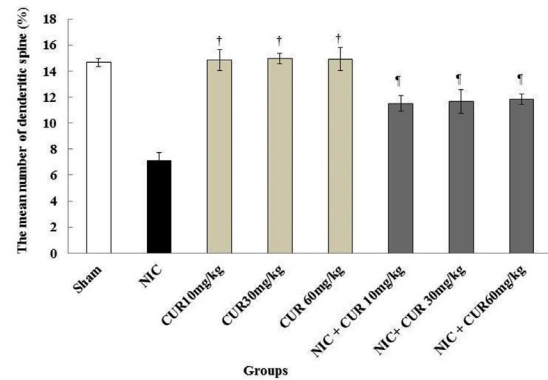

Figure 3. Comparison of nicotine, saline and CUR groups at the mean number of dendritic spines in hippocampal region DG; *Significant difference compared to the sham group ($P < 0.01$). †Significant difference compared to the nicotine group ($P < 0.01$). ¶Significant difference compared to the nicotine and sham groups ($P < 0.01$). CUR: Curcumin, Nic: Nicotine, DG: Dentate gyrus.
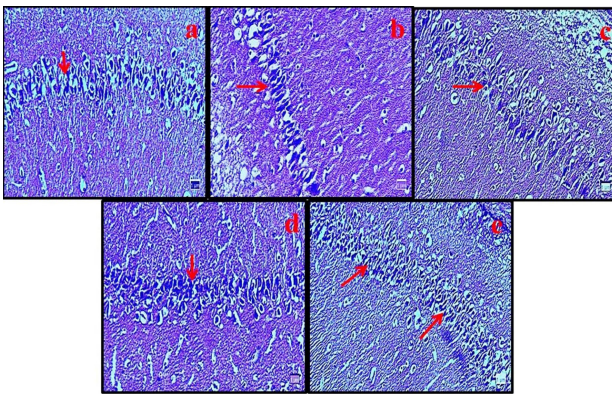

Figure 2. Microscopic images of dentate gyrus (DG) region in male rats in different groups (5 μm thick sections, Cresyl violet staining, magnification: ×100); Micrograph of the DG section in the sham groups (a), the normal number of neurons in the DG region. Micrograph of the DG section in nicotine group (b), decreased neurons cells due to the oxidative stress caused by nicotine. Micrograph of DG section in CUR (60 mg/kg) group (c), the normal number of neurons. Micrograph of DG section in nicotine + CUR (30 mg/kg) (d) and nicotine + CUR (60 mg/kg) (e) groups (d), normal number of neurons. Red arrows identify neuron cells. CUR: Curcumin, DG: Dentate gyrus.
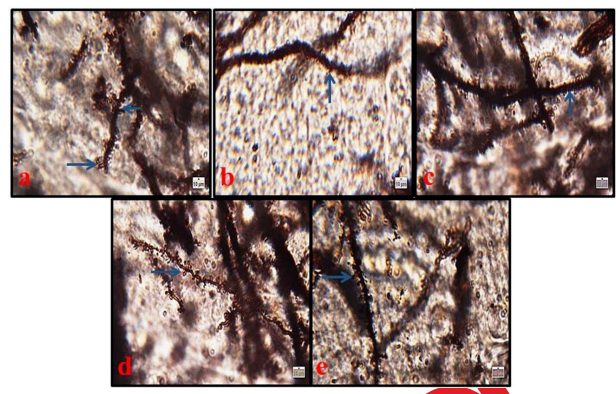

Figure 4. Microscopic images of neuronal dendritic spines in hippocampal dentate gyrus (DG) region in male rats in different groups (Five-micron thick sections, Golgi staining, magnification: ×100). Micrographs of the DG section in the sham group (**a**), normal structure. Micrograph of the DG section in nicotine group (**b**), decreased number of dendritic spines due to the oxidative stress caused by nicotine. Micrograph of the DG section in CUR (60 mg/kg) group (**c**), normal structure; Micrograph of DG section in nicotine + CUR (30 mg/kg) group (**d**) and (60 mg/kg) (**d**) groups, normal structure. Blue arrows identifies neuronal dendritic spines. CUR: Curcumin, DG: Dentate gyrus.

Further, the mean number of neuronal dendritic spines decreased significantly in all nicotine + CUR groups (dose 10 mg/kg = 11.50%, dose 30 mg/kg = 11.66%, and dose 60 mg/kg = 11.83%) compared to the sham group ($P < 0.01$) (Figures 3 and 4).

Nitrite oxide
The results of blood serum nitrite oxide measurement showed a significant increase in nicotine group compared to sham group (404.285 μm) ($P < 0.01$). The mean nitrite oxide in the blood serum was not significant in all CUR groups compared to the sham group ($P > 0.05$). Also, the mean level of nitrite oxide in blood serum declined

significantly in CUR (dose 10 mg/kg = 190.61 μm, dose 30 mg/kg = 193.83 μm, and dose 60 mg/kg = 190.69 μm) and nicotine + CUR groups (dose 10 mg/kg = 309.69μm μm, dose 30 mg/kg = 295.88μm μm, dose 60 mg/kg = 299.58μm μm) in all doses compared to the nicotine group ($P < 0.01$) (Figure 5).

Total antioxidant capacity
The total antioxidant capacity serum level reduced significantly in the nicotine group compared to the sham group ($P < 0.01$) and enhanced in all CUR (dose 10 mg/kg = 2.09 mmol/L , dose 30 mg/kg = 2.03 mmol/i, dose 60 mg/kg = 2.04 mmol/i) and nicotine + CUR groups (dose
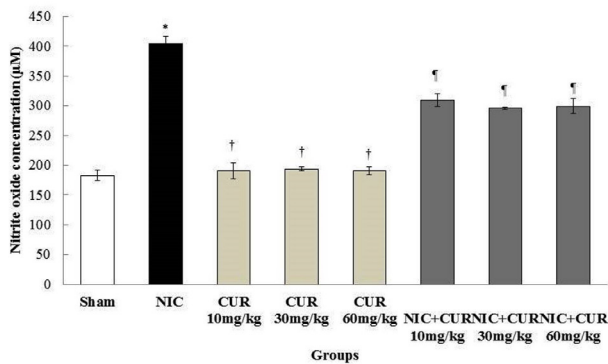
Figure 5. Effects of CUR, nicotine, and nicotine + CUR on the mean of the nitrite oxide levels; *Significant difference compared to the sham group ($P < 0.01$). †Significant difference compared to the nicotine group ($P < 0.01$). ¶Significant difference compared to the nicotine group ($P < 0.01$). CUR: Curcumin, Nic: Nicotine, DG: Dentate gyrus.

Figure 6. Comparison of total antioxidant capacity in nicotine, and sham and CUR and nicotine + CUR groups; *Significant difference compared to the sham group ($P < 0.01$). †Significant difference compared to the nicotine group ($P < 0.01$). ‡Significant difference compared to the nicotine group ($P < 0.01$). CUR: Curcumin, Nic: Nicotine, DG: Dentate gyrus.
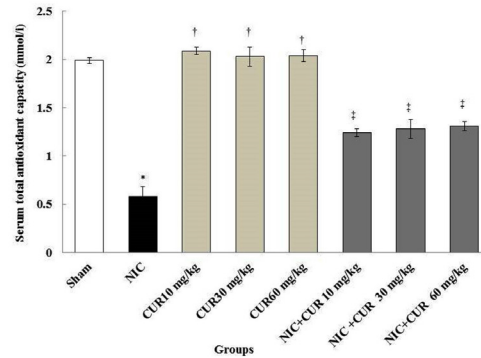
10 mg/kg = 1.24 mmol/i, dose 30 mg/kg = 1.28 mmol/i, dose 60 mg/kg = 1.31 mmol/i) compared to the nicotine group ($P < 0.01$) (Figure 6).

Discussion

The present study was aimed to investigate the effects of CUR on nicotine-induced disorders in the hippocampus DG region. The number of neurons and dendritic thorns decreased significantly in the nicotine group comparted to the sham group. In nicotine + CUR groups, there was a significant increase in the number of dendritic thorns compared to the nicotine group. The results may indicate the control of apoptosis and neurodegeneration by administering different doses of CUR. The results of Tewari et al. were consistent with those of the present study that showed nicotine could damage the cells in the hippocampus by increased protein accumulation in the membrane and reduced cell size (24). Similarly, based on the results of the current study, nicotine could decrease significantly the number of neurons in the hippocampus due to damaging the cells. It seems that nicotine induces oxidative stress and, consequently, the production of free radicals such as superoxide and hydroxyl radicals can cause cell damage (11). Generated free radicals following oxidative stress induction may have the potential to damage cellular compositions, including proteins, lipids, and DNA (22). Equally, in the current study, nicotine could decrease the number of neurons and dendritic thorns in the hippocampus due to oxidative stress caused by administration of nicotine. Exposure to nicotine can also increase the production of ROS and peroxidation of lipids and decrease the level of GSH antioxidant enzymes (16). The brain is one of the most important organs in the body which consumes a large amount of oxygen. Lipid in the membrane of the nerve cells has a high content of oxidized unsaturated fatty acids (25). Therefore, it seems that nicotine can produce ROS via P-450 enzyme and, by producing oxidative stress, cause the destruction of the

nucleus in neurons (7). Given that dendritic thorns play a major role in synaptic transmission, it is not surprising that many brain diseases are associated with changes in the morphology and density of dendritic thorns (21). Dendrite thorns are likely to be involved in memory as exon and dendrites interface (15). Nicotine can reduce the length of dendrites and the number of dendritic thorns in nucleus accumbens by affecting the neurotrophic factors in the striatum (26). The results of the study by Brown et al showed that nicotine injections with a dose of 0.7 mg/kg could reduce the length of dendrites and the number of dendritic thorns; which is consistent with the results of our study (27). Nicotine due to oxidative stress, could decrease the number of neurons and dendritic thorns in the hippocampus horns by β2-nAChRs deactivation in postsynaptic cells in the hippocampus region (28). Moreover, nicotine can reduce the number of thorns by deactivating α4β2-nAChRs in the pre-synaptic membrane and by disrupting the release of glutamate neurotransmitters (29). It seems that nicotine reduces the number of dendritic thorns in two ways. One way is the regulation of Glutamatergic synapses on pyramidal neurons. This mechanism is mainly based on the activity of α4β2 nAChRs on presynaptic glutamate terminus. The next way focuses works based on the activity of GABA in the internal neurons or interneurons (30). CUR is a purifier of ROS and has the potential to destroy oxidative stress and prevent lipid peroxidation (30). The results of the study by Shin et al confirmed those of the present study that CUR could prevent cell death from kainic acid due to oxidative stress in the hippocampus (31). CUR seems to inhibit lipid peroxidation of quinolinic acid and control the production of cyanide-induced superoxide in the brain, suggesting the protective properties of CUR. In addition, nicotine treatment increased lipid peroxidation and the levels of interleukin 1 beta (IL-1β), tumor necrosis factor-α (TNF-α), and BCL2-associated X protein (Bax), while reducing B-cell lymphoma 2 (Bcl-2),

cAMP response element-binding protein (CREB), and Brain-derived Neurotrophic factor (BDNF) levels in the hippocampus (32). CUR has been shown to significantly improve spatial memory impairment induced by human immunodeficiency viruses-1 (HIV-1) in rats, but the electrophysiological mechanism remains unknown (33). The results of a study by Pan et al, in line with the results of the present study, showed that CUR improved learning and memory in mice and could increase the neuroprotective effects in Alzheimer's modal rats (34). CUR can reduce neuropathological changes in the hippocampus and control apoptosis by increasing the density of Bcl-2 protein (35). The present study showed a significant increase in serum nitrite oxide level in the nicotine group compared to the sham group. In all nicotine + CUR groups, there was a significant decrease in serum nitrite oxide level in comparison to the nicotine group. Nitrite oxide is a free radical and can regulate angiogenesis, apoptosis, cell cycle, invasion, and metastasis in cells. Nitrite oxide seems to play a key role in the destruction of myelin in the central nervous system (1). Nicotine can stimulate nicotinic receptors in the brain and increase glutamate release and N-Methyl-D-aspartic acid (NMDA) activation. The activation of NMDA may increase the formation of nitric oxide in the hippocampus (36). The results of a study by Keser et al revealed that exposure to nicotine may increase the activity of nitrite oxide in the frontal cortex in the mouse brain. This result confirms the results of the present study (37). Also, the results of Salahshoor et al. are consistent with those of the results of the present study that showed CUR prescription significantly decreased the serum level of NO in the blood serum of mice (11). The results of this study showed that there was a significant decrease in total antioxidant levels in the nicotine group compared to the sham group. In all nicotine + CUR groups, there was a significant decrease in serum total antioxidant levels in comparison to the nicotine group. Total antioxidant level is significantly lower in smokers in proportion to non-smokers (38,39). The reduction in total antioxidant capacity level in this study shows the effects of oxidative stress from nicotine on the hippocampal neuron. This result is expressed as growth in the levels of ROS and lipid peroxidation and a reduction in the action of antioxidant enzymes such as total antioxidant capacity (40). CUR via activation of cAMP response element-binding protein - Brain-derived neurotrophic factor (CREB/BDNF) signaling pathway confers neuroprotection against nicotine-induced inflammation, apoptosis, and oxidative stress. In contrast, various doses of CUR attenuated nicotine-induced apoptosis, oxidative stress, and inflammation, while elevating CREB and BDNF levels. Thus, CUR via activation of CREB/BDNF signaling pathway confers neuroprotection against nicotine-induced inflammation, apoptosis, and oxidative stress (41,42). In the present study, improved levels of total antioxidant capacity in rats treated with CUR highlight the antioxidant and anti-lipid peroxidation effects of CUR.

Conclusion

It appears that CUR provides protection against oxidative stress resulting from nicotine. Such an ability of the CUR might be due to its strong potential antioxidant attributes. As a result, it leads to DG tissue recovery and prevention of nicotine adverse effects on total antioxidant capacity, nitrite oxide, number of neurons, and dendritic spines as evidenced in the abovementioned examination of the male rats. However, supplementary studies are essential to describe its molecular mechanism.

Authors' contributions
CJ conceived and designed the experiments, CJ and SR revised the manuscript, SR and BA analyzed the data and MRS wrote the paper. All authors read and confirmed the publication of the article.

Conflict of interests
The authors declare that there was no conflict of interest.

Ethical considerations
All investigations conformed to the ethical of research and were approved by the Ethics Committee of Kermanshah University of Medical Sciences (ethics certificate No.1396.562)

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