ORIGINAL ARTICLE

Veterinary Research Forum. 2024; 15 (9): 473 - 480
doi: 10.30466/vrf.2024.2018359.4107
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Curcumin alleviates inflammatory effects of ketamine anesthesia in postnatal rats

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Article Info

Article history:

Received: 20 December 2023
Accepted: 05 February 2024
Available online: 15 September 2024

Keywords:

Brain
Curcumin
Ketamine
Liver
Real-time polymerase chain reaction

Abstract

Curcumin has been employed in traditional medicine for over a millennium to treat various ailments, and its global use is now widespread. Chinese medicine relies heavily on curcumin as a primary element and uses it to cure infectious diseases, skin disorders, depression, and stress. It has cardioprotective, neuroprotective, and anti-diabetic properties, as well as pharmacological effects on disorders like type II diabetes, atherosclerosis, and human immunodeficiency virus replication. The anti-cancer activity of curcumin has been studied extensively with notable improvements in gastrointestinal, melanoma, urogenital, breast, and lung malignancies. We investigated the anti-inflammatory effects of curcumin on expression of *tumor necrosis factor* (*TNF*)-*α*, *c-Fos*, and *interleukin* (*IL*)-*6* genes in brain and liver tissue owing to the effects of ketamine anesthesia on postnatal rats. The thalamic and hepatic tissues were collected without anesthesia, immediately after anesthesia, and 4 and 12 hr after anesthesia in control and curcumin treated postnatal rats. The results showed that glucose, triglyceride, high- and low-density lipoprotein levels were lowered with curcumin treatment. We also found that ketamine increased *c-Fos* and inflammatory cytokines like *TNF-α* and *IL-6*, all of which contribute to inflammation. Brain and liver immunohistochemistry studies confirmed the real-time polymerase chain reaction findings. Curcumin injections alone may be effective in decreasing ketamine-induced inflammation in both brain and liver tissues.

Introduction

Today, the use of medicinal plants as an alternative medicine is considered due to biological safety.[1] *Curcuma longa*, also known as turmeric, is a flowering plant belonging to the ginger family.[2] In traditional medicine, turmeric as an herbal medicine has been used for strengthening the body's overall energy, improving digestion, regulating menstruation, dissolving gallstones, and decreasing arthritis.[3] Curcumin or diferuloylmethane is the principal bioactive component of turmeric[4,5] and due to its outstanding anti-bacterial, anti-inflammatory, and anti-tumor activities, it is used in the treatment of colitis, pancreatitis, osteoarthritis, rheumatoid arthritis, diabetes, and inflammatory bowel and cardiovascular diseases.[5-10] Lipoxygenase, cyclooxygenase-2, collagenase, phospholipase, hyaluronidase, and protein kinase-c are all involved in the inflammatory process, as are chemicals such as leukotrienes, thromboxane, prostaglandins, nitric oxide, interleukins (*IL-12*, *IL-8*, *IL-6*, *IL-2*, and *IL-1*), and tumor necrosis factor.[6,11] Curcumin may also enhance nervous system performance by altering the L and D isoenzymes of phenylalanine, decreasing pain transmission and dampening those pathways. Curcumin also contains cardioprotective, neuroprotective, and anti-diabetic effects. Type II diabetes, Alzheimer's disease, and atherosclerosis are pharmacologically affected by curcumin too.[12-14] The anti-cancer action of curcumin, which has just lately been widely examined, has been observed in studies on the digestive, gastrointestinal, genital, breast, and lung cancers, as well as melanoma.[15-17]

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Ketamine is a selective antagonist and non-competitive receptor of N-methyl-D-aspartate (NMDA) and derivatives of phencyclidine (N-1-phenycyclohexypiperidine), exerting its anesthetic effect by blocking the impact of excitatory neurotransmitter glutamate on these receptors.[18,19] It has been used almost for 53 years as a general anesthetic for humans and animals, and also used in several clinical conditions, including shock, cardiac tamponade, or status asthmaticus, as well as burn patients.[20,21] Previous studies have shown that low doses of ketamine and other NMDA receptor antagonists cause anxiolytic and anti-depressant effects in humans. In addition, the anesthetic dose of ketamine produced an acute and maintained anti-depressant effect in mice and *Wistar* rats.[22] Other studies have shown that ketamine prevents the exacerbation and extension of local inflammation without blunting the local process. Delaying inflammatory resolution also prevents the general anti-pro-inflammatory mechanisms from excessively over-coming the pro-inflammatory influences.[23] However, long-term use of ketamine hurts the brain. A study showed that long-term exposure to ketamine increased cell death in areas of monkeys' brains.[24] Other experimental studies have demonstrated that administering 30.00 mg kg^{-1} ketamine for 6 months in mice hyper-phosphorylates and aggregates tau protein, leading to apoptosis in the prefrontal and entorhinal cortex.[25] Various studies have also indicated the effect of different doses of ketamine. However, it is still unclear how the change in inflammatory cytokine factors level is involved in inflammation. Recent studies have reported that chronic ketamine administration at the dose of 60.00 mg kg^{-1} induces spatial recognition memory deficit and decreases anxiety-like behaviors in mice. They showed that ketamine could increase the levels of inflammatory cytokine *IL-6*, which would lead to neuro-inflammation.[26,27] Despite the common use of ketamine, few studies have been conducted on the side effects of this drug in long- and short-term exposures. Based on the given background, this study was carried out to assess the anti-inflammatory effect of curcumin in the expression of *TNF-α*, *c-Fos*, and *IL-6*, and anesthetic effect of ketamine in the postnatal rats through immunohistochemical tracing in the brain and liver.

Materials and Methods

Chemicals. Curcumin was graciously provided by the Exir Nano Sina Company (Tehran, Iran). Ketamine hydro-chloride was obtained from Pfizer Co. (New York, USA).

Animals. Postnatal rats (28 - 30 days old) weighing 60.00 - 80.00 g were purchased from the Pasteur Institute of Iran. Postnatal rats were maintained under standard conditions (temperature: 24.00 ± 1.00 ˚C; humidity: 60.00 ± 5.00%) with a 12 hr light/dark cycle. All animals were given a pellet diet and *ad libitum* water. The animals were acclimatized to the environment for 1 week before the start of experiment. The experimental design was performed according to the Animal Ethics Committee of Lorestan University, Lorestan, Iran (Number: LU. ECRA.2019.12).

Experimental design. Postnatal rats were randomly divided into eight groups of five. The 1st four groups (untreated groups; F-groups) including Fn1, Fn2, Fn3, and F-control, were given only normal saline (0.90% NaCl, Praspeyvand, Tehran, Iran) orally twice a day for 2 weeks. After the 2nd week, ketamine was injected intra-peritoneally (IP) at a dose of 75.00 mg kg^{-1}. In Fn1, post-natal rats were sacrificed immediately after anesthesia, in Fn2, postnatal rats were sacrificed 4 hr after anesthesia, in Fn3, postnatal rats were sacrificed 12 hr after anesthesia, and in the control of F-groups, postnatal rats were sacrificed without ketamine injection.[28,29] All animals were euthanized by cervical dislocation. The 2nd groups (S-groups) including Sn1, Sn2, Sn3, and S-control, were given 20.00 mg kg^{-1} curcumin diluted with normal saline for 2 weeks. Postnatal rats were anesthetized (IP) with ketamine at a dose of 75.00 mg kg^{-1} after the 2nd week. The doses of curcumin and ketamine, and the treatment duration were selected based on previous studies.[3,9,21,22] This study was carried out in compliance with Animals in Research: Reporting *In Vivo* Experiments guidelines.[30]

Biochemistry. Cardiac blood samples were taken following animals' euthanasia. Subsequently, factors such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), cholesterol, triglycerides, and blood glucose levels were estimated using commercial kits (Pars Azmoon, Tehran, Iran) and an enzymatic-colorimetric method using an auto-analyzer (Alpha Classic AT Plus, Esfahan, Iran).

Quantitative real-time polymerase chain reaction (RT-PCR) RNA analysis. The liver and thalamus of postnatal rats were removed and fixed in 10.00% buffered formalin solution for RT-PCR and immunohistochemical examinations. Total RNA was extracted from tissues using RNX-Plus reagent (CinnaGen Co., Tehran, Iran). The cDNA was synthesized using oligo dT and random hexamer primers and MLV reverse transcriptase (PrimeScript™ RT Reagent Kit; TaKaRa Bio, Kusatsu, Japan). The RT-PCR was performed with the SYBR Green I Master Mix Kit (TaKaRa Bio, Kusatsu, Japan) using the Rotor-Gene 6,000 instrument (Corbett Research, Sydney, Australia) according to the manufacturer's protocol briefly including 95.00 ˚C for 2 min, and 40 cycles including 95.00 ˚C for 15 sec and 60.00 ˚C for 15 sec. The melting cure was respectively 95.00 ˚C for 15 sec, 60.00 ˚C for 15 sec, and 95.00 ˚C for 15 sec, by designing forward and reverse primers using the NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer; Table 1). Moreover, the sizes of implicated fragments are provided at Table 1. The results were normalized by mRNA level of *HPRT-1* and calculated using the ΔΔCt standard method.[31,32]

Table 1. Designed primers for quantitative real-time polymerase chain reaction analysis.

Genes	Species/Accession No.	Sequence 5'-3'	Primer (bp)	Product length (bp)
IL-6	*Rattus norvegicus*/ NM_012589.2	**F:** GCCCTTCAGGAACAGCTATGA	**21**	80
		R: TGTCAACAACATCAGTCCCAAGA	**23**	
TNF-α	*Rattus norvegicus*/ NM_012675.3	**F:** TGGGCTCCCTCTCATCAGTTC	**21**	108
		R: TCCGCTTGGTGGTTTGCTAC	**20**	
c-Fos	*Rattus norvegicus*/ NM_022197.2	**F:** ACGGAGAATCCGAAGGGAAAGGAA	**24**	125
		R: TCTGCAACGCAGACTTCTCGTCTT	**24**	
HPRT-1	*Rattus norvegicus*/ NM_012583.2	**F:** CTCATGGACTGATTATGGACAGGAC	**25**	123
		R: GCAGGTCAGCAAAGAACTTATAGCC	**25**	

Immunohistochemistry. An immunohistochemical procedure was performed on 3.00 µm-thick paraffin-embedded liver and thalamus tissue specimens using *TNF-α* antibody (Abcam, Queretaro, Mexico) at 1:100 dilution. After dewaxing and rehydrating, sections were immersed in target retrieval solution (Tris-ethylenediamine tetra-acetic acid; pH: 9.00) and heat-mediated antigen retrieval was carried out in a water bath for 20 min at 98.00 ˚C to deliver unmasked antigens. The sections were treated with H_2O_2 (3.00%) in phosphate-buffered saline for 15 min to inhibit endogenous peroxidase and incubated for 1 hr with primary antibodies. After that, the sections were incubated with biotinylated goat anti-rabbit immuno-globulin G (20 min) and then with streptavidin horseradish peroxidase (pre-diluted; Biocare, Concord, USA) for 20 min. The antibody binding sites were visualized with 3,3'-diaminobenzidine chromogen substrate. Eventually, sections were counterstained with Mayer's Hematoxylin (Bio Optica, Milano, Italy).[31,33]

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 8.0; GraphPad Software Inc., San Diego, USA). The results were expressed as means ± standard error. The difference between the expressions of target genes among groups was followed by ANOVA, Tukey, and *t*-test. The $p < 0.05$ values were considered statistically significant.

Results

To consolidate the notion of a neuroprotective effect of curcumin against ketamine-induced neuro-inflammation, the effects of curcumin on biochemical factors, and *c-Fos*, *TNF-α*, and *IL-6* genes expression levels were evaluated between F-groups (untreated groups) and S-groups (curcumin-treated groups) of ketamine-anesthetized postnatal rats in three steps including n1 (immediately after anesthesia), n2 (4 hr after anesthesia), and n3 (12 hr after anesthesia), and controls (without ketamine injection).

Biochemistry. After administration of ketamine, the level of cholesterol significantly increased in Fn2 and Fn3 compared to the F-control ($p < 0.05$; Fig. 1A). No significant changes were observed in the F-groups (Fn1, Fn2, Fn3, and F-control) in comparison with the S-groups (Sn1, Sn2, Sn3, and S-control; $p < 0.05$). The level of glucose significantly decreased in Sn2 and Sn3 compared to the Fn2 and Fn3, respectively ($p < 0.05$). In addition, the glucose level significantly increased in Fn2 compared to the Fn1 ($p < 0.05$; Fig. 1B). At the beginning of the anesthesia, ketamine caused an increase in blood glucose levels. After administration of curcumin, the level of glucose was reduced in Sn2 and Sn3 compared to the Fn2 and Fn3, respectively. It showed the protective effect of curcumin, preventing a high glucose level at the beginning of anesthesia ($p < 0.05$; Fig. 2B). As demonstrated in Figure 1C, the triglyceride level was significantly reduced in Fn2 and Fn3 compared to the F-control. It seems that ketamine increased triglyceride levels at the beginning of anesthesia. However, the effect decreased slightly, and the triglyceride rose again. After administration of curcumin, the triglyceride level was slightly reduced in Sn1, Sn2 and, Sn3 compared to the S-control. After t administration of ketamine, at the beginning of anesthesia, the level of HDL was increased in Sn1 and Sn2 compared to the Fn1 and Fn2 ($p < 0.05$). As a result, curcumin could increase the level of HDL in the blood, which was also seen at the beginning of anesthesia (Fig. 1D). After ketamine injection, the level of LDL in Fn1 and Fn2 was increased compared to the F-control and then, reduced gradually in Fn3. After administration of curcumin, in Sn1 and Sn2, the level of LDL was significantly reduced compared to the Fn1 and Fn2, respectively ($p < 0.05$; Fig. 1E).

Real-time analysis of *c-Fos*, *TNF-α* and *IL-6* genes in brain. According to the Figure 2A, after ketamine administration, the expression level of the *c-Fos* gene was significantly increased in Fn1, Fn2, and Fn3 compared to the F-control ($p < 0.001$). Although ketamine raised the expression level of *c-Fos* gene in Sn1, Sn2, and Sn3, curcumin administration significantly reduced the expression level of *c-Fos* gene in Sn1 ($p < 0.05$), Sn2 and Sn3 ($p < 0.01$) compared to the Fn1, Fn2, and Fn3. The result of *TNF-α* gene showed that curcumin injection could modulate the level of *TNF-α* gene in Sn1, Sn2, and Sn3 compared to the S-control. Moreover, in Fn1, Fn2, and Fn3, ketamine increased the level of *TNF-α* gene compared to the F-control ($p < 0.01$; Fig. 2B). After ketamine administration, the *IL-6* expression level was increased in Fn1, Fn2, and Fn3 compared to the F-control. However, curcumin remarkably reduced the *IL-6* expression level in Sn3 and S-control compared to the F-groups (Fig. 2C).

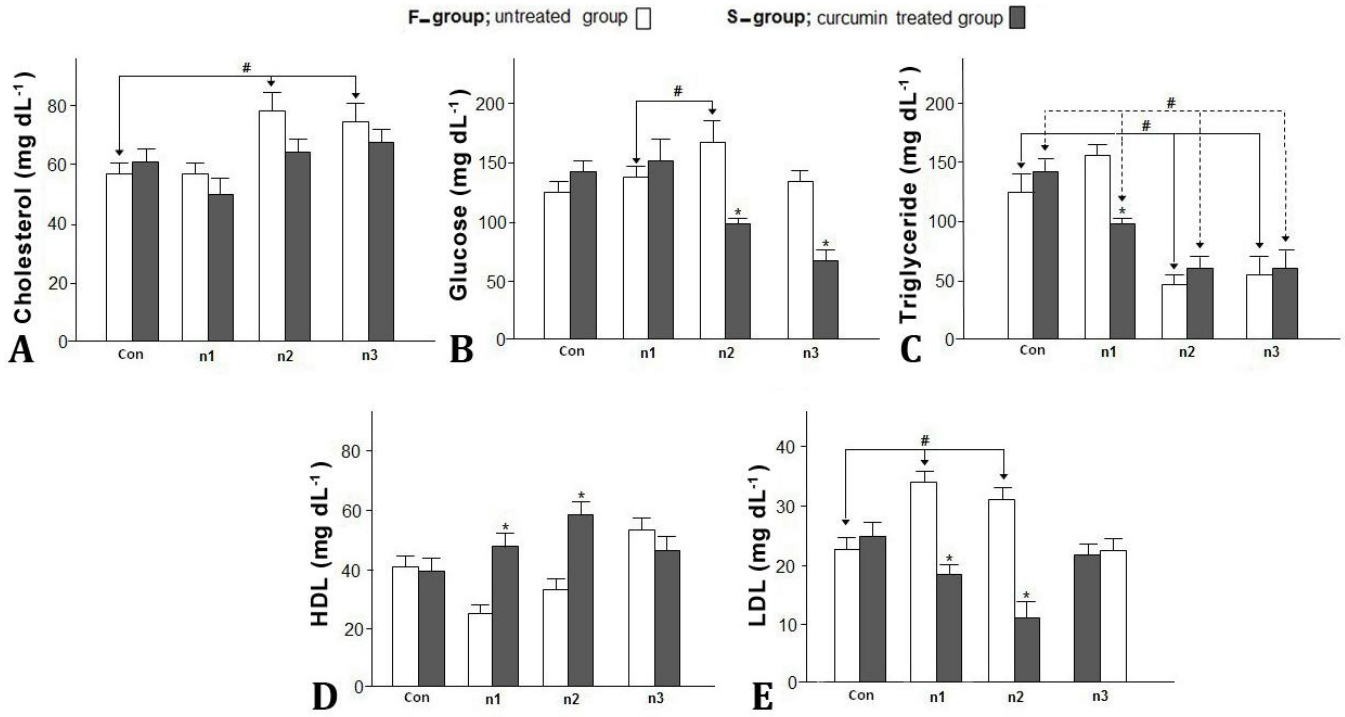

Fig. 1. Levels of **A)** cholesterol, **B)** glucose, **C)** triglyceride, **D)**, high-density lipoprotein (HDL), and **E)** low-density lipoprotein (LDL) in different groups. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a comparison between untreated groups (F-groups) and curcumin-treated groups (S-groups). # $p < 0.05$ and ##$p < 0.01$ indicate comparison with control.

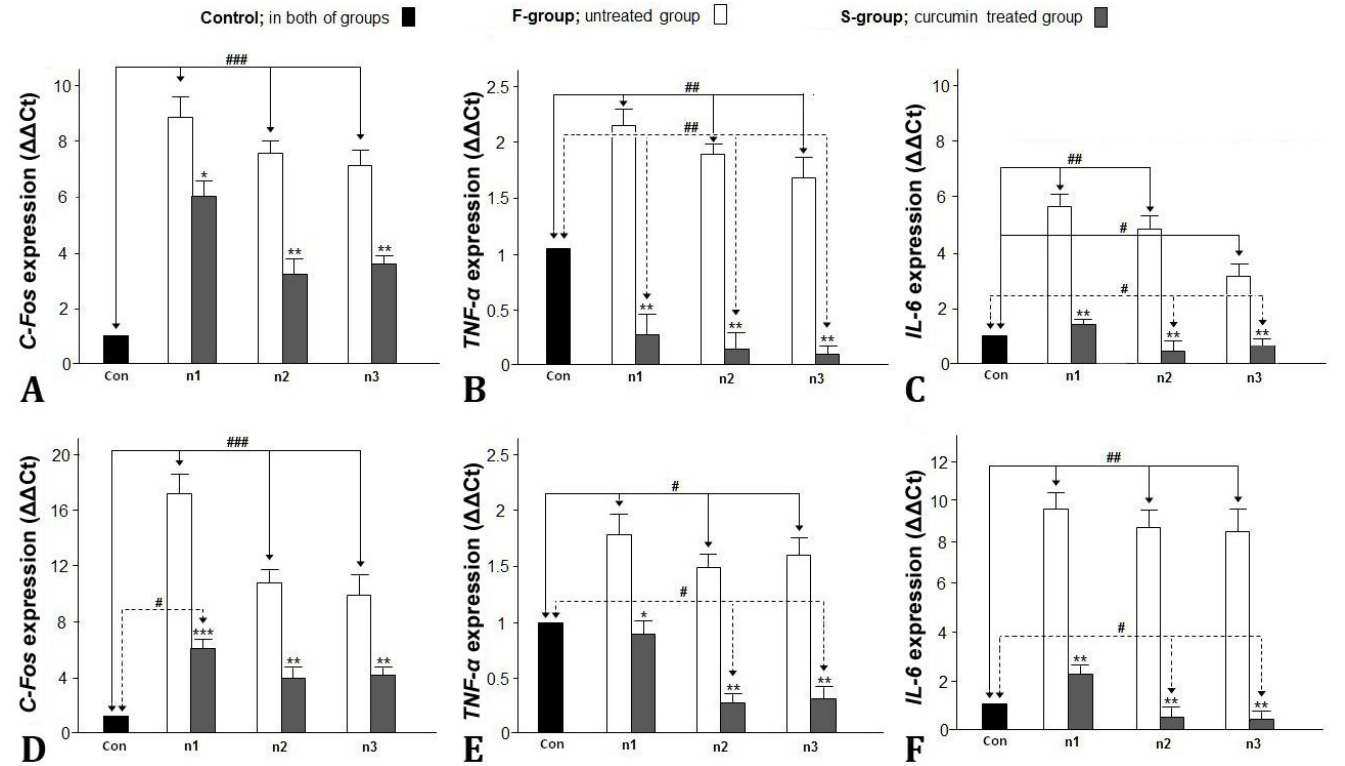

Fig. 2. The qPCR indicates mRNA expression levels relative to *GAPDH* of **A)** *c-Fos*, **B)** *TNF-α*, and **C)** *Interleukin-6* **(***IL-6)* in postnatal rats' brains and expression levels of **D)** *c-Fos*, **E)** *TNF-α*, and **F)** *IL-6* in postnatal rats' liver. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a comparison between untreated groups (F-groups) and curcumin-treated groups (S-groups). # $p < 0.05$, ##$p < 0.01$, and ##$p < 0.01$ indicate comparison with control. Con: Without ketamine injection in both groups; n1: Immediately after anesthesia; n2: 4 hr after anesthesia; n3: 12 hr after anesthesia.

Real-time analysis *of c-Fos*, *TNF-α* and *IL-6* genes in liver. Figures 2D, 2E, and 2F shows that curcumin treatment prevented the increase in *c-Fos*, *TNF-α* and *IL-6* genes expression levels after ketamine injection, showing the negative effect of ketamine on expression levels of *c-Fos*, *TNF-α* and *IL-6* gens in the liver. Also, as shown in these Figures, curcumin has a positive impact and considerably decreases the level of *c-Fos*, *TNF-α* and *IL-6* genes in Sn1, Sn2, and Sn3 compared to Fn1, Fn2, and Fn3.

Immunohistochemistry. Immunohistochemical tests were carried out for *TNF-α* protein in brain (thalamus; Fig. 3A) and liver (Fig. 3B) tissues in both of curcumin treated and the untreated groups in four groups including n1, n2, n3, and controls. The results did not show any significant alterations, which confirmed the findings of RT-PCR findings regarding *TNF-α* gene. Further, immunohistochemistry revealed no evidence of TNF protein accumulation in tissues.

Discussion

Ketamine is a phencyclidine derivative, a selective and non-competitive NMDA receptor antagonist exerting its anesthetic effect by blocking the stimulatory neurotransmitter effect of glutamate on these receptors.[18] It has been shown that low doses of ketamine can reduce pain during and after surgery.[19] However, chronic ketamine administration also increases the levels of inflammatory cytokines *TNF-α*, *c-Fos*, and *IL-6*, which would lead to neuro-inflammation and neuro-apoptosis.[24-26,34,35] In the present study, a high dose of ketamine administration at the dose of 75.00 mg kg^{-1} resulted in a significant increase in cholesterol, glucose, HDL, and LDL levels. On the other hand, it decreased triglyceride levels, indicating the negative effect of ketamine.

The *TNF-α* is an essential protein in cellular signaling pathway and plays a vital role in systemic inflammation and acute phase response. The prominent role of *TNF-α* is to regulate the immune system cells function.[36] The *IL-6* is a multi-functional cytokine regulating immune responses and acute phase reactions, playing a significant role in the host defense mechanism.[37] The protein product of *c-Fos* as a transcription factor is expressed in low levels in most normal cells.[38] However, oncogene *c-Fos* can be affected by a variety of inflammatory mediators and cells, and cytokines.[39] Our findings indicated that ketamine can increase the level of inflammatory cytokines, which would lead to neuro-inflammation and neurodegenerative damage

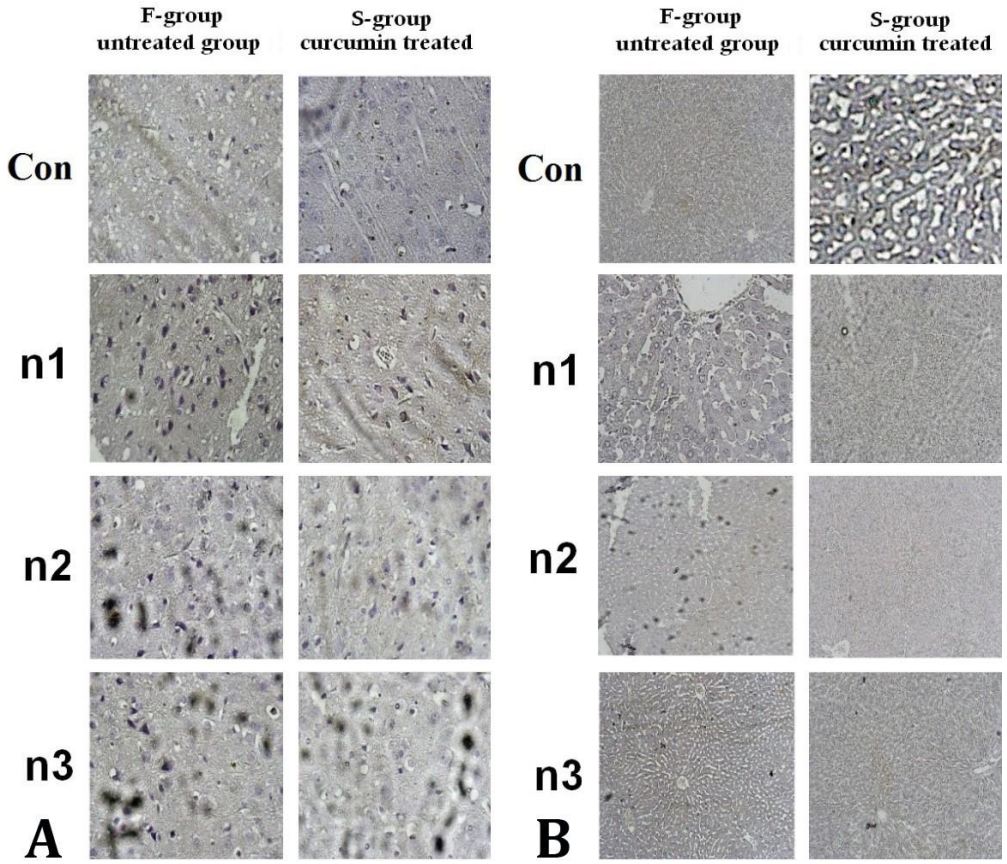

Fig. 3. Immunohistochemical staining of *TNF-α* in both untreated groups (F-groups), and curcumin-treated groups (S-groups) in, **A)** Brain (400×), and **B)** Liver (con, n2, n3: 100×, n1: 400×). No positive reaction was found. Con: Without ketamine injection in both groups; n1: Immediately after anesthesia; n2: 4 hr after anesthesia; n3: 12 hr after anesthesia.

in rats. Interestingly, the effects of ketamine on *TNF-α* level are contradictory in different conditions in brain and liver tissues, depending on dose and duration. Single-dose injection of ketamine increases the expression level of *TNF-α* in liver tissue, being different compared to the brain tissue and repeated injections.[24,26,27,34,40,41] Recently, the ability of curcumin to enhance a variety of pharmacological and biological activities, such as anti-inflammatory and anti-oxidant ones was approved.[42] It was reported that curcumin acts as a modulator of the glutamatergic neurotransmitter mission, and restores glutamatergic receptor levels, oxidative stress, and imbalanced glutathione metabolism. Moreover, curcumin inhibits glutamate release, blocks GluN2B receptors, and enhances neuronal survival against NMDA toxicity.[42] It also inhibits the production of cytokines (*IL-1*, *IL-6*, *IL-8*, and *TNF-α*) and various chemokines; curcumin also reduces inflammation and oxidative stress by inhibiting the Nrf-2 pathway and modulating glutathione peroxidase and malondialdehyde activities in the liver and kidneys.[43] Moghaddam *et al*. investigated the protective effects of curcumin on ketamine-induced schizophrenia-like behaviors and oxidative damage in male mice.[44] They found that curcumin improved ketamine-induced brain damage, oxidative stress biomarkers such as reduced glutathione, catalase, and glutathione peroxidase were significantly increased and malondialdehyde remarkably reduced compared to the control group. Also, memory deficits and anxiety-like behaviors were notably diminished in the treated group.[44] Pavlovic *et al*. have evaluated the effects of curcumin on ketamine-induced toxicity in rat thymocytes and revealed that curcumin significantly reduces apoptosis, cytotoxicity, reactive oxygen specious production, and caspase 3 levels in rat thymocytes.[45]

This study showed the ability of curcumin to prevent neuro-inflammation and oxidative stress induced by ketamine in rats. Curcumin injection had significant effects on reducing ketamine-induced inflammation in brain and liver tissues. The anti-inflammatory effects of curcumin at different hours of anesthesia reduced and normalized the expression level of inflammatory-associated genes up to 12 hr after anesthesia. Curcumin also significantly reduced glucose, triglyceride, LDL, and HDL levels.

The findings indicated that chronic ketamine injection at the dose of 75.00 mg kg^{-1} induced neurotoxicity in rats. In addition, it showed that ketamine could increase the levels of the inflammatory cytokines. The results of this study also demonstrated that treatment with curcumin protected the brain and liver against ketamine-induced neurotoxicity. Meanwhile, we found normalizations of several biochemical characteristics and inflammatory cytokines. We also postulated that the neuroprotective effects of curcumin may due to its free radical scavenging anti-oxidant activity. The underlying molecular mechanism by which ketamine affects the expression of inflammatory cytokines needs further investigation.

Acknowledgments

We would like to thank Dr. Azita Dilmaghani for her supportive help. This work was supported by Lorestan University, Khorramabad, Iran (Grant No. LU-9211501001-20) and Tabriz University of Medical Sciences, Tabriz, Iran (Grant No. 70949).

Conflict of interest

The authors declare that they have no competing interests.

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