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Colitis can reduce the cingulate cortex neuronal density in rats

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Abstract

Background and aim Hyperalgesia and hypersensitivity in patients with Inflammatory Bowel Disease (IBD) can be related to central nervous system (CNS) changes, particularly in the pain pathways. The objective of this study was to examine the neuronal density of the cingulate cortex area (CC) and amygdala in an animal model of colitis.

Materials and methods In this experiment, 13 male Wistar rats were subjected to study. Colitis was induced in the rats by transrectally administering 1 cc of acetic acid 3% under sedation with xylazine 10% (5 mg/kg). After 14 days of colitis, the rats were euthanized under high doses of anesthesia with ketamine (50 mg/kg), xylazine (10 mg/kg), and diazepam (2.5 mg/kg). Their brains were then removed surgically. Six-micrometer-thick brain slices were stained with *cresyl violet*, and the neuronal density of the amygdala, area 1 of the cingulate cortex area (CC1), and area 2 of the cingulate cortex area (CC2) was assessed via microscopic imaging.

Results The mean ± standard deviation (SD) of the neuronal density in CC1 was significantly decreased in rats with colitis compared to the control group in both the right CC1 (43.53 ± 9.63 vs. 62.7 ± 11.89; p-value < 0.001), and left CC1 (41.19 ± 9.05 and 63.1 ± 7.44; p-value < 0.001). Additionally, the neuronal density of CC2 in the colitis group was found to be significantly lower than that of the controls in both the right CC2 (57.8 ± 13.23 vs. 87.95 ± 8.76; p-value < 0.001), and left CC2 (55.42 ± 11.3 vs. 98 ± 8.99; p-value < 0.001). Furthermore, the amygdala had a lower neuronal density in both hemispheres in rats with colitis in comparison to the controls bilaterally: right hemisphere (24.51 ± 5.49 and 36.3 ± 7.44; p-value = 0.360), and left hemisphere (24.52 ± 5.53 VS. 35.25 ± 5.6; P-value = 0.869).

Conclusion This study showed that colitis can reduce the neuronal density within cortical areas and amygdala of both hemispheres. Considering the cingulate cortex's role in suppressing pain perception, any harm inflicted upon this region of the brain can has the ability to impact the cognitive and sensory aspects of pain.

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Key message

• What is already known?

Inflammatory Bowel Disease (IBD) can be related to central nervous system (CNS) changes.

• What is new here?

A reduction rate in the density of neurons in the cingulate cortex and amygdala area of the brain was observed in rats with colitis.

• How can this study help patient care?

The neuronal damage in the pain matrix can result in the alteration of pain perception in patients with IBD.

Keywords Colitis, Inflammatory bowel disease, Acetic acid, Gyrus cinguli, Amygdala, Chronic inflammation, Animal model

Introduction

Inflammatory bowel disease (IBD) is a long-term immune-mediated disorder that triggers ongoing inflammation in the gastrointestinal (GI) tract. It is primarily categorized into two types: Crohn's disease (CD) and ulcerative colitis (UC) [1]. While IBD predominantly affects the GI tract, patients may also experience a range of symptoms affecting other systems, including pulmonary, cardiovascular, ocular, dermatological, musculoskeletal, urinary, and neurologic systems [2–4]. This multi-organ involvement s in IBD stems from systemic inflammation resulting from an imbalance between pro-inflammatory and anti-inflammatory substances in the body.

The central nervous system (CNS) is often underestimated aspect that can be influenced by IBD, leading to various psychiatric, cognitive, or neurological symptoms [5]. Injury or inflammation of the GI tract can result in heightened sensitivity and pain in the intestine [6]. Additionally,, those with IBD frequently encounter other pain-related conditions, such as fibromyalgia, polymyalgia rheumatica, chronic pain syndrome, abdominal discomfort, myalgia, and arthralgia [7–9]. Various proposed mechanisms indicate an alteration in the functioning of the pain neuromatrix. These include persistent hyperexcitability in the viscerosensory nuclei of the brainstem and thalamus, hypothalamus, amygdala, hippocampus, anterior insula, and anterior cingulate cortical [10]. This amplified sensitivity to pain is largely attributed to neuroplastic alterations in the spinal cord and brain that arise from GI tract pathologies.

Melzack and Casey introducee the neuromatrix theory of pain to demonstrate the interconnectedness of various pain components, including cognitive, sensory, and affective elements. They suggested that various areas of the brain work together to create the overall experience of pain [11, 12]. A key player in this pain neuromatrix is the cingulate cortex (CC), which is essential for modulating pain-related behaviors and influencing activity in the spinal dorsal horn [13]. Research has shown that damage to the CC can significantly enhance nociceptive responses in animal models [14]. Additionally, studies have revealed that patients with IBD often exhibit a reduction in grey matter volume and alterations in various cortical regions of the brain [15]. As a result, injuries in the areas of the brain responsible for processing pain can interfere with the system's typical functioning, leading to alterations in the pain experiences of those affected by IBD.

The concept of the gut-brain axis (GBA) refers to the interaction between the brain and the GI tract in both normal and abnormal circumstances [16]. A growing body of evidence suggests that the GBA establishes direct links between the limbic system and the enteric nervous system of the GI tract [17]. The Amygdala, a vital element of the GBA, plays a crucial role in the two-way communication that occurs among GI functions, gut microbiota, and emotional response [18]. Furthermore, the amygdala is implicated in pain perception and related behaviors, as part of the pain neuromatrix [18]. Studies using functional magnetic resonance imaging (fMRI) have shown that the amygdala is impacted in patients with IBD [17]. This observation suggests that alterations in the CNS may play a role in heightened pain sensitivity experienced by IBD patients.

We proposed that the onset of systemic inflammation associated with IBD could result in neuronal damage within the brain. Such damage may affect the pain pathways in the CNS, particularly in areas like CC and amygdala, potentially altering how pain is processed.

Therefore, in this study we aimed to evaluate the neuronal population in CC and amygdala in both hemispheres of rats with colitis induced by the 3% acetic acid solution enema; then compare the neuronal density in rats with IBD and healthy group.

Materials and methods

Animals

In the present study, 13 male Wistar rats aged between 12 and 15 weeks, with an average weight of 250 ± 25 g, were employed. The animals were purchased from a branch of the Institute Pasteur of Tehran, Iran, which is responsible for the production and sale of animals for research, located in the north of Iran. Following their relocation

to the animal center of Golestan University of Medical Sciences (GOUMS) in Iran, the rats were subjected to standard conditions. The adaptation period to the new environment was 14 days. The animals were housed in plastic cages that provided an enriched environment. A 12-hour light-dark cycle and an ambient temperature of 22 to 24 °C were maintained for their continued care. Furthermore, the rats were provided with unrestricted access to both food and water. Before induction of colitis, the rats had only access to water for 24 h and their food was limited [19].

The study protocol has been approved by the local ethical committee of Golestan University of Medical Sciences (ID: IR.GOUMS.REC.1401.081). The experiments reported herein were precisely in accordance with the principles given by the National Committee for Research Ethics in Science and Technology, Guide for the Care and Use of Laboratory Animals in Iran and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [20, 21].

Design of the experiment

The colitis group consisted of nine rats (20% possible percentage decrease), which were administered 2 ml of a 3% acetic acid solution via transrectal injection on the first day of the experiment. This was done under sedation conditions, with the rats receiving an intraperitoneal (IP) injection of xylazine 2% (5 mg/kg; Alfasan, the Netherlands). The control group consisted of four rats that appeared healthy. They were treated with distilled water in the same manner as the group with colitis.

Inclusion and exclusion criteria

After adaptation of the animals, all apparently healthy animals with a weight range of 225–275 gr were included in the experiment into two groups. Furthermore, animals were remained in the colitis group if the colitis model induced successfully.

If the rats lost more than 30% of their initial body weight during the experiment or fever was detected in them, they were excluded from the study because of the increasing risk of death [19, 22].

Colitis induction

To prepare the 3% acetic acid solution, a mixture of absolute glacial acetic acid (CAS-Nummer:64-19-7, Merck, Germany) and distilled water was utilized. Each rat was then administered 2 ml of this solution via transrectal administration using a feeding tube with a diameter of 1.5 mm under conditions of animal sedation (xylazine 2% (10 mg/kg)). The tube was inserted into the rectum to a depth of approximately 8 cm, ensuring that it reached the splenic flexure of the colon. The solution was infused at a slow rate in the Trendelenburg position, and the rats were kept for one minute to prevent leakage [22]. After induction, the animals were observed by a veterinarian (H.S.) for one hour.

Each group of rats were kept in separate cages for 14 days [23] and have been monitored daily regards their weights and disease activity index as described in the next section. Following 14 days of colitis, all rats were euthanized and their brains and colons were collected for analysis, as described in Sects. 2–5 and 2–6.

Disease activity index (DAI)

The severity and activity of colitis were assessed by the Disease Activity Index (DAI). The DAI assessed aspects such as weight loss, stool consistency, and rectal bleeding [24]. To measure the DAI, a checklist was used daily. The checklist included the following items:

(1) Stool trait: It was rated as normal (0), soft (2), or diarrhea (4).
(2) Weight loss: It was categorized as none (0), 1–5% (1), 6–10% (2), 11–20% (3), or more than 20% (4).
(3) Rectal bleeding: It was rated determined as negative (0), positive occult blood test (2), and visible bleeding (4).

Colon evaluation

One day following the administration of acetic acid, one rat from the colitis group was placed in a carbon dioxide (CO_2) gas chamber for the purpose of sacrifice. Its intestine (colon portion) was then surgically removed. Upon examination, the colon exhibited significant inflammation (Fig. 1). The colon displayed complete involvement with hyperemia, inflammation, and ulcers, which can be indicative of colitis [25].

On the 14th day after the acetic acid enema, the colon of one other rat was surgically removed (euthanized as before in the CO_2 tank) and appeared non-hyperemic, but exhibited a 2-cm long ulcer, with very mild inflammation. Imaging studies depicted a severe and acute onset of colitis in the initial days; followed by a gradual reduction in its severity over time. Considering the evidence, it can be concluded that the induction of chronic colitis was successful in the colitis group.

Histological procedures

The other rats were first administered an intra-peritoneal (IP) injection of a cocktail including ketamine 10% (50 mg/kg; Alfasan, the Netherlands), xylazine 2% (10 mg/kg), and diazepam hydrochloride (2.5 mg/kg; Darupakhsh, Iran) drugs [20, 21], followed by decapitation to remove their brains from the skull [23]. The brains were then preserved in a solution containing 4% paraformaldehyde (DR Mojallali, Iran) for a duration of 14 days. Afterward, the brain tissues underwent preparation using

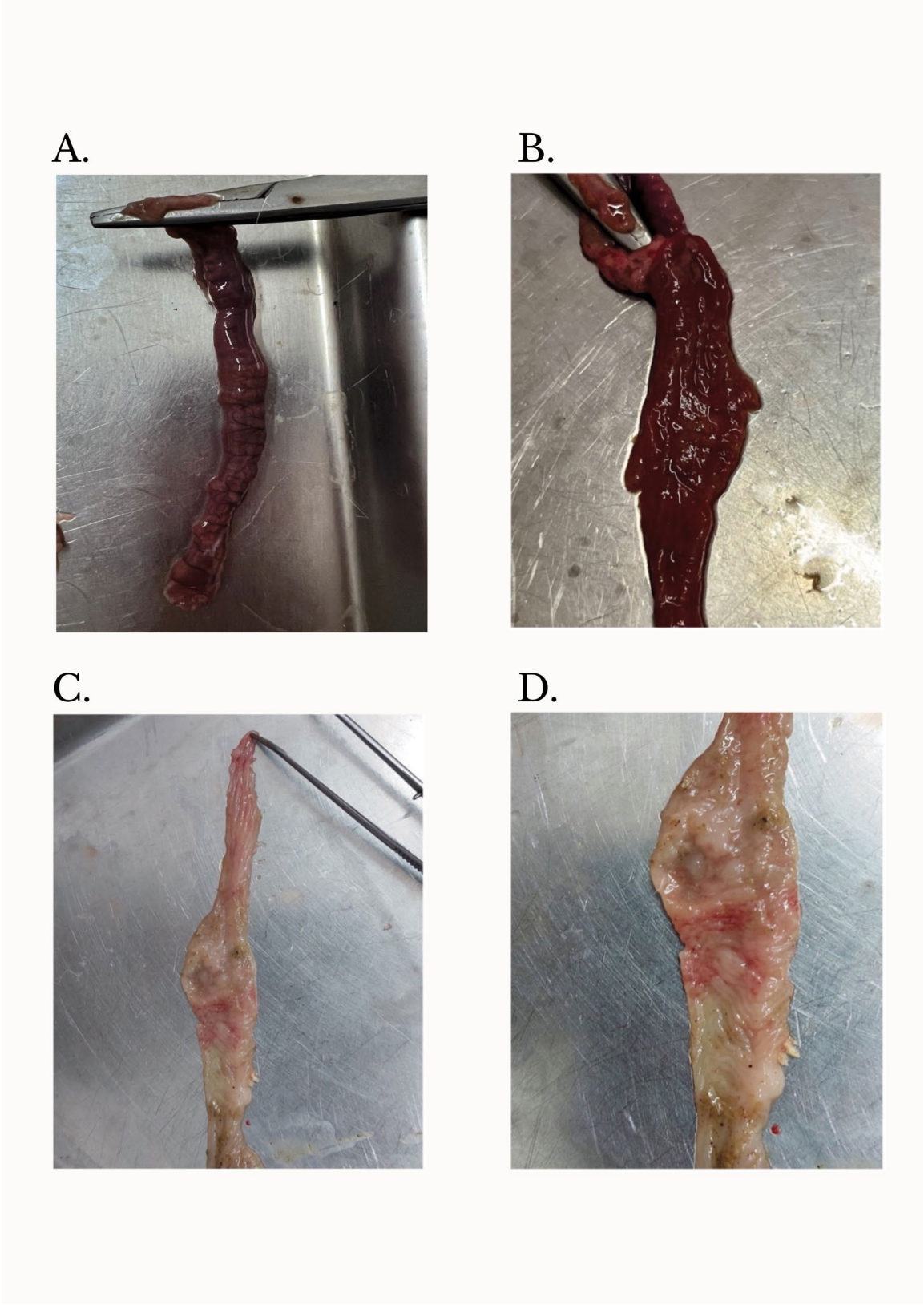

Fig. 1 The macroscopic evaluation of colon in rats with acetic acid-induced colitis. **A** and **B**: after one day of colitis induction, the colon was completely hyperemic and inflamed; **C** and **D**: 14 days later, the inflammation seemed to be decreased but large ulcers were seen

a tissue processor (D2 2080/H, Iran), involving dehydration using alcohol and clarification using xylol (DR Mojallali, Iran) [26]. Subsequently, the paraffin blocks of the brain were sectioned to 6 µm thickness sagittal planes serial approach using a rotary microtome (Pooyan MK 1110, Iran) [26].

Cresyl Violet staining

Deparafinized brain slices were rehydrated in a graded ethanol series and rinsed in distilled water. The brain sections' slides were then stained with the 0.02% *cresyl violet* (Sigma, USA) for 5 min [27], then dipped in distilled water (3 times), and dehydrated in a graded ethanol series (Merck, Germany), and finally washed out in xylene (DR Mojallali, Iran) and coverslipped with entellan (Merck, Germany).

Brain areas

The brain areas within the CC and amygdala are defined based on the Paxinos and Watson *The Rat Brain atlas* [28]. The CC is composed of two distinct areas, namely CC area 1 (CC1) and area 2 (CC2), which are situated adjacent to the midline. To locate the amygdala, one can trace the internal capsule on the lower section of the brain in both hemispheres.

Morphometry of neurons

To evaluate the density of neurons, a microscope (BX51, Olympus, Japan) along with a DP 12 digital camera was employed to capture an image from each location in both hemispheres. The images, magnified at 400×, were subsequently transferred to a computer for morphometric analysis [29]. The OLYSIA Autobioreport software was utilized to overlay suitable grids onto the images. On each image, a randomly selected area of 30,000 μm^2 was chosen [30]. As neuronal density refers to numerical density of neurons per unit area, our definition of neuronal density was the ratio between the sum of area covered by neurons divided by the whole frame surface. An operator, who remained unaware of the condition, manually counted the neurons, considering only those with distinctive cell bodies and/or nuclei.

Statistical analysis

The mean and standard deviation (SD) of CC1, CC2, and amygdala variables were calculated. The normality of the data was checked using the Shapiro-Wilk test and then, they were compared using the Welch t-test, student t-test, and Mann-Whitney u-test. The significance level of the tests was considered to be 0.05. Data were analyzed using SPSS 25 software.

Results

The group of rats with colitis showed a higher DAI compared to the control groups. The mean ± SD of DAI was 2 ± 1.08 in rats with colitis, whereas it was 0.33 ± 0.27 in the control group (p-value = 0.024). As it was expected, the colitis group had a higher DAI compared to control group. The neuronal density in Cingulate Cortex (CC) was found to be lower on both sides in rats with colitis compared to the control group (Fig. 2).

In the right CC1, the mean ± SD of neuronal density in the colitis group was 43.53 ± 9.63, whereas it was 62.7 ± 11.89 in the control group, showing a significant difference (p-value ˂ 0.001), which is detectable in image B comparing to image D in Fig. 3. Similarly, in the left CC1, the mean ± SD of neuronal density in the colitis group was 41.19 ± 9.05, while it was 63.1 ± 7.44 in the control group, again demonstrating a significant difference (p-value ˂ 0.001). Also, the image A showed lower population of neurons in CC compared to image C in Fig. 3. The neuronal density of CC1 between hemispheres was not significantly different in the colitis group (p-value = 0.333). However, in the control group, the neuronal density of CC1 in the left hemisphere was significantly higher than in the right hemisphere (p-value = 001). Figure 3 shows the microscopic image of CC1 in both hemispheres.

In the colitis group, the neuronal density in the right CC2 was significantly lower compared to the control group (57.8 ± 13.23 VS. 87.95 ± 8.76, respectively; p-value ˂ 0.001), which is detectable in image B and D in Fig. 4. Similarly, the mean ± SD of neuronal density in the left CC2 was 55.42 ± 11.3 in the colitis group and 98 ± 8.99 in the control group, showing a significant difference (p-value ˂ 0.001), as showed in the image A and C in Fig. 4. There was no significant difference in the neuronal density of CC2 between the hemispheres in rats with colitis (p-value = 0.056). Additionally, this measure was not significant in the control group either (p-value = 0.932). In Fig. 4 the microscopic images of this area are presented.

In both the left and right hemispheres, the density of the amygdala was observed to be lower in the colitis group compared to the control group (Fig. 2). Specifically, in the right amygdala (image B and D in Fig. 5), the mean ± SD neuronal density in the colitis group was 24.51 ± 5.49, while in the control group, it was 36.3 ± 7.44. However, there was no significant difference in the neuronal density between these groups (p-value = 0.360). Similarly, the neuronal density in the left amygdala was found to be lower in the colitis group compared to the control group (Image A and C in Fig. 5), but this difference was not statistically significant (24.52 ± 5.53 VS. 35.25 ± 5.6; p-value = 0.869). The microscopic images of the amygdala in both hemispheres are presented in Fig. 5.

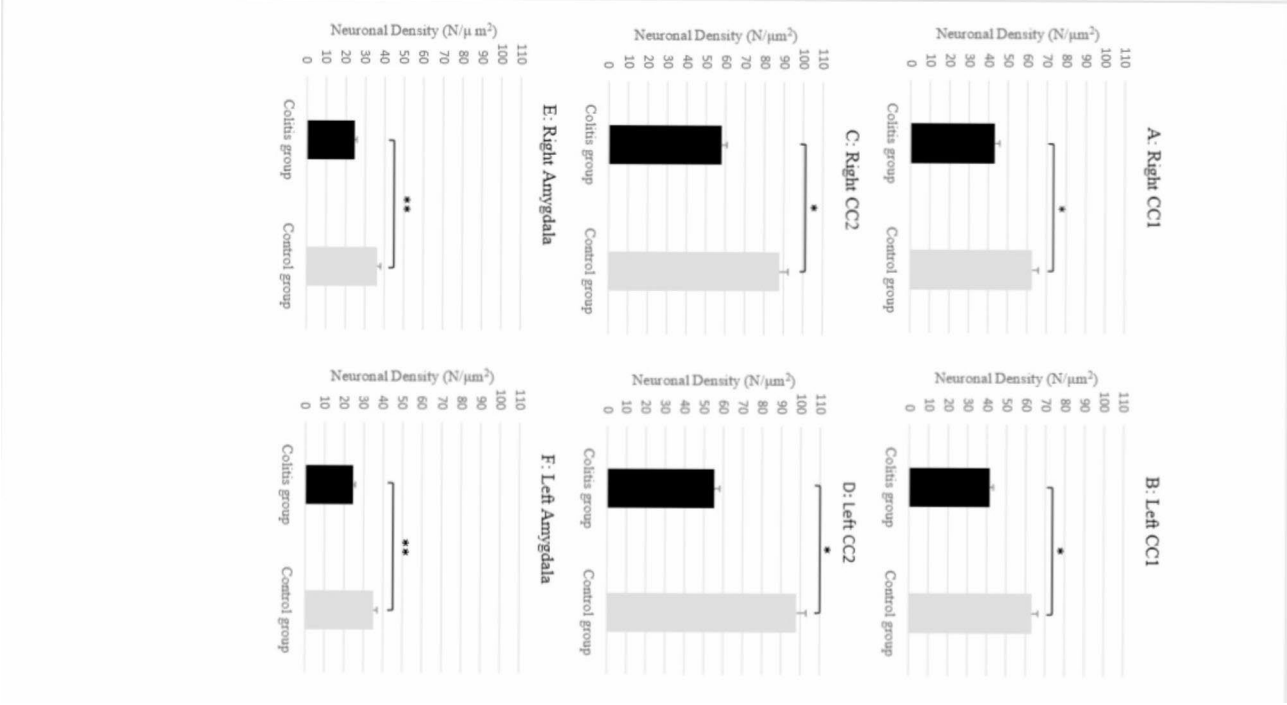

Fig. 2 Comparison of the neuronal density of cingulate cortex (CC) and amygdala in colitis and control group. **A**: CC area 1 in the right hemisphere; **B**: CC area 1 in the left hemisphere; **C**: CC area 2 in the right hemisphere; **D**: CC area 2 in the left hemisphere; **E**: amygdala in the right hemisphere; **F**: amygdala in the left hemisphere. *p-value < 0.001; ** p-value > 0.05

Discussion

The current research revealed a notable decline in neuronal density within the pain neuromatrix of the brain (specifically in CC areas 1 and 2) in rats with colitis induced by acid-acetic compared to the control group. However, the decrease in neuronal density observed in the amygdala did not reach statistical significance. These findings imply that colitis could negatively impact neurons in brain regions linked to pain perception.

Zikou et al. found comparable findings concerning alteration in the brain among IBD patients [15]. Using magnetic resonance imaging (MRI), they showed a decrease in gray matter volume across several brain regions in IBD patients including the inferior temporal gyrus, right precentral gyrus, right supplementary motor area, right middle frontal gyrus, and left superior parietal gyrus. In contrast to our observations, their results displayed a noticeable lateralization, while we identified a symmetrical decline in the population of cortical neurons. This suggests that the impact of colitis on the brain may be more widespread that previously understand.

The potential mechanisms underlying the neuronal damages associated with colitis primarily involve the GBA. Vasculitis of the small cerebral vessels, arises due to vascular blockage, increased coagulability, inflammation, and alteration in the muscle tone of the vessels, plus decreased oxygen supply to brain tissues can lead to valerian degeneration in neurons [15, 31]. Moreover, three to four times greater risk of thrombosis and intravascular coagulation have been seen in UC compared to the normal population [32]. These findings strongly suggest a contribution of vasculitis to the decline in neuronal density in rats with colitis, that would better to be investigated in further studies.

Another study conducted by Agostini et al. [17] used fMRI images in UC patients, identified a significant reduction in blood oxygen level-dependent (BOLD) activity in the amygdala, thalamic regions, and cerebellar area in IBD patients compared to controls in absence of volume difference. Our results also indicated nonsignificant changes in neuronal density of amygdala. But considering Agostini's investigation, the effect of IBD on the brain is not limited to neuronal deaths merely and can result in functional changes of such a brain area. These studies together highlight the substantial impact of IBD on various brain regions in affected individuals, which aligns with our findings.

Systemic inflammation caused by colitis has the potential to spread inflammation to the brain, resulting in neuroinflammation [33]. This fact is observed in different animal colitis models. In rats with colitis induced by Tri-Nitrobenzene Sulfonic Acid (TNBS) the expression level of pro-inflammatory cytokines such as interleukin 6 (IL-6) increases, in both colon and brain tissue (33–34). Tumor necrosis factor α (TNF-α), involved in the colitis pathology [1], was also found to be significantly increased

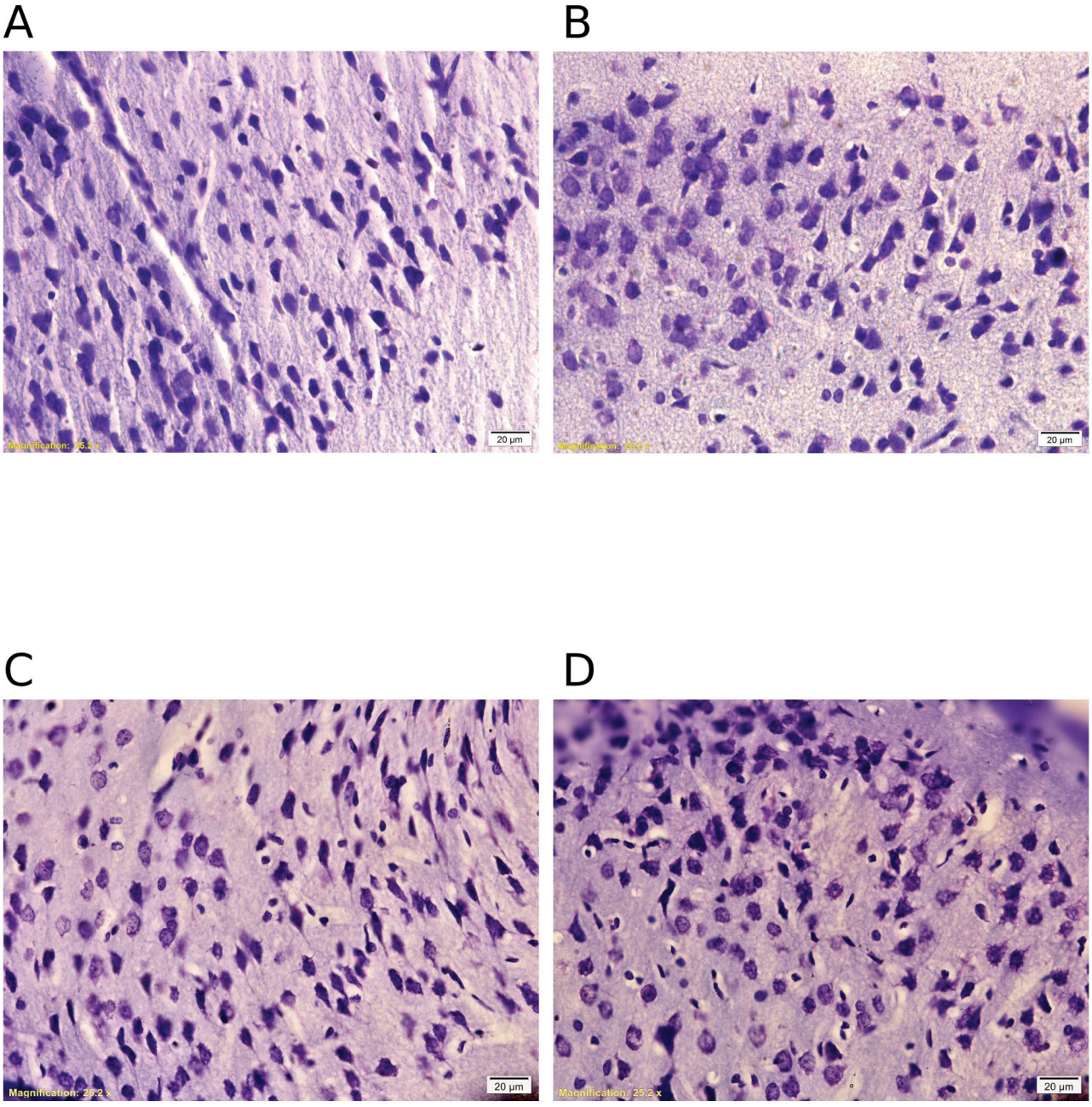

Fig. 3 Microscopic images (400 X magnification) of cingulate cortex area 1 (CC1) in rats with acid acetic-induced colitis shows a significant decrease level of neuron density compared to the control group. **A**: Left CC1 in colitis rats; **B**: Right CC1 in colitis rats; **C**: Left CC1 in control rats; **D**: Right CC1 in control rats

in Dextran sulfate sodium (DSS)-induced colitis model [34]. Additionally, the number of monocyte-derived macrophages was increased during colitis leading to significant changes in microglial activities [35]. These findings demonstrated how peripheral inflammation can result in neuroinflammation in the colitis animal model by altering the production of cytokines and inflammatory cells. These could somehow be the underlying reasons for decreased neuron density in the studied areas, although it would be better to investigate the changes in neuroinflammatory markers in further studies.

A noteworthy finding of this study was the insignificant decrease in neuronal density observed in the amygdala of rats with colitis, as compared to the control group. The activation of the hypothalamic-pituitary-adrenal (HPA) axis and the presence of inflammatory markers are additional factors contributing to neuroinflammation in an animal model of colitis. Rise in the levels of cortisol, corticosterone, and C-reactive protein (CRP), glial fibrillary

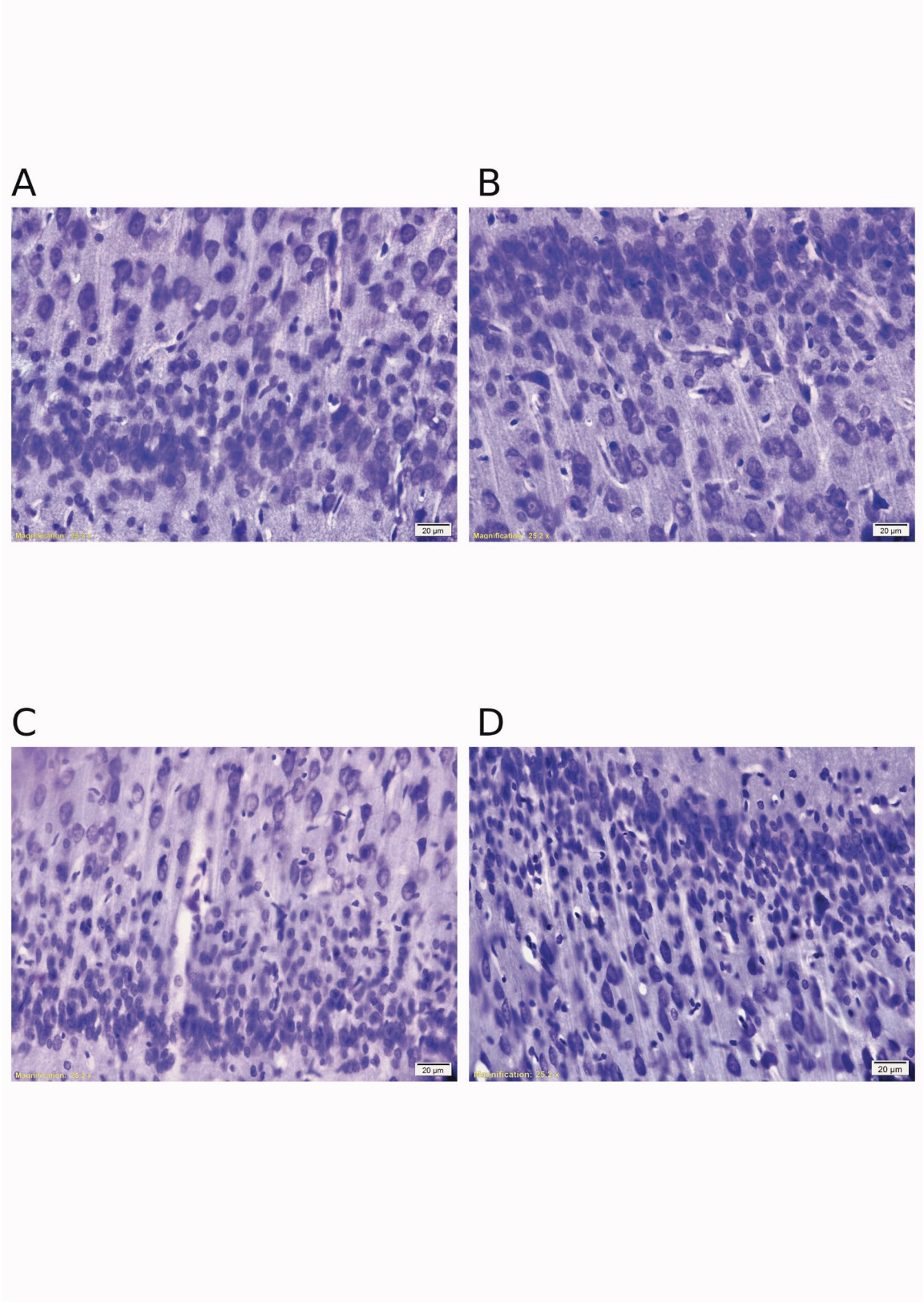

Fig. 4 Microscopic images (400· magnification) of cingulate cortex area 2 (CC2) in rats with acid acetic-induced colitis shows a significant decrease level of neuron density compared to the control group. **A**: Left CC2 in rats with colitis; **B**: Right CC2 in rats with colitis; **C**: Left CC2 in control rats; **D**: Right CC2 in control rats

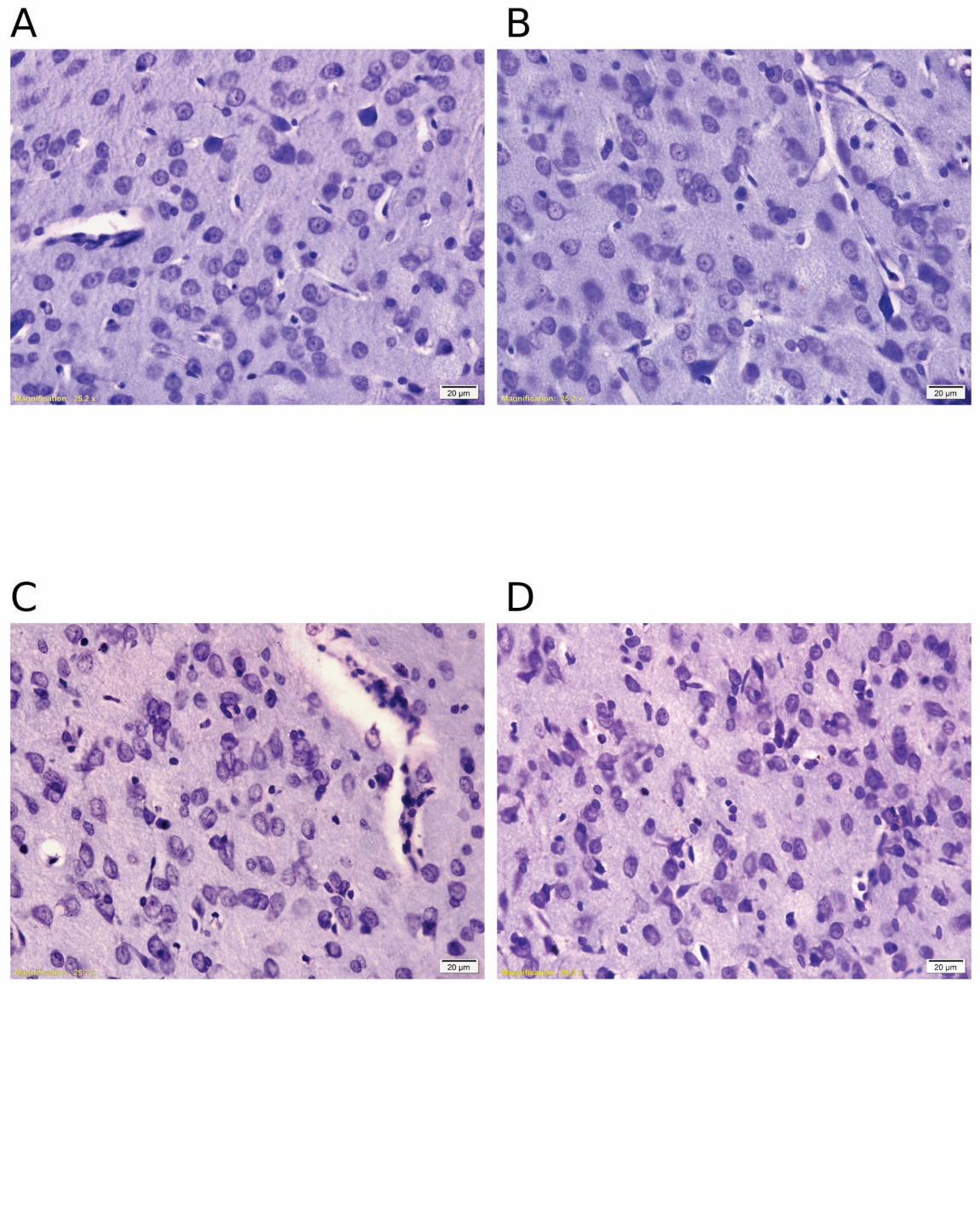

Fig. 5 Microscopic images (400· magnification) of the amygdala in rats with acid acetic-induced colitis shows decrease level of neuron density compared to control rats. **A**: Left amygdala in rat with colitis; **B**: Right amygdala in rat with colitis; **C**: Left amygdala in control rats; **D**: Right amygdala in control rats

acidic protein (GFAP) and cyclooxygenase 2 (COX-2) up regulation in the hippocampus and hypothalamus [36–38], increase brain-derived neurotrophic factor (BDNF), in the hypothalamic regions of mice with colitis, and down regulate COX-2 and BDNF in the amygdala of mice with colitis [36]. These findings may show that the amygdala exhibits a greater resistance to inflammatory changes when compared to cortical areas. Consequently, it is evident that colitis can induce inflammation in the brain through a bottom-up pathway.

The blood-brain barrier (BBB) can be disrupted as a result of peripheral inflammation, leading to changes in the normal condition of the central nervous system (CNS) by interrupting intercellular tight junctions (TJs). In the rodent model of colitis, the TJ protein expressions, including occludin and claudin-5, were significantly downregulated in specific regions of the brain, such as the cortex and hippocampus [34, 39, 40] On the other hand, Colitis can alter the permeability of the colon, allowing the release of the bacterial endotoxins into the bloodstream [41] and altering the composition of the gut microbiota and elevating levels of lipopolysaccharides [42, 43]. The leakage of endotoxins could potentially serve as another mechanism through which inflammation can spread to the CNS [34].

The question at hand pertains to the implications of a decrease in the density of neurons in the CC on the pain process that could be focused in humane and clinical studies in future. The CC plays a dual role in the pain process: directly inhibiting pain and exerting inhibitory effects on other brain regions involved in pain [13]. Activation of CC has been linked to a reduction in pain-related behavior in animal models [44]. Conversely, lesions in this area increase nociceptive responses [14, 45]. Electrophysiological investigations have revealed a larger population of inhibitory neurons compared to excitatory neurons in the CC [46]. Presence of GABAergic neurons and opioid receptors in the CC can lead to inhibitory role in pain perception [14]. The CC can alleviate pain by influencing other CNS regions such as the supra-spinal pain suppressor system, located at the level of the Periaqueductal gray (PAG) and inhibiting neurons in the dorsal horn of the spinal cord that respond to mechanical pain stimuli. (47–48). Therefore, it can be inferred that a decrease in the neuronal density of CC diminishes its inhibitory roles in the pain neuromatrix and perception of pain.

The study had a few constraints that can provide valuable insights for future researchers in their upcoming projects. Firstly, it would have been beneficial to include a larger number of animals to yield more reliable and conclusive outcomes. However, it is important to acknowledge that reducing the number of animals is a fundamental principle of bioethics in working with laboratory animals. Secondly, the study lacked access to animal imaging devices such as brain MRI, or fMRI for functional and volume evaluations. Lastly, this study did not evaluate inflammatory markers in the blood and brain. Assessing these markers would have been advantageous in elucidating the effects of colitis on the central nervous system. Therefore, it is suggested that future studies incorporate the assessment of inflammatory markers to gain further clarity in this regard.

Conclusion

As the results showed, colitis could reduce the density of neurons in the CC region of the brain in comparison to the control group. Considering the role of CC in suppressing pain perception, further studies on different parts of the brain are required to delve into the precise mechanisms underlying CNS damage in colitis.

Abbreviations

CNS	Central Nervous System
CC	Cingulate Cortex Area
SD	Standard Deviation
IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
UC	Ulcerative Colitis
GBA	Gut-Brain Axis
GI	Gastrointestinal
fMRI	Functional Magnetic Resonance Imaging
GOUMS	Golestan University of Medical Sciences
ARRIVE	Animal Research: Reporting of In Vivo Experiments
DAI	Disease Activity Index
CO_2	Carbon Dioxide
IP	Intra-Peritoneal
MRI	Magnetic Resonance Imaging
WMHIs	White-Matter Hyperintensities
BOLD	Blood Oxygen Level-Dependent
TNBS	Tri-Nitrobenzene Sulfonic Acid
IL-6	Interleukin 6
TNF-α	Tumor Necrosis Factorα
DSS	Dextran Sulfate Sodium
CD86	Cluster Of Differentiation 86
HPA	Hypothalamic-Pituitary-Adrenal
CRP	C-Reactive Protein
GFAP	Glial Fibrillary Acidic Protein
COX-2	Cyclooxygenase 2
BDNF	Brain-Derived Neurotrophic Factor
BBB	Blood-Brain Barrier
TJs	Tight Junctions
PAG	Periaqueductal Gray

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12876-025-03745-x.

Supplementary Material 1

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Author contributions
FA: Study concept and design,; acquisition of data; analysis and interpretation of the data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; All authors approved the final version of the manuscript. SB: study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content; drafting of the manuscript; administrative, technical, and material support; study supervision; All authors approved the final version of the manuscript. MJ: study concept and design; critical revision of the manuscript for important intellectual content; drafting of the manuscript; study supervision; All authors approved the final version of the manuscript. HSH: study concept and design; critical revision of the manuscript for important intellectual content; drafting of the manuscript; administrative, technical, and material support; All authors approved the final version of the manuscript. FN: analysis and interpretation of the data; drafting of the manuscript; statistical analysis. All authors approved the final version of the manuscript.

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Data availability
Data are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
The study protocol has been approved by the local ethical committee of Golestan University of Medical Sciences (ID: IR.GOUMS.REC.1401.081). The experiments reported herein were precisely in accordance with the principles given by the National Committee for Research Ethics in Science and Technology, Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines. It should be noted that these animals did not belong to any other private company.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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