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Original Article

# Effect of Intracerebral Hemorrhage on Anxiety and Depression-Like Behavior and Amygdala Glial Changes and Cell Death

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## Abstract

**Introduction:** Intracerebral hemorrhage (ICH) is a cerebrovascular disease with a high mortality and morbidity rate. The study aimed to investigate the relationship between amygdala and striatum changes and neuronal and glial changes post-stroke, as over two-thirds of stroke patients experience depression and anxiety.

**Methods:** The study involved 48 male Wistar rats, divided into six groups, including three sham (sham-7, sham-14, and sham-21) and three ICH (ICH-7, ICH-14, and ICH-21) groups. Behavior tests, such as the elevated maze test (EMT), open field test (OFT), and forced swimming test (FST), were used on the 7th, 14th, and 21st days following surgery. Next, the stereology technique was utilized to assess the neuronal density. Finally, the immunohistochemistry method was employed to evaluate alterations in astrocytes.

**Results:** The results indicated that the immobility time, according to FST, was significantly increased on the 21st day after ICH induction. Although the active time in this group decreased compared to the sham-21 group, based on the EMT, the time spent in the closed arm in the ICH-14 and ICH-21 groups had a significant upward trend. According to OFT, central crossing in the ICH-21 group demonstrated a considerable decrease. Nonetheless, neuronal density in the amygdala area displayed no significant differences between the groups. Eventually, glial fibrillary acidic protein-positive cells in the ICH-7 and ICH-21 groups were increased in the ipsilateral amygdala.

**Conclusion:** The results revealed that anxiety and depression behaviors occur 21 days after unilateral ICH is created in the striatum, possibly because the striatum-to-amygdala pathways are affected.

**Keywords:** Intracerebral hemorrhage, Depression and anxiety, Astrocytes, Amygdala

## Introduction

As estimated by research studies, 795,000 people annually suffer from a stroke; among them, about 610,000 cases are reported to experience just their first-time attack, and other cases have had a second stroke.[1] Two-thirds of stroke patients show various degrees of cognitive disorders. Mood disorder is one of the most common types of cognitive disorders. Nearly two-thirds and one-fourth of people demonstrate depression and anxiety after stroke, respectively.[2] The quality of life is lower, but the rate of mortality is higher in people who developed cognitive, functional, or communicative difficulties compared to other individuals.[3] Intracerebral hemorrhage (ICH) is a severe type of stroke, which is responsible for a high rate of morbidity and mortality. There is evidence of clear signs of depression in 20% of ICH survivors.[4] Nevertheless, it is controversial whether post-stroke depression (PSD) is the result of aroused biological factors by the pathophysiology of brain injuries or whether it is a psychological response to the cognitive, physical, and social disorders stimulated by the stroke.[5] Some studies have cautioned that PSD has a higher rate of incidence in the earliest year of the stroke.[6] Moreover, psychosocial factors and neurobiological dysfunctions are determinants associated with increased

risk factors of PSD.[7] Based on clinical and research studies, there is a relationship between PSD and cognitive disorders and brain injuries after stroke.[8] However, these relationships are not clearly defined. Thus, research in this field can be helpful.

Different parts of the brain are involved in emotions, including cortical areas (e.g., the frontal cortex) and subcortical areas (e.g., the striatum and amygdala).[9] The amygdala has a complex, diverse structure consisting of a group of nuclei. Special nuclei of the amygdala are responsible for sending differential projections to the ventral striatum.[10] The striatum projects to the cortex and plays a role in developing specific behavioral responses.[11] Evidence indicates that changes in dopamine neurons in the ventral tegmental area and striatum can lead to cognitive changes, such as schizophrenia or depression.[12] The amount of dopamine in these two regions significantly decreases in rats exposed to chronic stress.[13] In addition, positron emission tomography studies in patients with major depression have shown that dopamine receptors are abnormally distributed in the striatum, and these patients experience anxiety with depression simultaneously.[14] However, the mechanism of damage to dopamine neurons in the striatum and its effect on depression and anxiety are not fully understood.

Amygdala damage can cause serious cognitive problems, such as induced mood disorder, impaired emotional behavior, increased passivity, or decreased aggression.[15] Moreover, reduced amygdala volume has been investigated in people with traumatic brain injuries.[16] The basolateral amygdala contributes to regulating emotion and passion, and its neuroplastic changes significantly affect depression.[17] Destruction of the major inhibitory neurotransmitter of gamma-aminobutyric acidergic inhibition in the basolateral amygdala can be the underlying cause of behavioral hyperexcitability, which is characterized by excessive anxiety, emotional dysregulation, depression, seizures, and epilepsy.[18] It has been reported that the left hemisphere of the amygdala automatically responds to subliminally and supraliminally presented emotions of neutral, sad, and fearful expressions under the condition of major depressive disorder.[19] During ICH, as a subtype of cerebral stroke, there are disruptions to cell metabolism and activation of stress responses.[20] Furthermore, evidence suggests that the pathogenesis of depressive disorders (e.g., depression and schizophrenia) may be attributed to dopaminergic neuronal abnormalities in the ventral tegmental area.[12] The astrocytes, microglia, and endothelial brain cell types are involved in ischemic stroke-induced immune-inflammatory activation.[21] In inflammatory conditions, microglia and astrocytes release inflammatory factors and nitric oxide, respectively. Thus, in the amygdala and striatum, which are involved in the emergence of emotions, changes in the amount of glia and neuronal density can affect mood and behavior after a stroke.[22] Moreover, considering that the mechanism of cognitive impairments after stroke is stills not fully investigated, understanding the mechanism and pathogenesis of depression and anxiety after ICH opens new insights into finding effective treatments. Accordingly, the present study aims to evaluate astrocyte activity and cell density in the amygdala. With the bleeding in the striatum area and its connection to the amygdala as well as its role in cognitive behaviors, it is hoped that promising information will be obtained in this area.

## Materials and Methods

### *Animals*

Overall, 48 male Wistar rats (220–250 g) were used in this experimental study. The animals were kept in standard conditions (12-hour light/dark cycle with full access to food and drinking water). All procedures in this experiment were approved by the Research Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1400.403.) Animals were randomly divided into six groups (n = 8 per group), including three sham groups (Sham-7, Sham-14, and Sham-21) and three ICH groups (ICH-7, ICH-14, and ICH-21). The first three groups underwent surgery, but no ICH was induced in these groups. To induce ICH, the animals of the other groups underwent surgery, and 100 µL of autologous blood was injected into the left striatum. Behavioral tests were performed on the animals of these groups on days 7, 14, and 21 after the surgery, and finally, the brain was removed for histopathology studies.

### *Intracerebral Hemorrhage Induction Surgery*

Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and fixed on a stereotaxic apparatus (Stoelting Stereotaxic Instrument). Based on stereotaxic coordinates (AP = + 3.6, ML = ± 3.4, DV = 6), 100 µL of autologous blood, collected from the orbital sinus, was injected into the left striatum using a Hamilton needle (100 µL, 700 series, Hamilton Company, Switzerland) without anticoagulant at 2 µL/min. The needle was held in place for 20 minutes after the injection to prevent backflow.[23] Then, the burr holes in the skull were sealed with bone wax, and the wound was stitched. Sham groups were studied using the same approach as that described for the ICH groups, but they did not have blood injection into the left striatum; then, the animals were allowed to recover in rat cages with free access to food and water.

### *Neurologic Deficit Score and Bodyweight*

NDS in each group (n = 8) were assessed following the method described by previous researchers.[24] Using consisted of the six tests. All six parameters of the NDS included spontaneous activity, symmetry of movements, symmetry of forelimbs, climbing the wall of the wire cage, reaction to touch on either side of the trunk, and response to vibrissae touch.[24] NDS function was graded from 3 to 18 (18 = normal function; 3 = maximal deficit). NDS and body weight were measured on the day before surgery and

then on days 7, 14, and 21 after surgery.

### *Forced Swim Test*

The forced swim test was performed for all groups on days 7, 14, and 21 after surgery to investigate depressive-like behavior. Before the experiment, animals were placed in the behavioral room for one hour to acclimatize to the environment. Each trial began with the rat being gently placed in the center of a glass cylindrical tank (60 cm height, 38 cm diameter, and 40 cm depth) containing water at 24 ± 1°C and allowed to swim freely. The total duration of the experiment was 7 minutes; the first two minutes were spent to acclimatize the animals to water, and during the next 5 minutes, the animals' movement was tracked by a camera system. Finally, horizontal and vertical movements and immobility time were measured.[25]

### *Open Field Test*

OFT was applied to evaluate anxiety-like behaviors. All animals underwent the OFT test on days 7, 14, and 21 after surgery. The rats were individually placed into the center of the open field box (the device was 80 cm × 80 cm, and the bottom of the box was divided into 16 squares). Then, they were allowed to move freely in the environment for 5 minutes, and the camera was used to record and analyze every rat's movements. At the end of the trial, the rats were immediately removed from the box, and the box was cleaned with 70% ethanol solution. The number of movements and time spent in the central and peripheral zones were counted. Moreover, the passage of the animal with its four limbs from one zone to another was considered a movement unit.[26]

### *Elevated Plus Maze*

The EPM is one of the most extensively used tests to assess anxiety-like behavior in mice. The apparatus consists of 4 arms in a cross shape with a central zone in the middle, placed approximately 40 cm above the ground, a pair of open arms (50 cm × 5 cm) perpendicular to a pair of arms with walls but no ceiling (50 cm × 10 cm × 40 cm), and a connected central area (10 cm × 10 cm). The rats were individually placed in the center of the apparatus and explored freely for 5 minutes. The camera frequently recorded the rat entering and exiting closed arms, as well as the time it spent in open arms and closed arms.[27]

### *Histological and Stereological Examination*

After behavioral analysis, the animals were anesthetized and perfused through the left cardiac ventricle with saline solution and 10% formaldehyde solution. Next, the brains were removed from the skull. For the stereological study, paraffin-embedded brains were cut into 5 m sections. The sections were then stained with hematoxylin to count the cell number.

### *Counting Cell Number*

Total neurons, non-neuron cells, and dead neurons in both amygdalae were calculated. In this method, the brain tissue was cut serially, and a slide of the amygdala was prepared from each section based on the Paxinos atlas. Then, the slides were graded in ImageJ software ; neurons were randomly counted in 5 squares (4 to the corner of one center) in each slide.

Finally, the number of counted cells per surface unit was computed using the following formula:

$$ND = \Sigma Q / \Sigma frame \times V$$

where ND refers to the number of dark cells per unit area, and ΣQ is the sum of the calculated cells. In addition, Σframe and V represent the total sampling times in a sample and the volume of the sampling frame, respectively.[28]

### *Immunohistochemistry*

Glial fibrillary acidic protein (GFAP) expression was measured by immunohistochemistry staining in both amygdalae. The primary antibody (1:1000) diluted in phosphate buffered saline (PBS) with 1% bovine serum albumin was used after preparing 5 µm sections of the tissues and passing the hydration and washing steps with PBS. After placing the tissues in 3% hydrogen peroxide for 15 minutes at room temperature, they were washed twice, and then the secondary antibody (1:400) was applied to the slide, diluted in PBS with 1% bovine serum albumin, and incubated.

After 2 hours, the tissues were washed with PBS solution and dyed with chromogen solution comprising 30 mL (1 drop) of 3, 30-diaminobenzidine tetrahydrochloride (3,3′-diaminobenzidine chromogen) and 1.5 mL (50 drops) of 3,3′-diaminobenzidine substrate. The chromogen solution was applied to the mixture on the tissue section and incubated for ten minutes. Finally, they were washed in running water for 5 minutes and stained with hematoxylin dye. The number of GFAP-positive cells in the amygdala was counted by light microscope and ImageJ software.[29]

### *Statistical Analysis*

All data were analyzed with SPSS software, version 18.0. The normality was tested with the Shapiro–Wilk test to ensure that they were normally distributed prior to analysis. The data are expressed as means ± standard error of the mean. Moreover, the comparison between two groups was analyzed using an independent sample t-test. Ultimately, the Kruskal–Wallis test was used for the non-normal data, and $P < 0.05$ was assumed as significant.

## Results

### *Neurologic Deficit Score and Body Weight Changes:*

To evaluate brain damage, the NDS test was utilized on days 7, 14, and 21. The NDS of rats in the ICH-7 and ICH-14 groups was significantly lower than that of the Sham-7 and Sham-14 groups ($P = 0.007$ and $P = 0.002$, respectively;

Figure 1A). However, NDS in the ICH-21 group was not significantly different from the Sham-21 group. There was no significant change in the weight of animals in different groups (Figure 1B).

### *Open Field Test*

OFT is used to evaluate anxiety-like behaviors in rodents. In this test, the time spent in the peripheral zone shows the increase of anxiety in animals. Based on the results (Figure 2), there was no significant difference between the groups in terms of the central time, peripheral time, and peripheral crossing. Conversely, a significant decrease was found in the central crossing in the ICH-21 group compared to the Sham-21 group ($P=0.011$).

### *Elevated Plus Maze*

The result of the EPM test indicated no significant difference between the ICH-7 group in the time spent in the open and closed arms and open and closed entries compared to the Sham-7 group (Figure 3). The time spent in the close arm was significantly higher in the ICH-14 group compared to the sham-14 group ($P=0.045$), but no significant difference was observed for other parameters (Figure 3A). Moreover, the time spent in the close arm in the ICH-21 group was significantly higher ($P=0.004$) than the same sample in the sham-21 group (Figure 3A), and the time spent in the open arm in the ICH-21 group was significantly lower ($P=0.028$) than the same sample in the sham-21 group (Figure 3B). The total number of closed entries dramatically increased ($P=0.002$), and open entries decreased ($P=0.004$) in the ICH-21 group compared to the sham-21 group (Figures 3C and 3D).

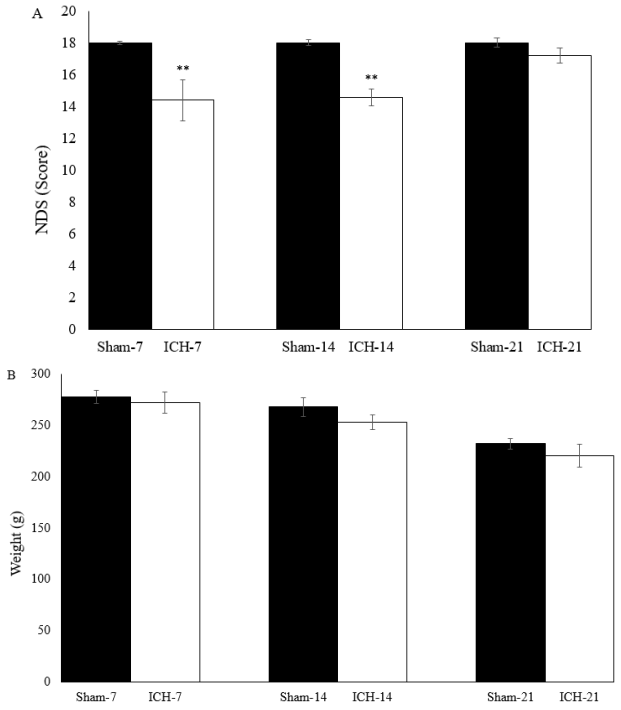


**Figure 1.** Comparison of Changes in NDS **(A)** and Body Weight **(B)** Between ICH and Sham Groups on Days 7, 14, and 21 After Surgery

*Note*. NDS: Neurologic deficit score; ICH: Intracerebral hemorrhage; SEM: Standard error of the mean. Data are shown as means ± SEM (n = 8). $^{**}P<0.01$ compared to the sham group

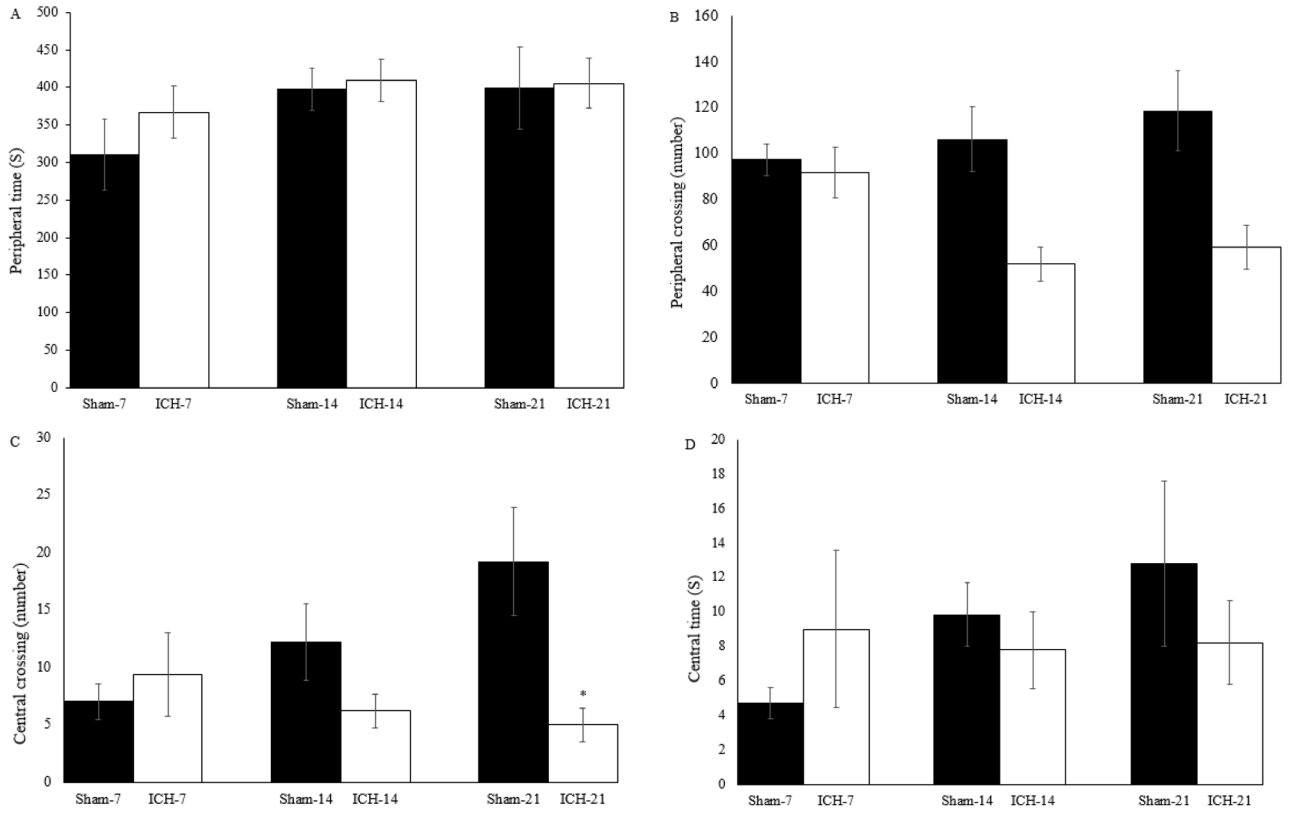


**Figure 2.** Comparison of Changes in Peripheral Time **(A)**, Peripheral Crossing **(B)**, Central Time **(C)**, and Central Crossing **(D)** Between ICH and Sham Groups on Days 7, 14, and 21 After Surgery

*Note*. SEM: Standard error of the mean; ICH: Intracerebral hemorrhage. Data are expressed as means ± SEM (n = 8). $^{*}P<0.05$ compared to the sham group

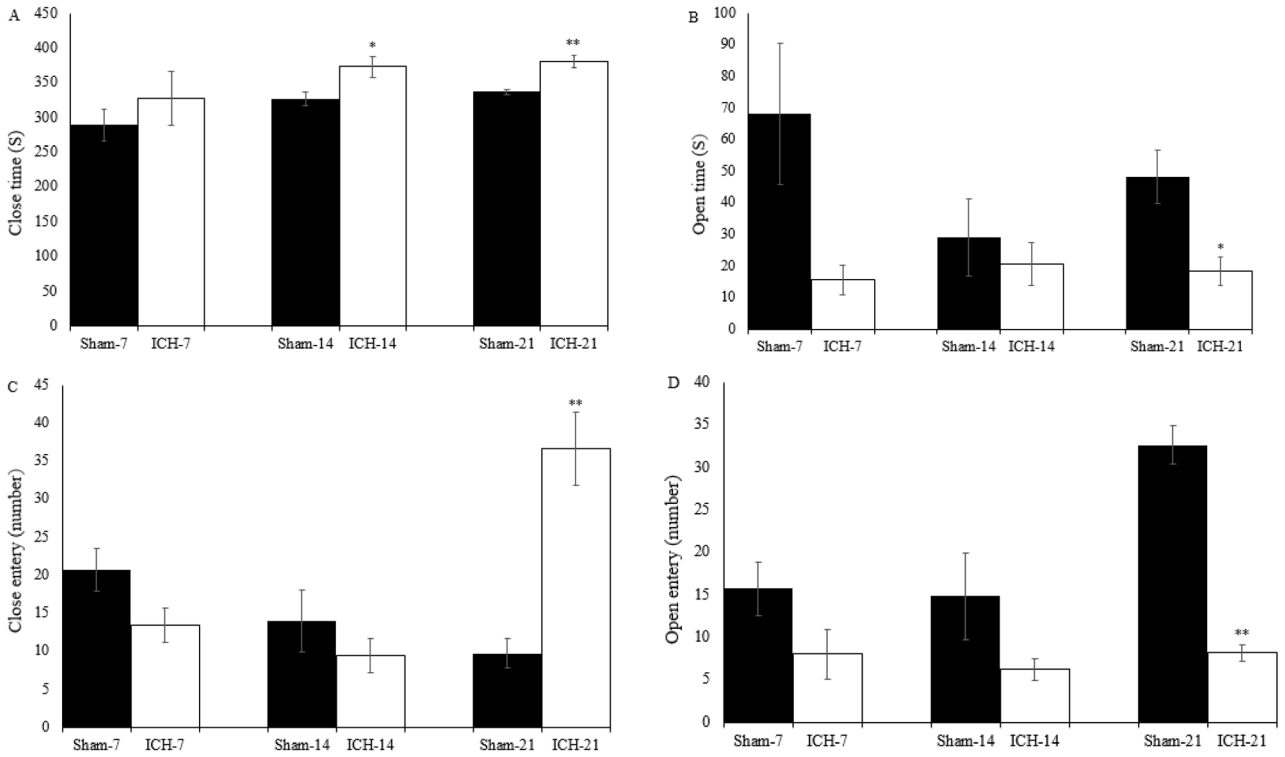


**Figure 3.** Comparison of Changes in the Time Spent in the Close Arm **(A)**, the Time Spent in the Open Arm **(B)**, Close Entry **(C)**, and Open Entry **(D)** Between ICH and Sham Groups on Days 7, 14, and 21 After Surgery
*Note*. ICH: Intracerebral hemorrhage; SEM: Standard error of the mean. Data are demonstrated as means ± SEM (n = 8). $^{*}P < 0.05$ compared to the sham group

### *Forced Swim Test*

FST was used to assess the level of depression in the animals. The results of this test indicated that immobility time and active time had no significant difference in the ICH-7 and ICH-14 groups compared to the Sham-7 and Sham-14 groups. There was a significant increase in immobility time and a significant decrease ($P = 0.047$) in active time ($P = 0.048$) in the ICH-21 in comparison to the Sham-21 group (Figure 4).

### *Estimation of Cell Number*

The number of cells in the amygdala region was measured to investigate the structural changes. No significant difference was found between the ICH and sham groups and the left and right amygdalae (Figures 5 and 6).

### *Amygdala Glial Fibrillary Acidic Protein Distribution*

The number of cells expressing the GFAP was estimated in the amygdala region. Based on the results (Figure 7), the expression level of the GFAP in the ICH-7 and ICH-21 groups in the ipsilateral amygdala was higher than the sham-7 and sham-21 groups ($P = 0.002$ and $P = 0.000$, respectively). However, in the contralateral amygdala, only the ICH-21 group showed a significant increase compared to the sham-21 group ($P = 0.000$). Figure 8 depicts the expression of the GFAP in the amygdala of both sides.

## Discussion

In the current study, the left striatal hemorrhage model and the ICH model were successfully established in rats, and anxiety and depression were assessed after 7 days, 14 days, and 21 days. The data supported depressive and anxious-like behavior after ICH, which significantly increased 21 days after the surgery. Furthermore, the results confirmed a significant increase in the number of GFAF in both amygdalae, indicating inflammatory responses on day 21 after ICH. Conversely, no change was observed in the

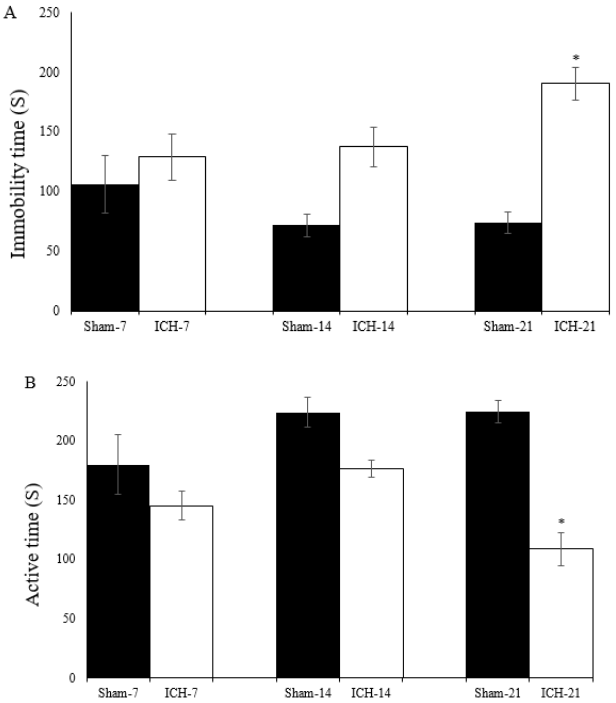


**Figure 4.** Comparison of Changes in Immobility Time **(A)** and Active Time **(B)** Between ICH and Sham Groups on Days 7, 14, and 21 After Surgery
*Note*. ICH: Intracerebral hemorrhage; SEM: Standard error of the mean. Data are shown as means ± SEM (n = 8). $^{*}P < 0.05$ compared with the sham group

number of neurons in the amygdala.

Neural pathway damage, whether inhibitory or excitatory, to different areas following stroke commonly occurs in the clinic, which can have different symptoms and treatments depending on the type of stroke and damaged areas.[30] The striatum is one of the most common areas found in people who have an ICH.[31] Based on clinical studies, individuals who suffered from ICH demonstrated different degrees of cognitive impairment due to damage to the subcortical pathways.[32] A stroke in the basal ganglia or striatum can cause damage to motor learning, so that people's motor performance remains poor even in response to post-stroke rehabilitation.[33] Likewise, some studies reported that severe striatum damage may lead to dopaminergic neuronal abnormalities and induction of depressive-like behaviors after ICH.[34,35] Additionally, in models of Parkinson's disease where the striatum is unilaterally damaged, inflammatory and neuronal changes have been detected in the contralateral striatum, representing the migration of inflammatory factors and glia to different areas of the brain.[36] In this study, a decrease in the NDS of the ICH groups compared to the sham groups was observed on days 7 and 14 after surgery, highlighting an increase in inflammation and its effects on the sensory and motor functions of the animals. However, as stated in other studies, after the 14th day, the brain goes toward recovery and the inflammation decreases; accordingly, no significant difference was found between the groups on the 21st day.[37,38]

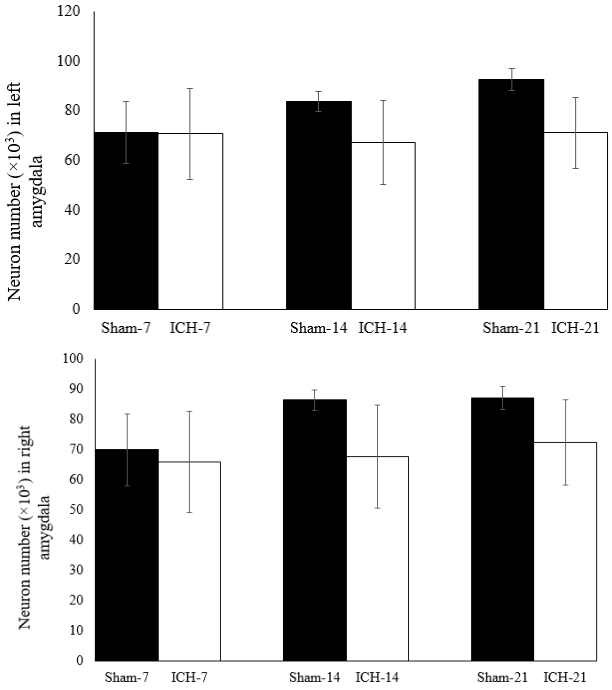


**Figure 5.** Comparison of Changes in Neuron Number in the Left **(A)** and Right **(B)** Amygdalae Between ICH and Sham Groups on Days 7, 14, and 21 After Surgery

*Note*. ICH: Intracerebral hemorrhage; SEM: Standard error of the mean. Data are displayed as means ± SEM (n = 8). $^{*}P < 0.05$ compared with the sham group

Clinical and preclinical research revealed that the amygdala plays a role in depression and anxiety reactions to stressful stimuli, and the amygdala-striatal networks support the regulation of emotional behaviors.[39-41] About one-third of survivors of stroke suffer from PSD, and patients with left-hemisphere lesions are more susceptible to PSD than those with right-hemisphere ones.[42,43] In addition, emotional disorders and depressive and anxiety behaviors are common in striatal ICH patients, especially in people with damage on the left side.[22] Similarly, in the present study, according to the result of behavioral tests, depressive-like behaviors occurred 7–21 days after ICH and were more visible on the 21st day. As mentioned, numerous clinical trials and studies have investigated PSD. However, the underlying mechanisms of its development after ICH or traumatic brain injuries are pathophysiologically unclear.

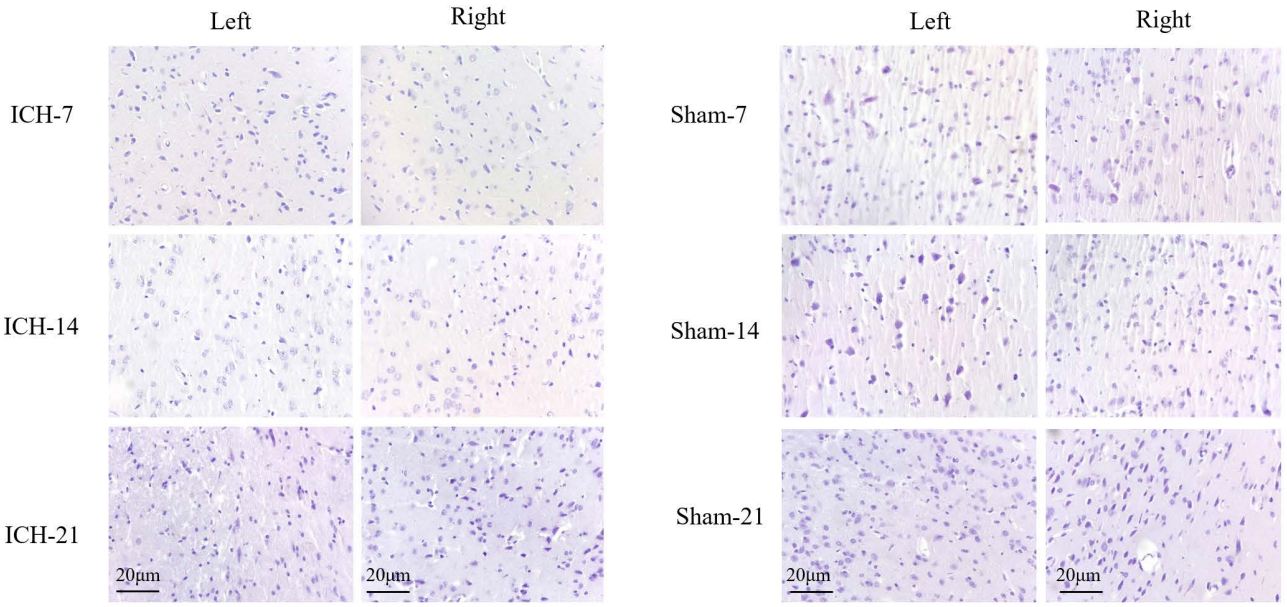


**Figure 6.** Photomicrographs Representative of Cell Number in Amygdala

*Note*. ICH: Intracerebral hemorrhage. There was no significant difference between the ICH and sham groups

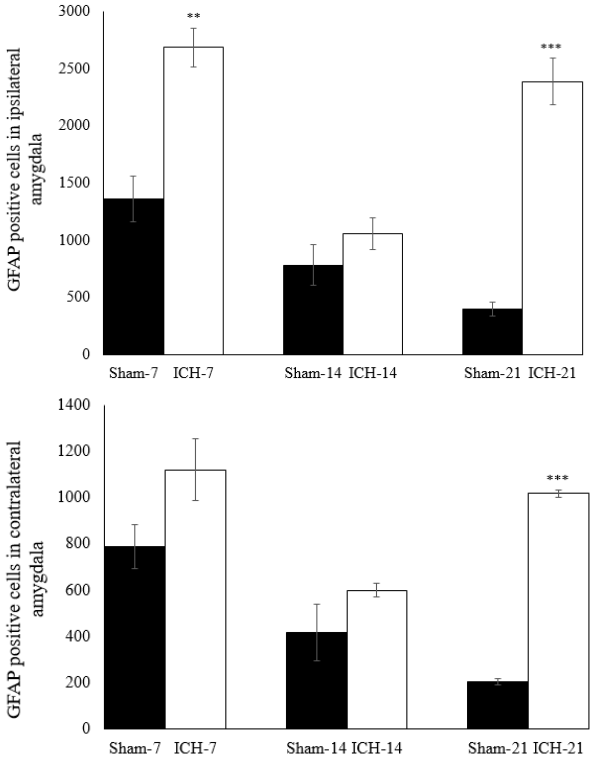


**Figure 7.** Comparison of Changes in Immobility Time **(A)** and Active Time **(B)** Between ICH and Sham Groups on Days 7, 14, and 21 After Surgery
*Note*. ICH: Intracerebral hemorrhage; SEM: Standard error of the mean. Data are shown as means ± SEM (n = 8). $^{*}P < 0.05$ compared with the sham group

Our results showed that ICH caused a significant increase in immobility time at 21 days after surgery in the FST, indicating an increase in the incidence of depressive-like behavior in the animals of this group.[44] The OFT is a valid tool for investigating anxiety-like behaviors and locomotor activities in animal models. In this experiment, animals are provided with a new environment to explore, so animals with anxiety-like behaviors tend to stay on the peripheral zone and do not search around much.[45,46] Based on the results, the amount of movement of the animals in the central zone decreased on day 21 after the surgery in the ICH group, which probably represents the beginning of anxiety-like behaviors. According to the result of the EPM test, the time spent in the dark room in the ICH group increased the most on day 21 after surgery compared to the sham group, which is consistent with the results of two behavioral tests and indicates an increase in anxiety-like behaviors in this group. Likewise, several other studies used the experimental model of ICH and reported similar results in some behavioral tests, such as the OFT and EPM.[35,47,48]

Astrocytes are responsible for maintaining homeostasis, protecting nutrition, and producing neurotransmitters. Some morphological and functional changes related to astrocyte hyperactivation (reactive astrogliosis) have been observed, which implies an increase in astrocyte

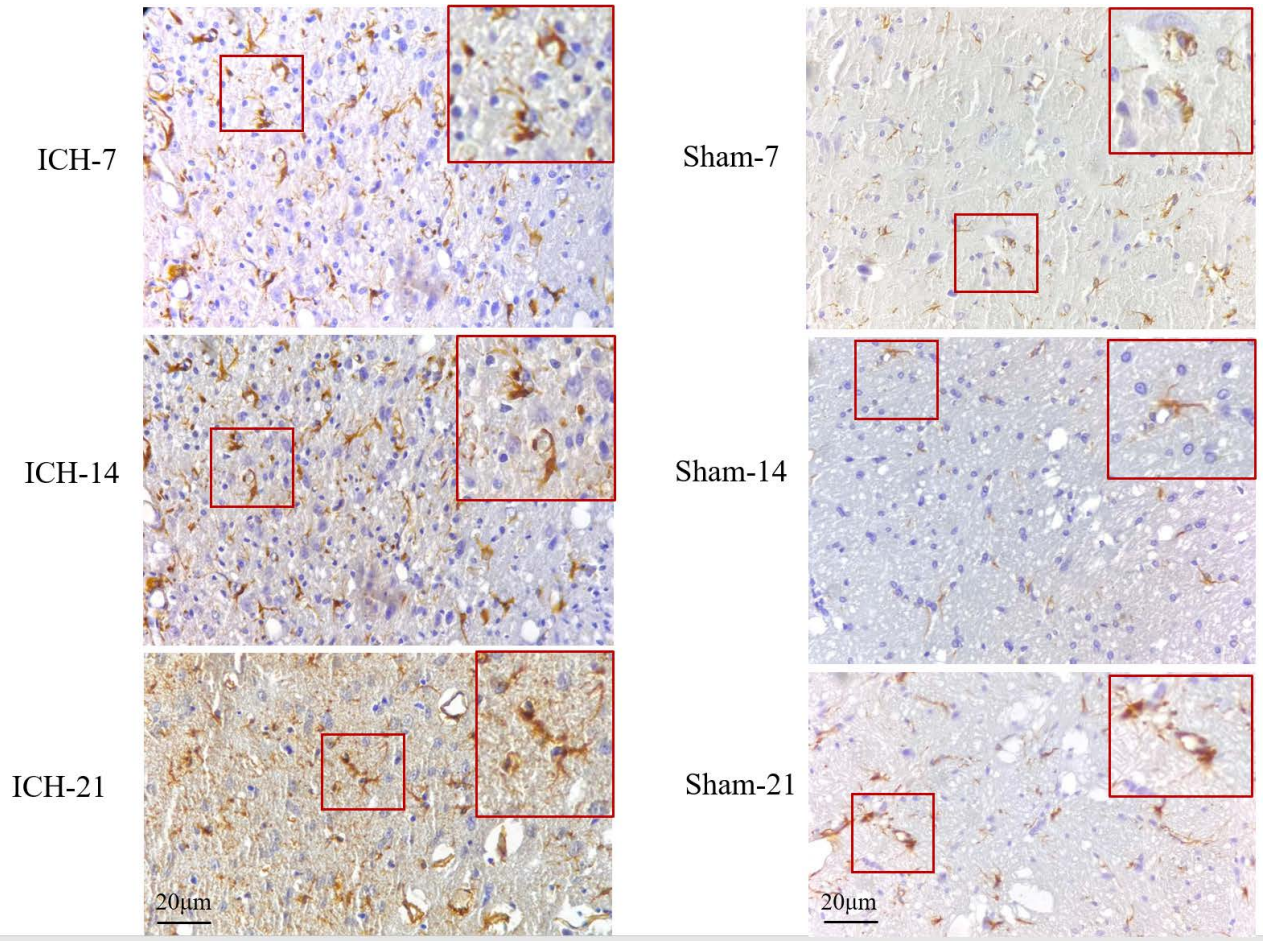


**Figure 8.** Photomicrographs Representative of GFAP Distribution in Amygdala
*Note*. ICH: Intracerebral hemorrhage; GFAP: Glial fibrillary acidic protein. GFAP distribution significantly increased in the ICH groups compared to the sham groups

proliferation in most models of central nervous system (CNS) injury or disease.[49,50] Astrocytes are a major component of the innate immune system and have a protective function against disease in its early stages. These immune cells regulate the uptake and release of interleukin-18 as a proinflammatory cytokine in the CNS.[51,52] Furthermore, GFAP is an intermediate filament protein expressed in glial cells, especially astrocytes, and detectable by immunohistochemical staining.[53] Generally, GFAP is used as a marker of astrocyte reactivity. Astrocytes increase during injury and secrete inflammatory factors that can lead to cell death.[54] In the present study, there was an increase in the level of GFAP in the ICH compared to the sham group on days 7 and 21 after surgery in the amygdala, so that only the ipsilateral amygdala was increased on the 7th day after the surgery. However, it increased in both amygdalae on the 21st day after the surgery. This change in the amount of the GFAP corresponds to the behavioral tests, as the results demonstrated that anxiety and depression-like behaviors occur on the 21st day after the surgery. This is accompanied by an increase in astrocytes and possibly an increase in inflammation on the 21st day after surgery in the amygdala.

Various studies have shown an increase in the GFAP level in different areas of the brain, especially around the hematoma area.[55,56] However, considering the lack of information about the relationship between the striatum and the amygdala in brain stroke, this study investigated the effect of striatum damage on subcortical areas. Our results confirmed that the amygdala will have inflammatory changes 21 days after the surgery, and one of these reasons could be the increase in the number of astrocytes. Nonetheless, there were no morphological changes or changes in the number of neurons in the amygdala. It is possible that more time is needed for morphological changes or plasticity changes. Accordingly, more studies are needed to thoroughly examine these relationships.

Due to the pathophysiology of ICH, the ultrastructural changes of hematoma tissues include axonal degeneration by the breakdown of axons into the injury site for their subsequent regeneration. More importantly, axons highly contribute to potential therapeutic interventions for long-term functional outcomes. It has been claimed that the improvement of axonal regeneration after CNS injury promotes functional recovery in ICH patients.[57-59] Another experimental study revealed that the volume of cortex and corpus striatum decreased in the ipsilateral part of the right hemisphere on day 22 after striatal ICH; meanwhile, the volume of hippocampus remained unchanged. This may have occurred due to continued post-ICH tissue loss.[59]

The diverse effects of depression (e.g., mood disorder, cognitive impairment, and chronic pain) must be attributable to the early or later involvement of neural systems at different time periods. The change in dopamine levels, due to dopaminergic mesolimbic neurotransmission, may lead to lack of motivation or loss of pleasure in the reward system.[60] The present model has reproducible parameters as predictors of hematoma expansion in the target region of the brain. To enhance the novelty of our model, tasks were considered to evaluate the severity of PSD. The rationale for the selection of striatal ICH as our PSD model lies behind the fact that the incidence of depression is closely related to the location of the hematoma in ICH patients.[4]

### Authors' Contribution

Data curation: Alireza Rahimi, Reyhane Vardiyan.
Formal analysis: Alireza Rahimi, Zahra Akhoond-Ali.
Funding acquisition: Zahra Akhoond-Ali, Reyhane Vardiyan.
Investigation: Mohamad Gharehbaghi.
Methodology: Azim Zaeimbashi.
Project administration: Arman Abroumand Gholami, Ali Nakhaei.
Resources: Arman Abroumand Gholami, Ali Nakhaei.
Software: Azim Zaeimbashi, Alireza Rahimi, Ali Nakhaei.
Supervision: Farzaneh Vafaee.
Validation: Hossein Akhtar, Mohamad Gharehbaghi.
Visualization: Hossein Akhtar, Mona Riyahi Rad.
Writing–original draft: Farzaneh Vafaee, Fatemeh Forouzanfar, Atiyeh Ghorbani, Shima Shirzad, Mona Riyahi Rad.
Writing–Review & Editing: Fatemeh Forouzanfar, Atiyeh Ghorbani, Shima Shirzad, Mona Riyahi Rad.

### Competing Interests

The authors declare that they have no conflict of interests.

### Ethical Approval

All procedures in this experiment were approved by the Research Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1400.403.)

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