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Effect of Adenosine Injection Following Cerebral Reperfusion Ischemia on A1AR Gene Expression and Apoptosis in Brain Hippocampal Tissue of Male Wistar Rats

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Abstract

Background: Apoptosis is a programmed cell death that occurs due to various factors such as reperfusion ischemia. As a purinergic receptor, A1AR acts as an oxidative stress sensor and an antioxidant during reperfusion ischemia.
Objectives: The purpose of the present study was to investigate the effect of adenosine injection following cerebral reperfusion ischemia on A1AR gene expression and apoptosis in the brain hippocampal tissue of male Wistar rats.
Methods: This experimental study was conducted at Shahid Mirghani Research Institute in Gorgan, Iran, from January 21, 2019, to March 18, 2019. The sample size was determined according to previous studies and a pilot study. Thus, 30 male Wistar rats were divided into three groups by simple random sampling (using a random-number table): Control group (n = 10), Reperfusion ischemia group (n = 10), and Reperfusion ischemia + adenosine group (n = 10). Ischemia was induced in animals by closing the carotid artery for 45 min. Adenosine was injected 24 h after ischemia. We measured A1AR gene expression, SOD protein expression, and apoptosis by real-time PCR, immunohistochemistry, and the TUNEL method, respectively.
Results: The results showed that cerebral reperfusion ischemia significantly increased A1AR gene expression (596%) and apoptosis (378%) and decreased SOD protein expression (72%) compared to the control group ($P < 0.001$). On the other hand, adenosine significantly reduced A1AR gene expression (46%) and apoptosis (69%) and increased SOD protein expression (94%) compared to the ischemic group ($P < 0.001$).
Conclusions: Ischemic brain reperfusion causes oxidative stress. Also, adenosine injection seems to be effective in reducing oxidative stress and apoptosis induced by cerebral reperfusion ischemia.

Keywords: Apoptosis, Oxidative Stress, Hippocampus, Brain Ischemia, Adenosine

1. Background

The cerebral ischemia (brain ischemia) is the third leading cause of death, after cancer and cardiac disease, in the world. Ischemia is often followed by reperfusion that causes other problems, such as inflammatory response, apoptosis, and nerve damage (1). Neurons are sensitive to changes in oxygen and the ischemia-induced hypoxia causes cell death (2). In the brain, the hippocampus is more susceptible to ischemia. Among hippocampal neurons, CA1 neurons show selective vulnerability to ischemic injury. Ischemia reduces the oxygen concentration of the cell and consequently decreases ATP levels. Decreased cellular energy consumption during ischemia and reperfusion results in the accumulation of Reactive Oxygen Species (ROS) and subsequently leads to cell apoptosis (3).

Apoptosis is a programmed cell death that prevents the release of organelles such as lysosomes to the extracellular matrix, resulting in secondary damage to tissues through inflammation. Most apoptotic deaths occur when there is enough energy (e.g., during reperfusion) (4). Disturbance in the cellular redox balance and anti-apoptotic defense of the cell leads to changes in the oxidation of biomolecules and cellular damage, which may ultimately lead to cell death (5).

As mentioned above, various factors, including oxidative stress and inflammation, play important roles in controlling the apoptotic process, and the apoptosis process can be controlled by factors affecting the expression of

anti-apoptotic and pre-apoptotic genes (6). Various studies have suggested the use of antioxidants to protect nerve cells against oxidative stress and eventually apoptosis (7). Adenosine acts as an antioxidant (8, 9) and plays an important role in hypoxia and ischemia but the antioxidant role of adenosine is not yet fully elucidated. Adenosine is produced under oxidative stress conditions and its effects are mediated by interacting with a variety of Adenosine Receptor (AR) subtypes, including A1, A2A, A2B, and A3AR (10). The subunit of the A1 Adenosine Receptor (A1AR) is a member of purinergic receptors and it has several actions in the central nervous system, such as cell-protection (11, 12) and anti-apoptosis (13) after ischemia and trauma.

Currently, the role of extracellular adenosine in the inflammation from hypoxia during ischemia has been indicated (14). Energy metabolism and neuronal activity are correlated with the extracellular levels of adenosine in the hippocampus (15). An increase in endogenic adenosine was reported after cerebral reperfusion ischemia and along with the increase in oxidative stress in the brain (14). Rudolphi et al. (16) showed that during in vivo ischemia, the A1AR accumulation protects the brain against damage. In opposition to the protective role of adenosine and A1AR, some evidence indicates that an increase in A1AR can develop neurodegeneration (17-19). One study reported that the increase of adenosine could cause global damage to the brain following stroke and enhancing neuronal death (18). A vitro study showed that the use of A1AR induced enhanced neuronal death in the rat hippocampus (20). Due to the antioxidant properties of adenosine and changes in oxidative stress during cerebral ischemia, changes in SOD are investigated as an antioxidant factor.

2. Objectives

Recently, researchers have paid more attention to cerebral reperfusion injury because of the complex mechanism and the social and economic consequences of this disease (21). On the other hand, due to the different effect of adenosine on cerebral ischemic-reperfusion disease, its antioxidant properties and the role of apoptosis in this injury, in the present study, we investigated the effect of adenosine injection following cerebral reperfusion ischemia on A1AR gene expression, apoptosis, and SOD protein expression as an antioxidant marker in the brain hippocampal tissue of male Wistar rats.

3. Methods

Male Wistar rats were used for this study. The present study was conducted at a private institution, Shahid Mirghani Research Institute, in Gorgan, Iran, from January 21, 2019, to March 18, 2019. Ethical approval for the study was obtained from the Committee on Animal Care at Islamic Azad University, Tehran Science and Research Branch, with code IR.IAU.SRB.1397.134 (January 2019). All variables were measured by standard and calibrated equipment.

3.1. Animals

The sample size was determined according to previous studies and a pilot study (test power = 0.95). First, a pilot study was performed. After analyzing the results of the pilot study and based on previous studies, 36 adult male Wistar rats (weight 250 to 280 g, age 10 to 12 weeks) were purchased. All rats were kept in polycarbonate cages (five rats per cage) at 22 $\pm$ 2°C, 55% humidity, and 12/12 h of light/dark cycle, without restriction in water and food. First, male Wistar rats were matched according to their weight and then divided into three groups by simple random sampling (using a random-number table): Control group (n = 10, receiving no intervention), Reperfusion ischemia group (n = 10), and reperfusion ischemia + adenosine group (n = 10; receiving adenosine 0.4 mg/kg).

3.2. Inclusion and Exclusion Criteria

According to inclusion and exclusion criteria, all rats should be healthy, of Wistar race, male, with the weight 250 to 280 g and age of 10 to 12 weeks. Furthermore, 24 h after the induction of cerebral reperfusion ischemia in the intervention groups, rats reminded in the study if they turned around themselves and were not able to walk spontaneously. Therefore, six rats were omitted during the study.

3.3. Cerebral Ischemia

Rats were first anesthetized by the intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg) provided by Alfastan Company. Rats were fixed on the surgical bed and the anterior part of the neck was clean-shaven. Then, under sterile conditions, a shear of 1 - 5 cm was given in the middle part of the neck. With the identification of the carotid sheath, the vagus nerve was removed from the pod and the common carotid arteries were occluded in two ways for 45 min by clamps. Finally, the clamps were removed and blood flow was returned. Then, all rats were kept in separate cages for 24 h.

3.4. Adenosine Administration

Adenosine (3 mg/mL) was purchased from the Pharmacy Faculty of Tehran University of Medical Sciences. Adenosine was injected into all rats 24 h after ischemia-reperfusion. Adenosine was administered with intraperitoneal injections (0.4 mg/mL/kg) for one week (22). The dose of adenosine was determined based on 50% of Lethal Dose (LD).

3.5. Tissue Preparation

Brain samples were collected one week after the induction of ischemia-reperfusion. Rats were anesthetized by ketamine (150 mg/kg i.p.) and xylazine (15 mg/kg i.p.) 48 h after the last injection of adenosine. Their brains were perfusion-fixed in formaldehyde 4% as a fixative solution. Finally, at the end of perfusion, the brain came out of the skull and we placed it in a similar fixative for three days (23).

3.6. Measurement of A1AR Gene Expression by Real-Time PCR

The expression of the *A1AR* gene in the brain hippocampal tissue was assessed by the Real-time PCR (ABI Stepone Company). First, primer design was performed and then the entire RNA was extracted from tissues and converted to cDNA. Then, cDNA was replicated using the QIAGEN RNeasy Mini Kit (50) by the PCR method and examined for expression of the mentioned gene. The *RGap* gene was used as a reference gene and the numbers from the replication chart of the target gene were normalized in each sample compared to the reference gene. In this study, the results were calculated using the Pfaffle formula and the $2^{-\Delta\Delta CT}$ method. The primers used in this study are presented in Table 1.

3.7. Apoptosis Measurement by TUNEL Method

In this study, TUNEL staining was used to measure the number of apoptotic cells. In this regard, the staining of the hippocampus was performed using the In Situ Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. After de-paraffinization, samples were placed in xylol for 10 min. Then, the slides were fluxed in 90%, 80%, and 70% alcohols, in sequence. Afterward, the slides were washed with PBS and then incubated in Proteinase K at 37°C for 20 min.

The tissue slides were then incubated for 10 min with a permeation solution and rinsed again with PBS. In the next step, a 50 μL TUNEL solution was poured onto each tissue sample and incubated for one hour at 37°C; after the final washing, the observation was done via a Zeiss LSM 5 fluorescent microscope.

For counting dead cells, five fields were selected in each group. A surface of 1 mm^2 in each group was considered for counting. In this protocol, TUNEL-Enzyme solution and TUNEL-Label solution at a ratio of 1 to 9 were used to stain positive control samples, while for negative control samples, only was TUNEL-label solution used. Apoptotic cells in this tissue were observed as bright spots indicating apoptotic cells marked during TUNEL staining. The core of the cells appeared red with Propidium Iodide (PI) 1 mg/mL as a storage solution that was diluted 500 times at the time of use; green cells of the positive TUNEL reaction after combination with the red color of the core appeared orange, being recognizable and countable compared to healthy red cells.

3.8. Measurement of SOD Protein Expression by Immunohistochemistry Method

Immunohistochemistry of CA1 was based on the EnVision antibody method using a specific antibody A1A (orb338920; Biorbyt, England). First, samples were washed with Phosphate-Buffered Saline (PBS) in four steps of 5 min apart. To retrieve the antigen on samples, 2 N hydrochloric acid (HCl) was poured over 30 min. Borate buffer was added to neutralize the acid for 5 min, and then cells were washed with PBS. Triton 0.3% was used for 30 min for the permeability of the cell membrane, followed by PBS washing. Then, a 10% goat serum was added as the additional background color for 30 min to block the secondary antibody response.

The primary diluted antibody (1 to 100) was added to the sample with PBS and after creating a wetting medium, it was placed in a refrigerator overnight at a temperature of 2 to 8°C to prevent tissue dehydration. On the next day, the tissue container was removed from the refrigerator and then washed four times with PBS for 5 min each time. Subsequently, a secondary antibody at a dilution of 1 to 150 was added to the sample and incubated at 37°C for 90 min in the dark. After that, the sample was transferred from the incubator to a dark room and after four times washing, DAPI was added, followed by PBS after 5 min. Finally, the sample was observed by an Olympus fluorescent microscope with a 400 mm lens for the confirmation of markers.

3.9. Statistical Analysis

All variables were evaluated for normality using the Shapiro-Wilk test and normal distribution of all variables was confirmed. One-way ANOVA was used for analyzing the differences in the mean values of groups. The Bonferroni's post hoc test was used to determine the points of difference in the groups. The significance level for all statistics was considered at $P < 0.05$. All analyses were performed

Table 1. Primers Used in the Study

Gene	F Primer	R Primer
RGap	AAG TTC AAC GGC ACA GTC AAGG	CAT ACT CAG CAC CAG CAT CAC C
A1AR	GCA GGT GTG GAA GTA GGT CT	GTG CTT CAT CGT GTC ACT GG

using SPSS version 22 software and figures were drawn using Microsoft Excel version 16.

4. Results

All data were presented as the mean ± SD in Table 2. The results of one-way ANOVA showed that there was a significant difference in A1AR gene expression (Figure 1) between control, ischemia, and ischemia + AD groups ($P < 0.001$). Based on the results of the Bonferroni post hoc test, there were significant differences between control and ischemia ($P < 0.001$), control and ischemia + AD ($P < 0.001$), and ischemia and ischemia + AD ($P < 0.001$) groups (Table 2).

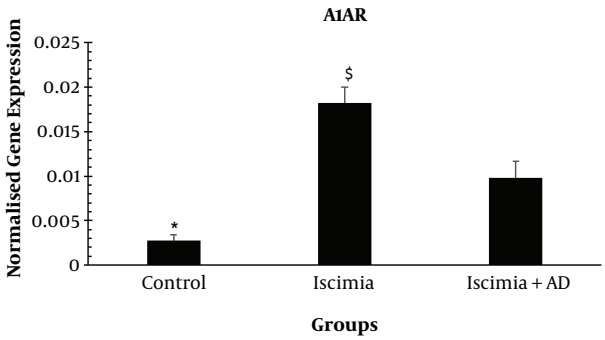

Figure 1. Means ± SD of A1AR gene expression in all groups. *Significant difference between the control group and ischemia and ischemia + AD groups. $Significant difference between ischemia and ischemia + AD groups. AD, adenosine.

The results of the TUNEL test for the evaluation of the anti-apoptotic effect of adenosine in hippocampal cells in the control condition (Figure 2A), non-adenosine, ischemic condition alone (Figure 2B), and ischemia with adenosine treatment (Figure 2C) are shown in Figure 2A-C. The percentages of apoptotic cells in the control group (13.3 ± 5.8), ischemia group (66.3 ± 4.3), and ischemia + AD group (19.3 ± 2.8) showed a significant difference ($P < 0.001$) between groups that emphases the protective effects of adenosine in hippocampal cells (Figure 3). The results of the Bonferroni post hoc test revealed that there were significant differences between control and ischemia groups ($P < 0.001$) and ischemia and ischemia + AD groups ($P < 0.001$) but control and ischemia + AD groups ($P = 0.401$) showed no significant difference (Table 2).

The results of the immunohistochemistry test for the evaluation of the reaction of SOD protein expression in hippocampal cells in control conditions (Figure 4A), non-adenosine, ischemic condition alone (Figure 4B) and ischemia with adenosine treatment (Figure 4C) are shown in Figures 4A-C. The percentages of apoptotic cells in the control group (65.6 ± 2.2), ischemia group (19.7 ± 0.9), and ischemia + AD group (32.1 ± 1.1) showed significant differences between the groups that emphases the protective effects of adenosine in hippocampal cells (Figure 5). Based on the results of the Bonferroni post hoc test, there were significant differences between control and ischemia groups ($P < 0.001$), control and ischemia + AD groups ($P < 0.001$), and ischemia and ischemia + AD groups ($P < 0.001$) (Table 2).

5. Discussion

Cerebral ischemia-reperfusion injury has ranked second among all diseases in the world. Stroke seriously affects the quality of life of the patient and imposes a lot of economic burden on the family and community. The onset of ischemic stroke results in brain tissue necrosis due to the lack of brain blood and oxygen support. Cerebral ischemia-reperfusion can promote tissue damage and impairment (24). On the other hand, a few studies have investigated the mechanisms of the antioxidant and anti-inflammatory effects of adenosine in the hippocampal tissue of the brain. Since neuronal apoptosis is one of the consequences of oxidative stress and inflammation and neuron regeneration does not occur, investigating the effects of adenosine and its receptors, especially A1, is important to reduce the damage caused by cerebral reperfusion ischemia.

The results of this study showed that cerebral reperfusion ischemia significantly increased A1AR gene expression (596%) and apoptosis (378%) and decreased SOD protein expression (72%) in hippocampal CA1 region neurons. Adenosine injection significantly decreased A1AR gene expression (46%) and apoptosis (69%) and increased SOD protein expression (94%) in comparison to the ischemic group in hippocampal CA1 region neurons. The ROS can regulate the expression of immune and inflammatory genes and play an important role in neural death (11).

Table 2. Mean ± SD of A1AR Expression, TUNEL Test Results, and SOD Protein Expression in Control, Ischemia, and Ischemia + AD groups[a]

	Control	Ischemia	Ischemia + AD
A1AR gene expression, %	0.00278 ± 0.00064	0.01821 ± 0.0018[b]	0.00981 ± 0.00189[b, c]
Anti-apoptotic effect of adenosine, %	13.33 ± 5.83	66.67 ± 4.47[b]	20.04 ± 2.78[c]
SOD protein expression, %	64.21 ± 4.27	18.12 ± 3.91[b]	36.89 ± 2.49[b, c]

[a] Values are expressed as mean ± SD.
[b] Significant difference from the control group.
[c] Significant difference from the ischemia group.

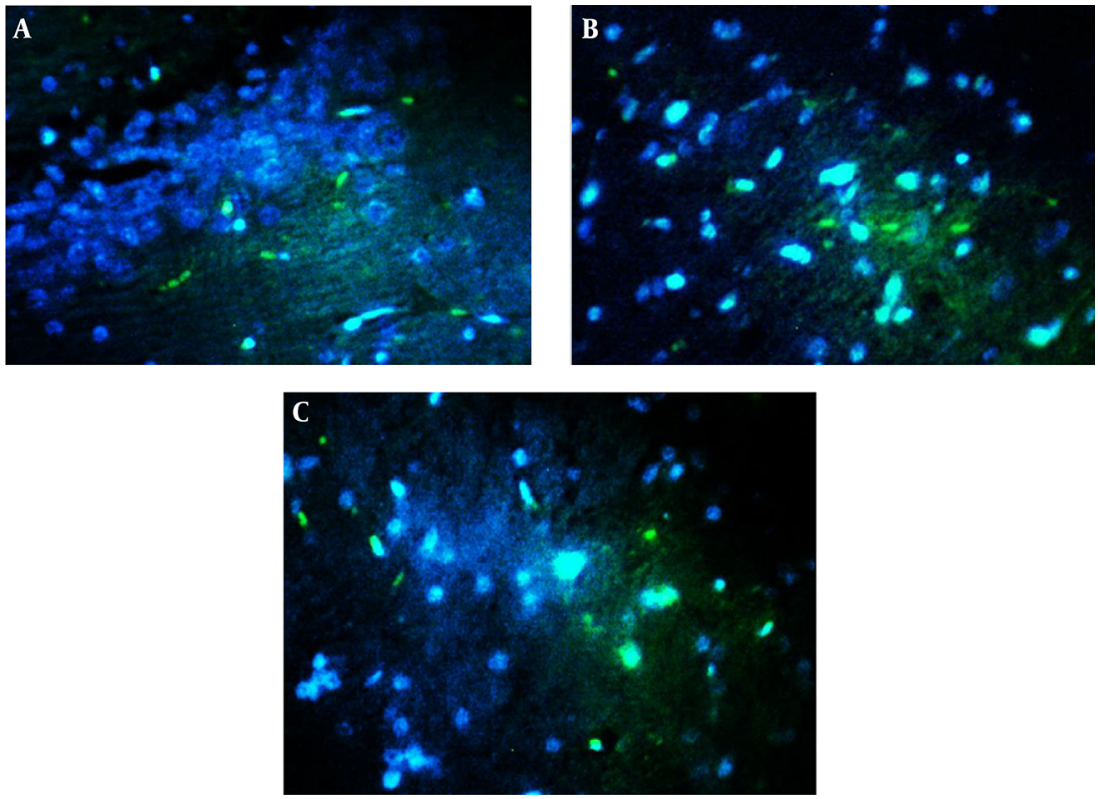

Figure 2. Photomicrographs of TUNEL staining in the hippocampus after transient global cerebral ischemia. A, control group; B, ischemia group; C, ischemia + AD Group (magnification 400×).

As previously mentioned, cerebral ischemia followed by reperfusion can induce a significant increase in ROS production (1). Since the brain is the largest oxygen user in the body, ischemia, ROS, and oxidative stress have the greatest impact on this part of the body. As a result of increased oxidative stress, an inconsistency between antioxidants and oxidative stress occurs and the process of degradation of the nervous system begins (25). The brain is protected against oxidative stress by endogenous antioxidant enzymes such as superoxide dismutase. A study showed that the activation of SOD protein could prevent the increased tissue destruction due to ischemia (26), which was in line with our results. The increased SOD protein expression in the ischemic, adenosine-injected group was associated with a decrease in the apoptosis in hippocampal CA1 region neurons.

Increasing the SOD protein activity decreases the damage induced by ischemia and reperfusion (27) and inhibits the fragmentation of DNA (28). Hence, the SOD protein plays an undeniable role in the phenomenon of ischemic tolerance. Fujimura et al. (29) stated that an increase in SOD prevents mitochondrial cytochrome C release and subsequently the fragmentation of DNA after ischemia and reperfusion and hence, reduces apoptosis.

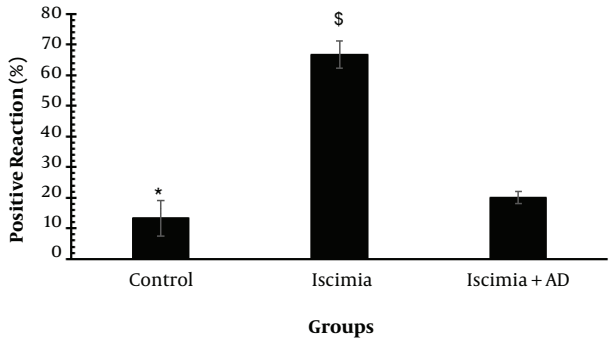

Figure 3. Means ± SD of the positive reaction of adenosine protein activity in all groups. *Significant difference between control and ischemia groups. $Significant difference between ischemia and ischemia + AD groups. AD, adenosine.

Adenosine is an endogenous metabolite with various functions ranging from the neuronal transmission to neural protection in the nervous system (30). Adenosine levels are low, but cell damage and metabolic stressors, such as ischemia and hypoxia, increase it and elicit adaptive responses by A1AR (31). The results of this study showed that A1AR gene expression and apoptosis were significantly higher in the ischemic group than in the control group, but the injection of adenosine significantly decreased them. Due to the antioxidant, anti-inflammatory, and anti-apoptotic roles of adenosine, Saransaari and Oja (32) showed that the stimulation of adenosine release provided nerve protection effects against excitotoxicity under conditions of cellular damage. Early studies showed that A1AR was significantly regulated by oxidative stress. It is known that A1AR acts as an oxidative stress sensor. For example, A1AR mRNA and its protein increased after the induction of cerebral ischemia in rats (33). Hu et al. (34) showed that A1AR could induce toleration to cerebral reperfusion ischemic injury, which was associated with the reduction of oxidative stress, inflammation, and maintenance of endogenous antioxidants. Also, nitric oxide increases following cerebral reperfusion ischemia, which can increase the expression of A1AR in neurons through NF$\kappa\beta$ (23); it can be another reason for the A1AR increase in the ischemia group.

Moreover, adenosine performs a cytoprotective role by increasing the activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase through the activity of protein kinase C-mediated phosphorylation (35). In the present study, SOD protein expression also increased with adenosine injection. Probably, this mechanism attenuated ROS and neuronal cell death, which is consistent with our results. Park et al. (36) also argued that the activity of A1 receptors in the kidney protected this tissue against cell death, apoptosis, and inflammation induced by ischemic reperfusion injury.

Generally, the injection of adenosine following the cerebral reperfusion ischemia decreased the apoptosis through antioxidant (increase of SOD and decrease of ROS) and anti-inflammatory (probably, reduction of NF$\kappa\beta$) properties of adenosine. Adenosine injection prevents the increase of ROS or oxidative stress after cerebral reperfusion ischemia.

Based on the obtained results of A1AR and SOD protein expression in the present study, further research is needed to better understand the mechanism of the effect of adenosine on A1AR and SOD. Also, it was better to evaluate the effect of adenosine on the studied variables at different times of ischemia and adenosine injection before ischemia induction.

5.1. Conclusions

The findings of this study showed that ischemia caused a significant increase in A1AR gene expression and apoptosis and a decrease in SOD protein expression. The group of ischemia with adenosine injection showed a significant decrease in A1AR gene expression and apoptosis and a decrease in SOD protein expression. Thus, adenosine injection may be an effective method of decreasing ischemic lesions.

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Footnotes

Authors' Contribution: Atossa Jozaei was involved in the study concept, design, analysis, interpretation of data, and drafting of the manuscript. Monireh Movahed, Maryam Khosravi, and Fereshteh Golab supervised the conduct of the study.

Conflict of Interests: The authors declare that they have no conflicts of interest.

Ethical Approval: The study protocol was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran (IR.IAU.SRB.REC.1397, 134 code- January 2019).

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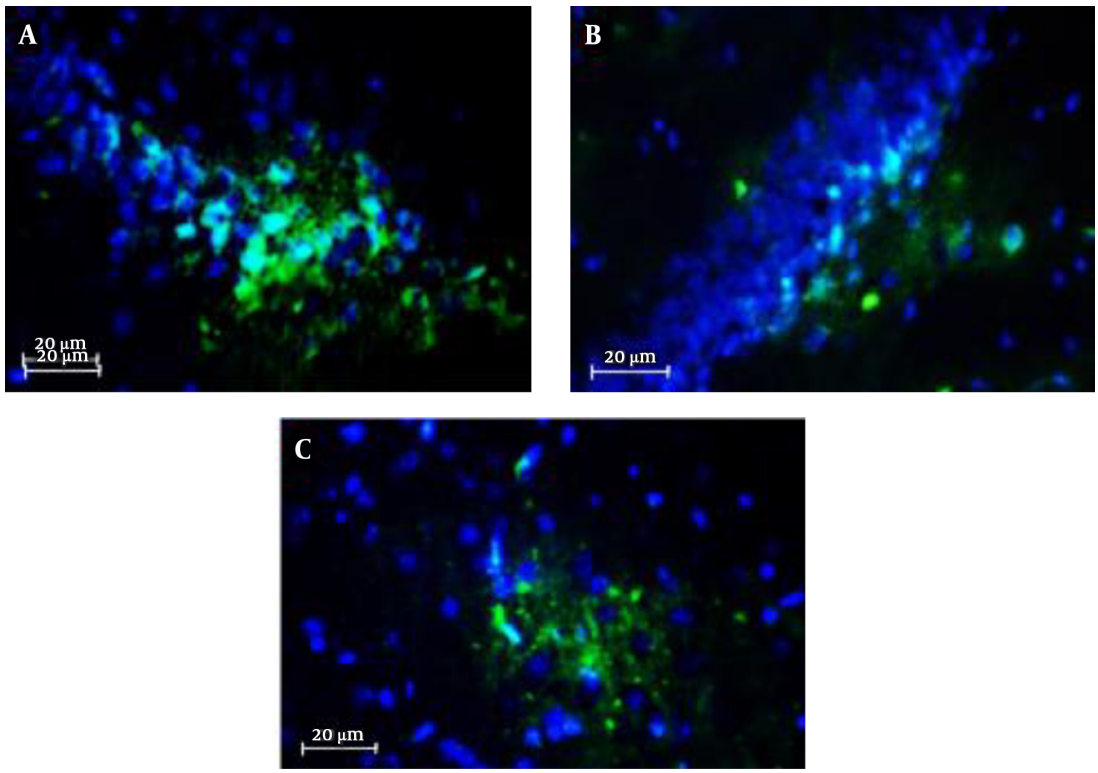

Figure 4. Photomicrographs of SOD protein expression in the hippocampus after transient global cerebral ischemia. A, Control group: 65% positive reaction; B, ischemia group: 15% positive reaction; C, Ischimia + AD group: 30% positive reaction (magnification 400 ×).

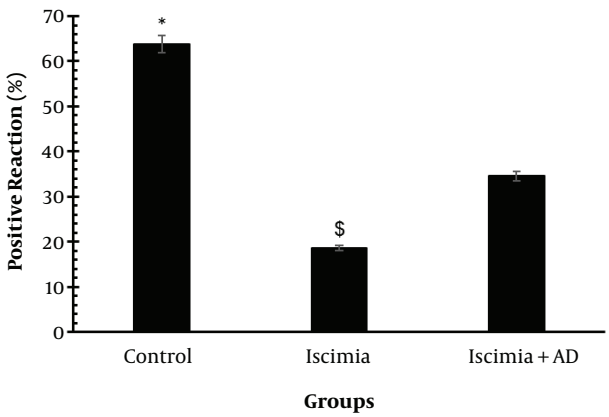

Figure 5. Means ± SD of the positive reaction of SOD protein expression in all groups. *Significant difference between control and ischemia groups. $Significant difference between ischemia and ischemia + AD groups. AD, adenosine.

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