Exercise Before and After Cerebral Ischemia-Reperfusion Inhibits Inflammation and Reduces Apoptosis in the Hippocampus in Rats

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Abstract

Background: Cerebral ischemic/reperfusion (I/R) causes severe damage. The purpose of this study was to evaluate the effect of exercise before or/and after I/R on inflammation and apoptosis in the CA1 region of hippocampus in rats.

Methods: Male Wistar rats were assigned to six groups (sham, exercise, ischemia, exercise+ischemia, ischemia+exercise, exercise+ischemia+exercise). Rats in the exercise groups received progressive treadmill training for 8 weeks, 5 days a week prior or/and after ischemic stroke. Ischemia was induced by occlusion of both common carotid arteries for 45 min. Cell apoptosis were detected by TUNEL staining. Caspase-3, NF-kB and TNF-α were determined by immunohistochemistry, and motor function was evaluated by ladder test.

Results: The cerebral ischemia caused neuronal loss through apoptosis in the hippocampus and reduced sensorimotor functions in lesion animals. The results of TUNEL and immunohistochemistry methods showed that rats in exercise+ischemia+exercise group had more significant decrease in the number of apoptotic cells and inhibited the expression of caspase-3, NF-κB and TNF-α compared to other groups ($p < .05$). Also, the results of Ladder test at the end of the eighth week showed significant improvements compared to 2 and 24 hours ($p < .05$).

Conclusion: This study showed that performing treadmill exercise, whether as a preconditioning or following cerebral ischemic event, could promote neuronal and motor recovery. However, such a protocol imposes more significant effect if conducted both pre and early after ischemia compared to each one alone.

Introduction

Stroke is the most important consequence of cerebral ischemia which occurs when a major cerebral artery or its branches are blocked, leading to lack of oxygen and energy to brain tissue and secondary tissue injury [1]. About 80% of strokes result from vascular obstruction leading to physical disabilities [2]. Exercise training promotes angiogenesis, neurogenesis, neuroprotection, and accelerates recovery of central nervous system dysfunction [3]. Both animal and clinical studies have shown that aerobic exercise reduces brain lesion volume and improves multiple aspects of cognition and motor function after stroke [4]. However, a few reports contradict the functional benefits of exercise in stroke [3]. For instance, in cerebral ischemia-induced damage, physical activity has been shown to increase the expression of proinflammatory cytokines and cortical tissue injury [5].

The role of exercise in conditioning as well as maintenance, promotion and regaining physical function before/after the diseases such as ischemic stroke has been extensively studied. In this case, some of these studies applied exercise protocols for the aim of preconditioning as a means of neuroprotection [6–9]. and some others as a recovery following physical disability [3, 5]،[10]،[11]. However, positive effects of exercise is not well-understood. Preconditioning exercise, defined as performing systemic exercise before ischemic

event, has the potential to induce ischemic tolerance against secondary cerebral damage following ischemic-reperfusion and may possibly provide neuroprotection [12]. Early exercise right after stroke is highly recommended by clinical guidelines in order to accelerate recovery following physical disability [13], [14],[15]. Yet, in the literature, there is lack of studies investigating the role of exercise both pre and post ischemic stroke, in comparison with preconditioning or recovery protocol alone.

Evidence demonstrates that inflammation plays a key role in the pathogenesis, but many reports indicate that inflammatory cells are involved in a multiphasic role (beneficial and harmful) where inhibiting the same pathway at the wrong time could exaggerate the pathogenesis [16]. The inflammation response emerged during ischemia occurs through cascade, the Nuclear factor-kappa B (NF-κB) signaling pathway and the cytokine molecules are essential for the occurrence of this process [17]. NF-κB is an essential transcription factor widely expressed in the central nervous system (CNS) and is reported to be involved in both inflammation and expression of apoptosis-related genes; it begins transcription of cytokines such as TNF-α and influences molecular pathogenesis of ischemic damage [18].

Tumor necrosis factor-alpha (TNF-α) is an inflammatory mediator after ischemic damage in the cerebral region which triggers apoptosis signaling pathway reaction mediated by the caspase-3 [19]. Its molecular activities have significant effect on cerebral response to stroke. It is able to provide either trophic or toxic effects on neuronal tissues, depending on its concentration [20]. TNF-α inflammatory cytokine would decrease pro- and increase anti-apoptotic protein expression [21]. This cytokine would encourage reduction of MMP9 levels (shown to be increased in the ischemic core 1-2 hours following cerebral ischemia [22] helping integration of blood-brain barrier [9].

Therefore, the aim of this study was to examine the effects of exercise prior or/and after cerebral ischemia-reperfusion on NF-κB, TNF-α, caspase-3, and apoptosis in the hippocampus of male Wistar rats. We hypothesized that exercise training pre/post-ischemic stroke compared to preconditioning or recovery protocols alone, has more prominent effect on reducing inflammatory cytokines and apoptosis as well as improving behavior and histopathological outcome in cerebral artery occluded rats.

Materials And Methods (Experimental Procedures)

Animals

Adult male Wistar rats (weighing 200–300 g, n = 48) were purchased from Shahid Mirghani Research Institute of Iran and food and water were freely available in standard cages and in controlled environments (temperature of 23±1°C under a dark/light cycle 12:12 h). Rats were randomly divided into six groups: sham (n=8), ischemia (ISC, n=8), exercise (Exe, n=8), ischemia+exercise (ISC+Exe, n=8), exercise+ischemia (Exe+ISC, n=8), and exercise+ischemia+exercise (Exe+ISC+Exe, n=8). This study was approved by the Ethics Committee of University of Mazanderan, Mazanderan, Iran (IR.UMZ.REC.1400.003) and all experiments were performed in accordance with the Declaration of Helsinki.

Induction of Transient Brain Ischemia

To induce transient brain ischemia [23], rats were anesthetized with intraperitoneal injection of ketamine/xylazine (40 mg/kg) and were fixed in supine position and middle incision was made in the neck. Both common carotid arteries were exposed, then the vagus nerves were separated. Both common carotid arteries were occluded for 45 minutes using surgical clamps followed by recirculation through removing the clamps. Restoration of blood flow in the carotid arteries was confirmed by observation. Their rectal temperature was monitored to remain at about 37°C during the surgery by using a heating pad. Animals were returned to their home cage after the surgery with free access to food and water.

Tissue preparation

Brains were excised and fixed in 4% paraformaldehyde in 0.1M phosphate buffer (7.4pH) for 3 days and embedded in paraffin. According to coordinates by Paxinos and Franklin, paraffin-embedded coronal sections were cut (7 μm thick) by a microtome for histological staining.

Exercise protocol

A motor-driven treadmill was used for exercise training. All animals performed treadmill exercise at speed of 15 m/min, for 15 min per day for 3 days prior to experiment (accommodation period). In order to avoid possible stress effects, electric shock was not administered.

During the experiment, animals in the exercise groups received progressive treadmill training for 8 weeks, 5 days a week prior or/and after ischemic stroke. The intensity and duration were 18 m/min without inclination for 20 min/day at first week. Then, speed, duration and inclination were gradually increased up to 30 m/min, 50 min, and 10% at 8th week (Table 1).

Table 1
Progressive treadmill exercise protocol

	Adaptive	Week1	Week2	Week3	Week4	Week5	Week6	Week7	Week8
inclination (degree)	0	0	0	0	5	5	5	10	10
duration (min)	15	20	20	25	30	30	40	40	50
speed (m/min)	15	18	20	20	20	25	25	30	30
frequency	3	5	5	5	5	5	5	5	5

Assessments of sensorimotor function

Horizontal ladder test. The ladder rung walking test was performed using an apparatus comprising sidewalls constructed of transparent panels and metal rungs [24]. Rats walked across a ladder that was irregularly shaped with varying distances between rungs from 1 to 3 cm at each crossing. Rats were trained for postoperative days 2, 24 h, and 8 weeks. At the end of the test, we counted the numbers of steps and

correct placements (the midportion of the palm of the forelimb was placed on the rung). The rates and average values of correct for these steps were calculated and indicate the average of three trials [11].

Immunohistochemical examination

Immunohistochemistry of coronal sections was based on the EnVision antibody method using a specific antibody Caspase-3, anti–NF-κB (1:100 dilution; Santa Cruz Biotechnology) and TNF-α (orb338920; Biorbyt, England). Briefly, the samples were washed in three stages with phosphate buffered saline (PBS). The secondary antibody was added to the sample at a dilution of 1:150, and then the samples were incubated at 37°C for 1 h and 30 min. The sample was then transferred from the incubator to the dark room and after 4 times washing, DAPI was added followed by PBS after 5 min [25]. Eventually the samples were viewed by a Labomed tcs400 fluorescent microscope, photographs were taken from each slide and analyzed using ImageJ software (version 1.49, NIH).

DNA fragmentation by TUNEL staining

For quantification of apoptosis-related DNA fragmentation was detected by TUNEL using In Situ Cell Death Detection Kit (REF:300155400,50 Tests, LOT:384830, GmbH&Co.KG). In brief, after deparaffinization, rehydration, permeabilization and blocking, tissue sections were treated with 50 ml of TUNEL reaction mixture that was prepared on ice with 10% enzyme solution and 90% label solution. They were incubated at 37 ° C for 10 minutes and washed twice with PBS wash buffer. In the next step, 50 µl of TUNEL Kit dye solution was poured on each tissue sample. The nuclei of the cells were stained with 4',6-diamidino-2-phenylindole (DAPI), and washed twice with a buffer. The number of TUNEL positive apoptotic cells were counted in randomly selected microscopic fields under a fluorescence microscope (x400 magnification) (LABOMED. TCM 400).

Statistical analysis

The collected data were analyzed using SPSS software version 22 (SPSS Inc., Chicago, IL). One-way Analysis of Variance (ANOVA) and Tukey post hoc test was used to examine between-group differences. The significance level was determined to be P<.05.

Results

Exercise Reduced Apoptotic Cells after Ischemia

We detected the distribution and the number of apoptotic cells in the hippocampus following ischemia/reperfusion using TUNEL staining. Quantitative analysis showed that hippocampus ischemia markedly induced cell apoptosis. Exercise significantly reduced cell death in the hippocampus (Fig. 1). As shown in TUNEL assays, apoptotic cell death was significantly increased in ISC group (P<.001). Following exercise post-ischemia, cell death was significantly reduced compared to non-exercise stroke group (P<.001).

Exercise prior to ischemia induction as a preconditioning had neuroprotective effect (P<.001). In other words, exercise before ischemia could prevent apoptosis progression. Moreover, combined pre and post-ischemia exercise encouraged apoptosis reduction as well (P<.001). Interestingly, this protocol demonstrated better results compared to pre-ischemia and post-ischemia exercise groups. In this case, mean apoptosis value in this group differed significantly with preconditioning and recovery alone groups (P<.05 and P<.01, respectively).

These findings indicate that the hippocampus ischemia caused cell death in the CA1 region. Comparison of mean cell death in hippocampus in six groups is presented (Fig. 2).

Levels of Inflammatory Proteins

Nuclear factor-kappa B level

The results showed a significant difference in NF-κB levels between experimental groups (P<.001). Ischemia induction increased this protein in the hippocampus (P<.001). Pre-ischemia exercise had protective effect and reduced the levels of this inflammatory protein (P<.001). Besides, post-ischemia exercise as a recovery tool, hindered this protein to increase (P<.01). No significant differences were observed between these two groups, whereas combination group showed less inflammation not only compared to ischemia, but interestingly to preconditioning (P<.01) and recovery (P<.01) groups. In other words, exercise protocol pre and post-ischemia provided better protection for CA1 cells against ischemia-induced inflammation. Specifically, significant difference was observed in NF-κB levels between Exe and ISC groups (P<0.05). In addition, results showed significantly higher NF-κB level in the ISC compared to the sham and Exe (P<.001). Exercise training significantly decreased NF-κB level in the CA1 in comparison to the ISC (P< .01) (Figure 3). Comparison of mean NF-κB in CA1 in experimental groups is presented (Fig. 4).

Tumor necrosis factor-alpha level

One-way ANOVA revealed significant difference between groups in the TNF-α level (P<.0001). In addition, results showed significantly higher TNF-α level in the ISC compared to the sham (P<.001). The findings indicate reduction effect of exercise pre (P<.001) and post (P<.001) ischemia on TNF-α levels. Besides, combination group showed more reduction effects compared to pre (P<.01) or post (P<.001) ischemia groups. These findings highlights positive influence of combination protocol on the inflammation induced by hippocampus ischemia (Fig. 5). Comparison of mean TNF-α in CA1 in different groups is presented (Fig. 6).

Proapoptotic Protein Expression

Caspase-3 expression in the cortex

Photomicrographs of caspase-3 cells in the cortex are presented in Fig. 7. The ischemia caused inflammation leading to increase in caspase-3 expression (P<.001), while exercise after ischemia suppressed this inflammation-induced caspase-3 expression in the hippocampus of rats (P<.001). Exercise preconditioning exerted similar inhibitory effects on the caspase-3 expression (P□.001, Fig. 7). Moreover,

treadmill exercise for eight weeks prior to and eight weeks following ischemia remarkably affected the reduction of this protein in CA1 neurons (P<.001). In addition, compared to Exe+ISC (P<.001) and ISC+Exe (P<.001), combination group had more significant reduction effect on caspase-3. Furthermore, comparison of mean Caspase 3 in hippocampus in experimental groups is presented (Fig. 8).

Exercise promotes the recovery of locomotor function post-ischemia

The neurological defect of the involved area is one of the major symptoms induced by ischemia. Our results showed that ischemia induction led to significant decrease in ladder rung walking test among experimental groups. The effects of exercise on motor function have been evaluated through ladder rung walking test at 2 h, 24 h and 8 weeks after ischemia. Locomotor function revealed significant difference between groups following induction of cerebral ischemic damage after ischemia (P< .001). In the exercise groups, the number of errors has been decreased significantly after 8 weeks post-ischemia compared to ISC (Fig. 9). Exercise groups showed no significant difference after ischemia (Fig. 9). In other words, exercise program as preconditioning, recovery or in combination had significant effects on ladder rung walking test of subjects at the end of eight weeks and no difference has been observed between two groups.

Discussion

We hypothesized that exercise before and/or after ischemia can improve hippocampus inflammation, cell apoptosis, and motor function in rats. For this purpose, we analyzed the expression of NF-κB, TNF-α, caspas3 in the CA1, and the ladder rung walking test to assess sensorimotor function. The present findings revealed that treadmill exercise pre and/or post ischemia, has significant effect on inflammation response, neural cell death and motor function recovery. Therefore, exercise before and after ischemic stroke leads to decrease in inflammatory proteins and reduces cell apoptosis. The most remarkable finding of the present study is that eight-week pre and eight-week post-exercise in combination provides better results compared to each protocols alone.

The molecular underpinnings of inflammatory damage following cerebral ischemia have been well established. Secondary brain injury is shown to be caused predominantly by brain inflammation during reperfusion through intensifying both the accumulation of inflammatory cytokines and microvascular dysfunction throughout involved areas of the brain tissue [26]. Ischemia results in a change in transcriptional activity of many inflammatory components through NF-κB that increases the expression of a large number of genes, including those associated with inflammatory responses [27],[28],[17]. The TNF-α factor is a particularly important component in the inflammatory cascades that play a pivotal role in neurodegeneration and generally associated with initiation of apoptosis [29],[30]. This is a major inflammatory cytokine, molecular activities of which deeply affects brain response to stroke. TNF-α has toxic and trophic effects on neuronal tissues which is concentration-dependent. It is suggested that chronic increase of TNF-α in low levels, observed in preconditioning exercises, may improve tolerance to this cytokine [31] [32]. This gradual increase in TNF-α expression, triggered by preconditioning exercise, is

correlated with brain damage following ischemia in rats [33]. In addition, TNF-α receptors are shown to be down-regulated through preconditioning exercise [34]. As a result, chronic stimulation by low levels of TNF-α may cause desensitization of TNF-α receptors.

Consistent with these studies are findings that shows exercise down-regulates the overexpression of NF-κB following cerebral ischemia in rats [35] and therefore inhibits TNF-α. For example, rehabilitation achieved through exercise promotes neuroprotection against cerebral ischemia–reperfusion injury via down-regulation of the expression of pro-inflammatory mediators [36]. In hippocampus the inflammatory cytokines were found down-regulated on day 14 but again interestingly elevated on day 21, which may be linked to repair mechanisms following ischemia [37]. Cytokines including TNF-α was found to be at their maximum level on day 7, 14 and remained elevated till day 21 following ischemia [38]. While opposite changes in the BDNF levels were observed as BDNF expression was elevated on day 14 and down-regulated on day 21, the down-regulation of BDNF plays a major role in the development of inflammation [39]. It is reported that expression and trophic levels in the blood and brain after a variety of physical activities in human and animal subjects is increased [40]. Trophic factors are found in large quantities in the hippocampus. So, increased levels of BDNF might have led to a decreased in the levels of inflammatory cytokines in the hippocampal region of the rat brain [41].

Based on the results of the present study, it appears that pre and/or post-stroke exercise may adjust the inflammatory injury associated with ischemia by decreasing levels of TNF-α and NF-kB that may reduce apoptosis. The results of our study confirms that intervention containing eight-week exercise before and after ischemic stroke can dramatically decrease inflammatory proteins and cell apoptosis in subjects compared to preconditioning and recovery exercise alone. This non-pharmaceutical and non-invasive strategy, thus, may be the most helpful way to prevent possible consequences of ischemia and has the potential to provide better recovery following ischemic conditions.

This is suggested that increase in NF-kB and TNF-α levels causes endothelial up-regulation and facilitates adhesion of leukocytes to the microvascular endothelium, congestion of the microcirculation, and infiltration of leukocytes into the parenchyma, all of which are likely to exacerbate ischemic injury [5]. During the process of neuroinflammation, apoptosis-regulatory proteins are repeatedly implicated in the susceptibility of neuron. Markers of apoptotic cell death include caspase-3, a terminal protein in the apoptotic cascade inducing irreversible fragmentation of DNA [42]. In the present study, exercise suppressed brain inflammation-induced TUNEL-positive cells and caspase-3 expression in the cortex. From these results, it can be suggested that exercise has ameliorating effect on brain inflammation-induced apoptotic neuronal cell death in CA1.

In this regard, there is no difference between exercise pre and post-ischemia, although combination of these exercise programs has significant effects.

Apoptosis appears to play an important role in neuronal cell death induced by brain inflammation [43]. TNF induces phosphorylation on apoptotic genes such as caspase-3, triggering cellular deterioration [44]. TNF-induced increase of TUNEL staining and caspase-3 expression caused degenerative change in the

hippocampus [45]. Exercise training may improve cerebral blood flow by enhancement of endothelium-dependent laminar shear stress, diminishing cerebral microvascular endothelial cell apoptosis in mice, which may be one of the possible protective mechanisms of exercise training (within 24 h post-stroke) [46].

The results regarding motor functional recovery tests (horizontal ladder) showed that 2 h after the stroke there was no significant difference between ischemic groups but the difference was significant after 24 h and 8 weeks. Eight-week exercise, either as preconditioning or as recovery, demonstrated optimal results. However, combination of exercise before and after ischemic stroke provides effects that are more significant. One of the potential mechanisms may be the duration of exercise program that was sixteen weeks compared to eight weeks for other groups. In the present study, we used progressive and a more vigorous level of exercise training (longer duration, increased speed, and greater incline).

Conclusion And Limitations

Our study revealed that performing treadmill exercise, whether as a preconditioning or following brain ischemic event, could promote neuronal and motor recovery. However, such a protocol imposes more significant effect if conducted both pre and early after ischemia compared to each one alone. In addition, overall trend of inflammatory cytokines and cell apoptosis indicated an increase following ischemic stroke, while exercise training could prevent this increase. As a result, this study suggests that decreasing the levels of these cytokines and cell apoptosis through exercise training can beneficially protect neurons against ischemia-induced damages and improve their recovery.

The present study has some limitations. First, high rate of fatality along the experiment caused reduction of sample size in each group. Second, our subjects were only young male rats (8 weeks). And finally, we did not measure the infarct volume.

Declarations

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Behzad Dehqanizadeh, Ziya Fallah Mohammadi and Mohammad Fallah Mohammadi. The first draft of the manuscript was written by Behzad Dehghanizadeh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available on reasonable request.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

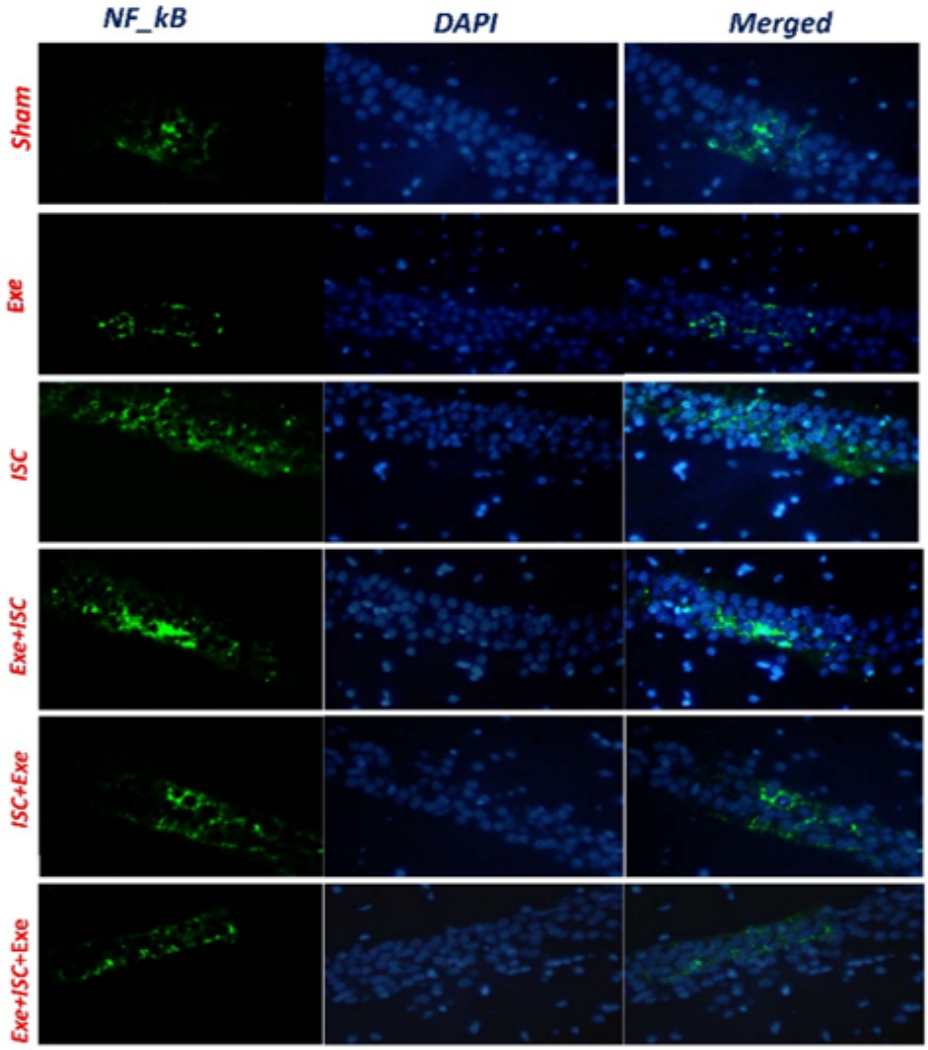

Figure 1

Evaluating and counting cells by a fluorescence microscope in the sham, exercise, ischemia, ischemia + exercise, exercise+ischemia, and exercise+ischemia+exercise groups. The first column images are related to the connecting of the primary antibody to the NF-κB protein. The second-column images are related to staining the all-cell nuclei by DAPI. And the third-column images are related to merging the first and the second-row images. Reactivity and expression of cells were evaluated and counted by fluorescence microscope with a magnification ×400.

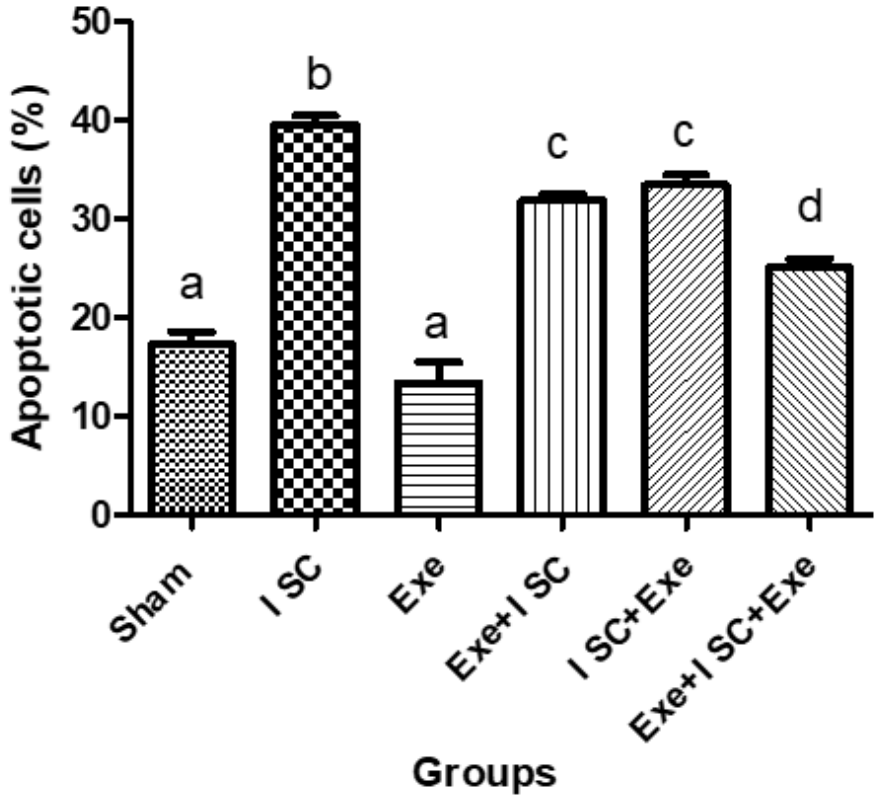

Figure 2

Comparison of mean cell death in CA1 among experimental groups.

a: Significant difference between sham and Exe with other groups (P < .05).

b: Significant difference between ISC with other groups (P < .05).

c: Significant difference between Exe+ISC and ISC+Exe with other groups (P < .05).

d: Significant difference between Exe+ISC+Exe with other groups (P < .05).

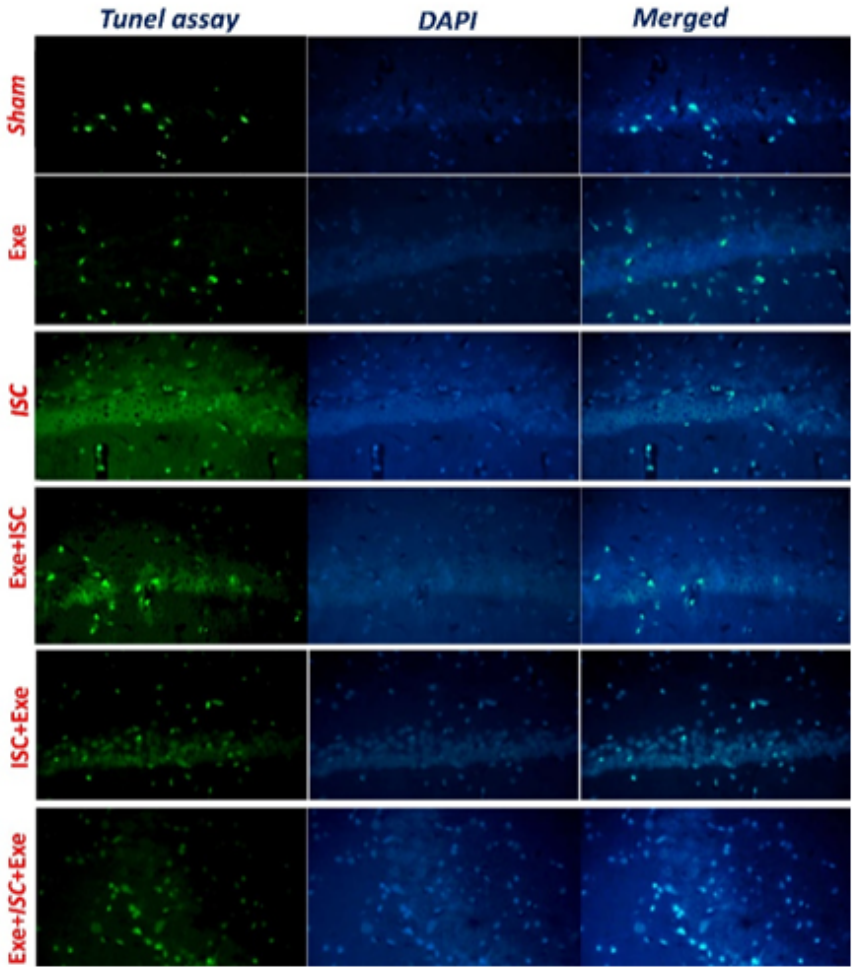

Figure 3

Evaluating and counting cells by a fluorescence microscope in the sham, exercise, ischemia, ischemia + exercise, exercise+ischemia, and exercise+ischemia+exercise groups. In all groups, the first column images are related to the connecting of the primary antibody to the NF-κB protein. The second-column images are related to staining the all-cell nuclei by DAPI. And the third-column images are related to merging the first and the second-column images. Reactivity and expression of cells were evaluated and counted by fluorescence microscope with a magnification ×400.

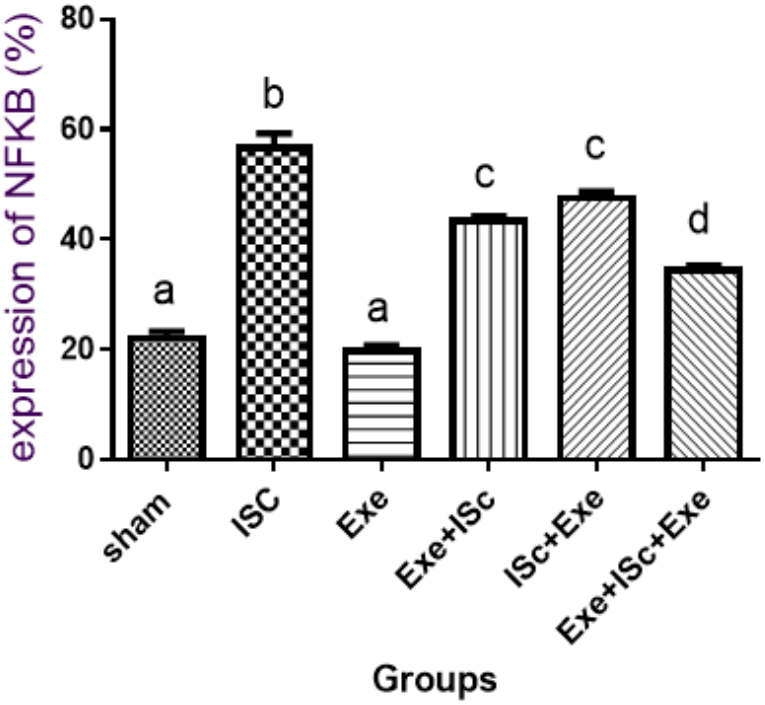

Figure 4

Comparison of mean NF-κB in CA1 among experimental groups.

a: Significant difference between sham and Exe with other groups (P < .05).

b: Significant difference between ISC with other groups (P < .05).

c: Significant difference between Exe+ISC and ISC+Exe with other groups (P < .05).

d: Significant difference between Exe+ISC+Exe with other groups (P < .05).

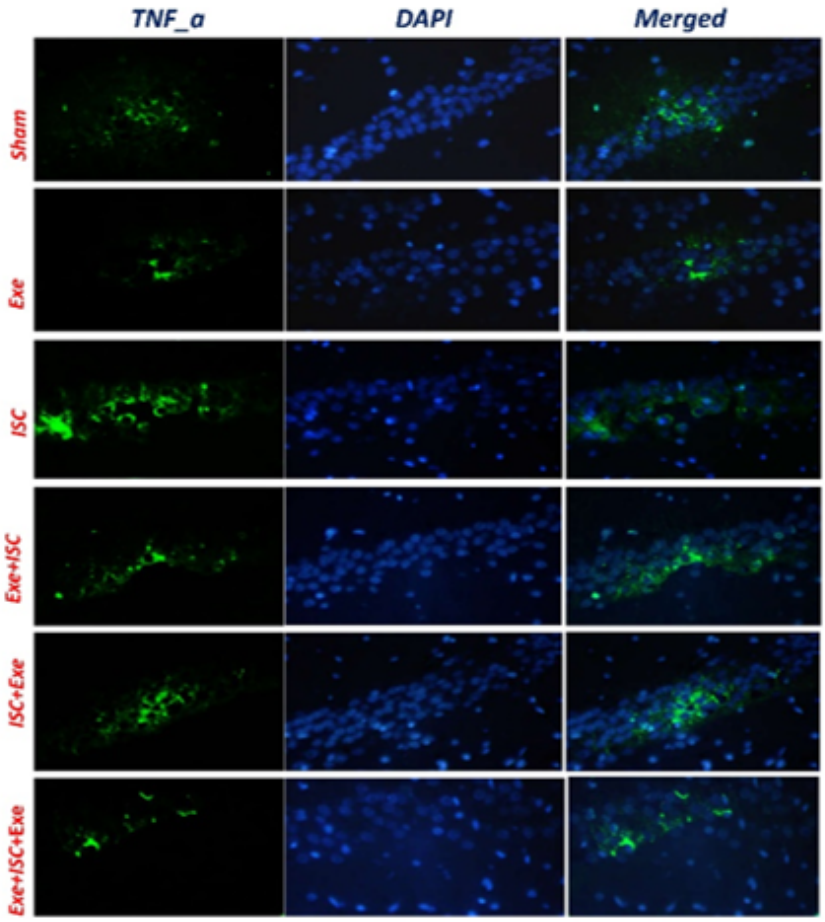

Figure 5

Evaluating and counting cells by a fluorescence microscope in the sham, exercise, ischemia, ischemia + exercise, exercise+ischemia, and exercise+ischemia+exercise groups. In all groups, the first column images are related to the connecting of the primary antibody to the TNF protein. The second-column images are related to staining the all-cell nuclei by DAPI. And the third-column images are related to merging the first and the second-column images. Reactivity and expression of cells were evaluated and counted by fluorescence microscope with a magnification ×400.

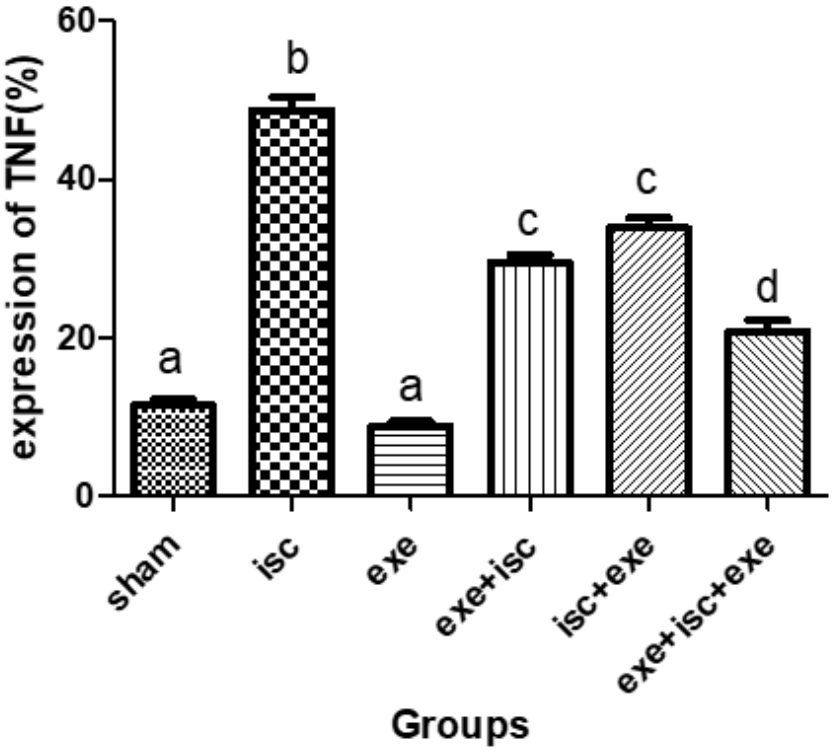

Figure 6

Comparison of mean TNF-α in CA1 among experimental groups.

a: Significant difference between sham and Exe with other groups (P < .05).

b: Significant difference between ISC with other groups (P < .05).

c: Significant difference between Exe+ISC and ISC+Exe with other groups (P < .05).

d: Significant difference between Exe+ISC+Exe with other groups (P < .05).

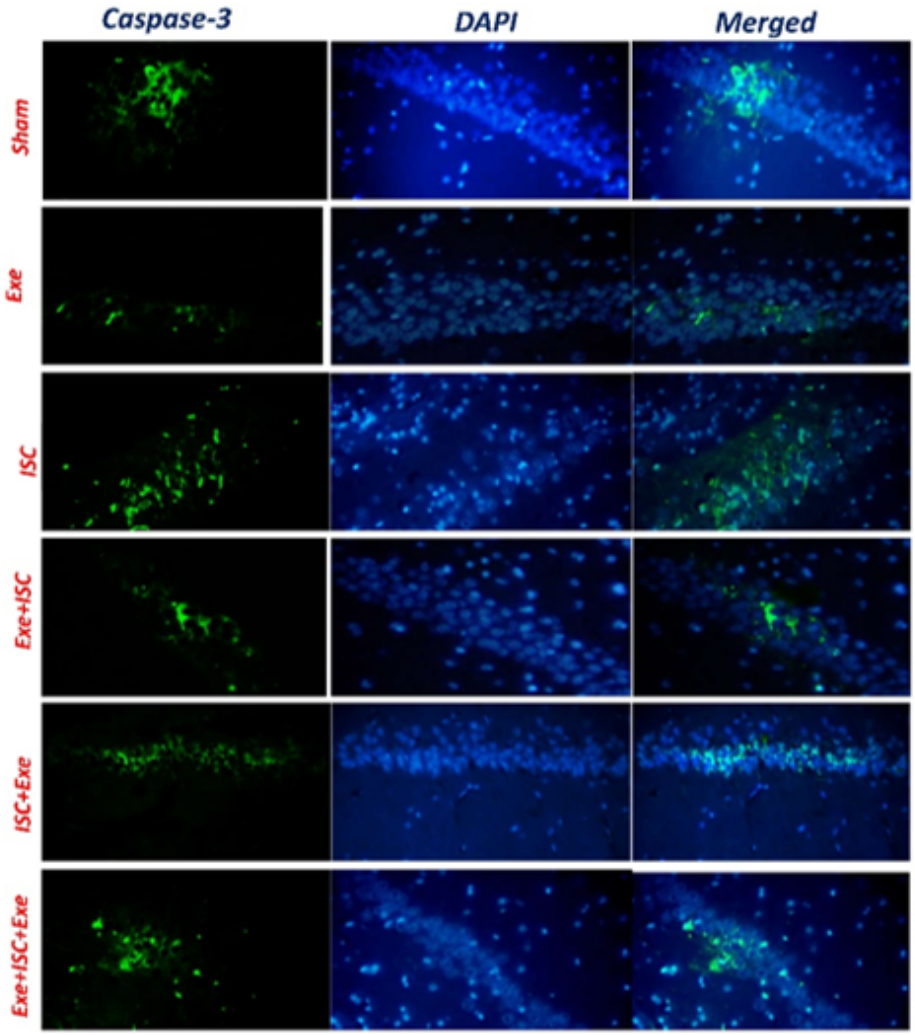

Figure 7

Evaluating and counting cells by a fluorescence microscope in the sham, exercise, ischemia, ischemia + exercise, exercise+ischemia, and exercise+ischemia+exercise groups. In all groups, the first column images are related to the connecting of the primary antibody to the Caspase 3 protein. The second-column images are related to staining the all-cell nuclei by DAPI. And the third-column images are related to merging the first and the second-column images. Reactivity and expression of cells were evaluated and counted by fluorescence microscope with a magnification ×400.

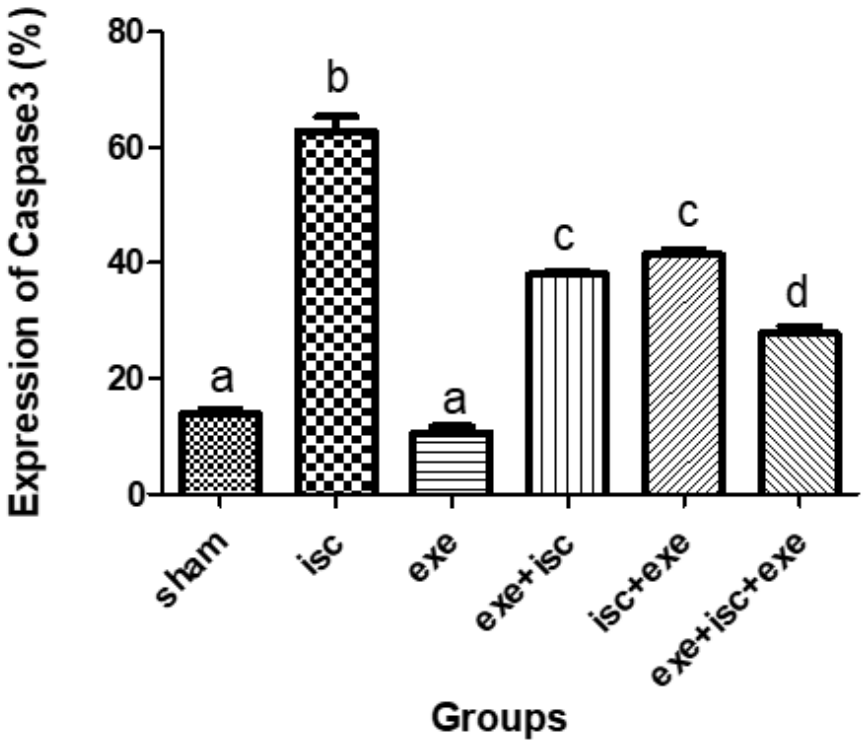

Figure 8

Comparison of mean Caspase 3 in CA1 among experimental groups.

a: Significant difference between sham and Exe with other groups (P < .05).

b: Significant difference between ISC with other groups (P < .05).

c: Significant difference between Exe+ISC and ISC+Exe with other groups (P < .05).

d: Significant difference between Exe+ISC+Exe with other groups (P < .05).

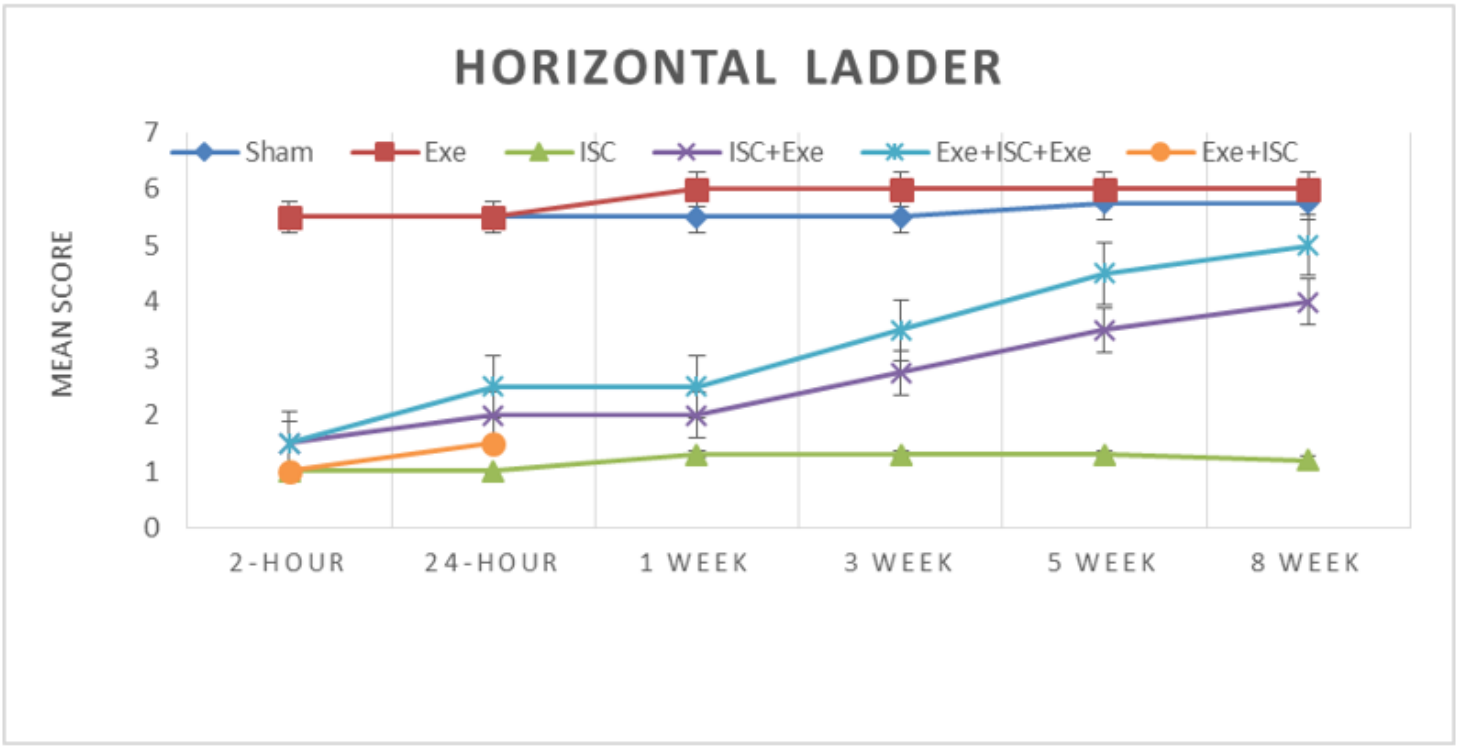

Figure 9

Mean number of scores in the ladder rung walking task. It shows significant improvements compared to ischemia groups ($P < .05$). In the exercise+ischemia+exercise group, the number of scores has been increased significantly from 2 h post-ischemia to the end of the study compared to exercise+ischemia and ischemia+exercise groups ($P < .05$).