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The role of endurance exercise and adenosine on monocyte chemotactic protein-1 (MCP-1) gene expression in male rat brain ischemia-reperfusion

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ABSTRACT

A suitable exercise program helps to improve physiological performance and improves aerobic capacity, blood absorption, and sufficient oxygen for the brain and muscles. This study investigated the effect of endurance training and adenosine drug injection on the MCP-1 gene after cerebral ischemia and perfusion in male rats. For this purpose, 48 male Wistar rats were selected, and after ischemic induction, they were placed in endurance training/adenosine/ischemia, ischemia/adenosine, endurance training/ischemia, and control/ischemia groups. After induction of ischemia, an endurance exercise protocol was performed. Real-Time PCR technique was used to evaluate monocyte chemotactic protein-1 (MCP-1) gene expression. The results showed a significant difference between the control/ischemia group and the adenosine/ischemia group in the expression of the MCP-1 gene among male rats. Also, there was a significant difference in MCP-1 gene expression between the control/ischemia group and the endurance exercise/ischemia group. On the other hand, there was no significant difference between the control/ischemia group and the endurance training/adenosine/ischemia group in MCP-1 gene expression in ischemia-reperfusion male rats. Overall, it is likely that preconditioning with endurance exercise and adenosine drug up-regulates MCP-1 gene expression before the ischemic stroke. Therefore, training and adenosine may be helpful as preventive stimuli against ischemic stroke.

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Introduction

Regular training and proper exercise programs are influential factors in absorbing enough blood and oxygen for the brain and muscles; therefore, it is necessary to find strategies that increase the resistance of neurons against neurodegenerative damage caused by ischemia (1). The evidence shows that previous sports activity or pre-preparation can lead to reduced infarct volume, improved neurological and functional rehabilitation, modulated risk factors, and endogenous neuroprotection and preservation of neuron survival in conditions of ischemia damage (2).

Pre-training with endurance exercises can cause neuronal protection and the expression of vascular growth factors; however, due to the presence of stress in forced exercise such as treadmills, the recovery of cerebral ischemia injuries is discussed (3). Some studies in this regard have introduced adenosine as a potent endogenous physiological mediator that exerts a wide range of physiological processes through cell surface protein-coupled receptors (4,5). These receptors are called adenosine receptors, which have different types (such as A1, A2A, A2B, and A3) and are coded by separate genes. Due to several mechanisms, neuroprotection strengthens the brain's neuronal network and increases resistance to damage caused by ischemia (5). The factors involved in providing the substrate, the experimental factors in metabolizing the substrate inside the cell, and the production of adenosine triphosphate (ATP) as a result of pre-training with exercise activity can reduce metabolic and bioenergetic disorders in neurons after ischemia damage and re-blood supply and lead to the reduction of neuronal apoptosis after ischemic injury (2). Therefore, pre-preparation with sports activity in people prone to ischemic brain damage or with a history of mild ischemic brain damage and before brain surgery interventions may reduce the disorder and improve the neurological results after ischemic injury to the blood supply (3).

The brain's immune system mainly includes astrocytes, microglia, and other immune cells and is activated in response to pathophysiological events such as ischemia, trauma, inflammation, and infection. Chemokines are proteins that are released from the immune (6). Monocyte chemoattractant protein-1 (MCP-1) is CCL_2, which serves the monocytes of macrophages. Its gene expression is activated when reperfusion lesions are damaged due to the return of blood flow (ischemia, inflammation, and oxidative lesions) (7). The expression of the MCP-1 gene is under the effect of adenosine, an endogenous purine nucleoside, produced under stress conditions and interacts with G protein receptors to regulate the function of the brain's immune system. Reports show that MCP-1 is expressed by brain

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microglia and helps alleviate neurological diseases (6).

One of the known effects of adenosine is the ability to control central nervous system tumors in both physiological and pathophysiological states. Adenosine interacts with four receptors A1, A2A, A2B, and A3 (7). The activation of some of these receptors aggravates and alleviates brain damage. Adenosine is effective in neuroprotection and brain concentration. Adenosine signaling is designed to control the flow of information between nerve cells in the brain (8). Adenosine exists in low concentrations in the extracellular space, but its extracellular levels increase in stress conditions. Metabolic stress associated with hypoxia, ischemia trauma, stimulants, and excessive nerve activity causes a sharp increase in extracellular adenosine concentration, which is essential in controlling subsequent tissue damage (9).

In some cases, adenosine receptor stimulation causes tissue damage (9). Liebelt et al. (10) investigated the effect of exercise pre-conditioning on reducing the effects of ischemia. Their results showed that longer exercise duration was insufficient to minimize stroke complications. Svensson et al. (11) also investigated the effect of exercise training on brain damage after the induction of global cerebral ischemia. Using a forced treadmill after induction of ischemia, the level of anxiety, depression, and cognitive behaviors were studied. Their results showed that forced treadmill caused stress response and increased anxiety is associated with increased corticosterone level; Hence, to determine the effect of endurance training and adenosine consumption on cerebral ischemia, the question is whether the interaction of exercise and medicine can cause MCP-1 gene changes and synergism; Therefore, this study aimed to investigate the effect of endurance training and adenosine drug injection on MCP-1 gene index of brain tissue after cerebral ischemia-reperfusion in male Wistar rats.

Materials and Methods

The current research was experimental in sterile laboratory conditions. The statistical population consisted of 8-10-week-old male Wistar rats weighing 200-230 grams and was affected by endurance training and adenosine in a post-test design. Forty-eight rat heads were prepared as research samples. The environmental conditions were controlled with a temperature of 25 degrees Celsius and a humidity of 45-50%. Then they were randomly divided into four groups of 12:

1. Control/ischemia group.
2. Ischemia/endurance training group.
3. Adenosine/ischemia/drug group.
4. Endurance exercise/adenosine drug/ischemia group

During the research, the mice were kept in transparent polycarbonate cages with dimensions of 15 x 15 x 30 average temperature of 223 degrees Celsius, manufactured by Razi Rad Company. The living conditions of the mice were designed according to the principles of care of laboratory animals and the ethics committee, and there was a 12-hour period of sleep and wakefulness. The air humidity of the mouse house was controlled at +50% with proper ventilation. For every 100 grams of the weight of each mouse, 5 grams of food was placed in the cage once a week based on weighing. In this research, the required water was freely available in a 500 ml bottle for laboratory animals.

In the last week, in the training groups, adenosine (Wockhardt Company, England) in the amount of 0.4 mg/ml/kg was injected subcutaneously into each rat once a day, 3 hours before training. After 48 hours after the last training session, blood sampling was done from the rats in a fasting state.

Exercise Training

First, five preliminary and pilot study rats performed an exercise program for three familiarization sessions and five main activity sessions. This study's exercise intensity basis was based on previously designed studies. Its specifications included 30 m/min 70 maximum oxygen consumption of rats and according to the Vo2max standard model in the Bedford study. Rats reached the maximum oxygen consumption at a speed of 42.66; Therefore, in this study, training groups trained for one week for three alternate days to familiarize themselves with sports activities and the treadmill at a speed of 15 meters per minute for 10 to 15 minutes (approximately 40 maximum oxygen consumption).

After approval, the program was implemented after a day of rest, a protocol of five sessions per week. According to the principle of gradual increase in intensity and volume, all the rats in the training groups performed the endurance training protocol with a speed of 18 meters per minute and a duration of 20 minutes in the first week of the start.

Then their training gradually reached a speed of 30 meters per minute and duration of 50 minutes with an incline of 10 degrees in the eighth week, equivalent to 70% of maximum oxygen evaluated in Bedford's study as the desired intensity in endurance training groups. Of course, the training protocol also had a warm-up phase. The warm-up phase of the running program was considered for 3 minutes with an intensity of 10 meters per minute, followed by 2 minutes with an intensity of 15 meters per minute. Also, after performing the main exercise in each group, the rats participated in the cooling protocol for one minute with an intensity of 15 m/min and then for 2 minutes with an intensity of 10 m/min. The Vozniax determination test was performed to determine the operated rats' capacity. No electric shock or stimulation other than touching and rubbing the tail was used as a stimulus.

The rats of each group were randomly divided into the required groups after familiarization with the new environment and activity on the treadmill. The main training program was for eight weeks, which included a training protocol of intermittent aerobic endurance activity (5 sessions per week, six sets of 2.5 minutes, 2 minutes of rest in each set, and 40m/min speed for mouse training) (Table 1). Exercise intensity was controlled by a heart rate monitor (Polar, Finland). The exercise protocol was considered based on the scientific principles of the American Association of Sports Medicine. To comply with the ethical principles of working with animals, an electric current was not used to continue the activity of the mice.

Procedure

First, ischemic induction was performed. Before surgery, health and safety measures and behavioral measures (natural movement of organs) were implemented. Then, according to the pilot conducted to investigate the amount of damage caused by the duration of the blockage of the

Table 1. Endurance training protocol.

Variable	Practice Duration	Practice Speed	Treadmill Incline
First Week	15 min	15 m/min	0 degree
Second to Seventh Week	Gradually increasing	Gradually increasing	0 degree
Eighth Week	60 min	30 m/min	10 degree

arteries and also by better and more accurate examination of the endurance activity and adenosine, the common carotid arteries after observation and separation from the vagus nerve using microsurgical clamps and was blocked for 45 minutes. Ischemia occurs by blocking common carotid arteries, Common Carotid Artery, and CCA. To create local ischemia in rats, the filament method blocked the middle cerebral artery. First, rats were anesthetized with ketamine xylazine (ketamine 10% with a dose of 50 mg/kg and xylazine 2% with a dose of 10 mg/kg (Alfasan Company, Netherlands).

Each rat was fixed on a unique surgical table. Using a surgical microscope, an incision was made in the front of the animal's neck, and the muscles of this area were removed to see the common carotid artery. Then the common carotid artery and its external and internal carotid branches were separated from the muscles and nerves. In addition to closing the internal carotid artery on the side where ischemia is to be caused, other branches and the side's main carotid root must be permanently closed with sutures. Otherwise, bleeding from the vessels may occur during surgery and cause the animal's death.

Using a microscope, the surgeon inserted a nylon thread with numbers 0-3. Its tip was rounded in front of the flame) through a small incision in the external carotid artery into the internal (ICA) artery. The nylon thread was slowly guided from the bifurcation of the common carotid artery along the internal carotid artery to the inside of the brain and the circle of Willis.

In this way, the blood flow in the middle artery of the brain was cut off, and ischemia was created in the brain's area supplied by this artery. The central cerebral artery occlusion or ischemia period is often reported between half and three hours. After the completion of the ischemia period, the nylon thread was slowly removed, and the blood flow was re-established in the middle cerebral artery and the ischemic area. After the end of the time, the microsurgery was removed, and the blood flow of the common artery was established. To check the extent of injury and damage caused by 45 minutes of cerebral ischemia, reperfusion of the brain was performed using the Ladder walking test one hour after the ischemia.

After ischemic induction, the rats were placed in their groups. The control/ischemia group was kept in cages without activity and taking adenosine for eight weeks. After ischemic induction, the ischemic endurance training group only performed endurance training in the designed protocol. The Adenosine/ ischemia group was injected with 1 mg of adenosine daily after ischemic induction in the 8th week. The course and injection dose choice was determined based on 50% LD with a lethal dose. The endurance exercise/adenosine/ischemia group performed endurance exercise and designed protocol after ischemic induction, and they were injected with 1 mg of adenosine every day. The practice followed the principle of gradual increase and drug injection in the form of ready-to-inject syringes. A behavioral test assessed and measured the rats with ischemia through carotid artery occlusion. Eight weeks after ischemic induction and training protocol and 48 hours after the last training session, skilled technicians took blood samples from rats in a fasting state. Mice were anesthetized by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (4 mg/kg). Blood samples were collected in falcon tubes and centrifuged at a speed of 3000 rpm for 15 minutes. The serum was separated and transferred to a freezer with a temperature of minus 70 degrees Celsius for the following stages of research and measurement of the desired variable. To homogenize the tissue, the target tissue was first taken out of the freezer and weighed using a digital scale with an accuracy of 0.001 grams. It was then placed inside the Falcon 15 test tube, and 200 microliters of single-phase lysing solution were used for every 0.5 grams of tissue. The tissue was homogenized for five minutes at 8000 rpm using a homogenizer. The obtained solution was centrifuged for 15 minutes at a speed of 3000 rpm. The sampler transferred the supernatant solution into the microtube, and the remaining sediment was discarded. Using laboratory methods, blood samples were taken, and the expression of the CCL gene in the brain tissue of rats was measured.

Gene expression measurement

Gene expression was measured by the $2^{-\Delta\Delta CT}$ or Livak method. To measure the number of target and reference gene copies, the comparative threshold cycle method was used. Finally, the amount of $2^{-\Delta\Delta CT}$ is compared with the ratio of the target gene to the reference gene (GAPDH). In Table 2, primer sequences, product length, and annealing temperature for the CCL_2 gene and reference gene are mentioned. RNA isolation was performed using the QIAGEN kit (RNAsy Mini Kit catalog number 74104) according to the manufacturer's instructions. QuantiTect Reverse Transcription Kit cDNA synthesis (Qiagen) was used to make cDNA according to the manufacturer's instructions as Real-Time PCR. A thermal CyclerTM BIO-RAD (C1000) device was used to measure gene expression.

The heating schedule of the device was done in three

Table 2. Primer sequences, product length, and annealing temperature for the MCP-1 gene and reference gene.

Gene	Primer Sequence (5'-3')	Product length	Annealing temp.
GAPDH	F: CGACTTCAACAGCGACACTCAC	180bp	65°C
	R: CCCTGTTGCTGTAGCCAAATTC		
MCP-1	F: GCGCCGGAAAGCTGTAGATG	98bp	50°C
	R: TTTGCTTGTCCAGGTGGTC		

stages. The first step, which leads to the denaturation of DNA molecules and the activation of the polymerase enzyme, included heating the solution for 3 to 5 minutes at 94°C to 98°C. In the second step, the temperature of the solution was reduced from 50°C to 65°C degrees for 20 to 40 seconds. At this temperature, the two strands of each molecule can be connected again. The binding temperature is about 3-5°C lower than the melting point of primers. The dissociation curve was drawn from 55°C to 95°C with an increase of 0.5 degrees in 5 seconds. Real-time PCR reactions were performed in a final volume of 10 microliters in 96-well plates.

Quantitative gene expression studies were done using the Real-time RT-PCR method with the Quantitative PCR technique. The replication of DNA fragments was checked at the same time as the experiment using fluorescent reporters. Before starting the Quantitative PCR process, RNA was extracted and converted to cDNA. The relationship between the amount of transcribed RNA and the expression level of the A2B gene was checked.

After cDNA synthesis, the sample was prepared and added to the colored DNA sample (SYBR Green) to enter the polymerization cycles. In the following, the real-time PCR device started the fluorescence reading by the optical system, and finally, the graph reached its threshold, which was the gene expression level in each sample. The GAPDH gene was used as a reference gene, and the numbers obtained from the target gene amplification graph in each sample were normalized to the reference gene. To check the efficiency of primers, a standard graph was drawn using five logarithmic concentrations, and the slope of the graph was obtained.

Data Analysis

The mean and standard deviation were used to determine the central index. Checking the normality of data distribution, which ultimately determines the choice of parametric and non-parametric tests, was determined through the Shapiro-Wilk test and using Levine's homogeneity of variance test. The results obtained in this study are based on at least two replications. One-way ANOVA was used to determine the significance of independent variables' effect on the research's dependent variables and to examine the differences between groups. Data analysis was done with SPSS version 22 software, Bonferroni post hoc test was used to determine the significance of the results ($P<0.05$).

Results

After induction of ischemia and after eight weeks of exercise protocol and adenosine injection, the mean weight changes of mice were compared among the four experimental groups, which is presented in Figure 1. A comparison of the average weight of rats in experimental groups showed weight loss in all four groups. Using the correlated t-test, the weight change of mice before and after the research period was investigated and compared. The results showed that this weight loss was significant only in the endurance training/ischemia group ($P=0.044$) and the endurance training/adenosine drug/ischemia group ($P=0.023$), which is probably caused by endurance exercise protocol in these two groups and ischemia probably had an effect on delivering oxygen consumption and metabolic reactions and energy generation (Figure 1).

The average changes in MCP-1 gene expression among the four experimental groups are compared and presented in Figure 2. The results of the Shapiro-Wilk test showed that the research variables are standard. Levine's test also confirmed the homogeneity of variances. The comparison between groups with a one-way analysis of variance test showed that there is a significant difference between the groups in the MCP-1 variable ($P<0.05$), and adenosine supplement and endurance training were able to have effects on the dependent variables ($P=0.0001$).

The results of the Bonferroni test showed a significant increase in the expression of the MCP-1 gene in the ischemia-reperfusion of male rats between the ischemia adenosine group and the ischemia control group ($P=0.004$). A significant increase in MCP-1 gene expression was also observed between the ischemia endurance training group and the ischemia control group ($P=0.0001$). There was no

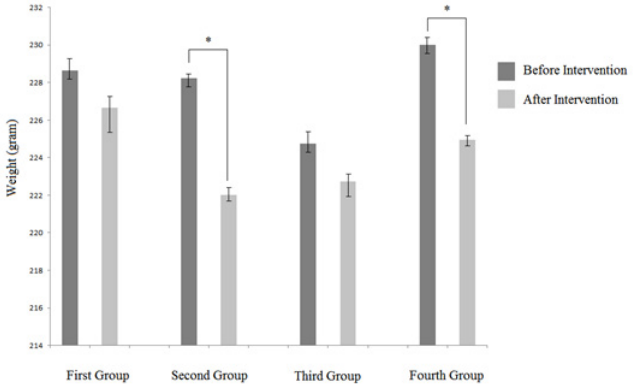

Figure 1. Checking and comparing the weight of rats in experimental groups; First Group: Control/ischemia group, Second Group: Ischemia/endurance training group, Third Group: Adenosine/ischemia/drug group, Fourth Group: Endurance exercise/adenosine drug/ischemia group; *: $P<0.05$ Significant decrease in the weight of the training/adenosine/ischemia group and the training/ischemia group compared to before the training protocol and Adenosine supplement.

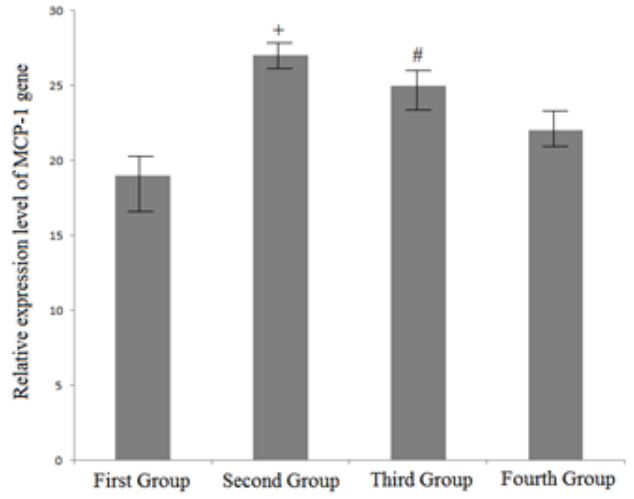

Figure 1. The relative expression of the MCP-1 gene in different groups; First Group: Control/ischemia group, Second Group: Ischemia/endurance training group, Third Group: Adenosine/ischemia/drug group, Fourth Group: Endurance exercise/adenosine drug/ischemia group; #: $P<0.05$, the significant increase in gene expression in the adenosine drug/ischemia group compared to the control/ischemia group; +: $p<0.05$, the significant increase in gene expression in the control/ischemia group compared to the exercise/ischemia group.

significant difference between the ischemia control group and the adenosine ischemia endurance training group in the expression of the MCP-1 gene in ischemia-reperfusion male rats (P=0.144) (Figure 2).

Discussion

The research results showed that sports activity could increase the expression of the MCP-1 gene (12). Macrophages and satellite cells influence the increase in MCP-1 gene expression (13). The role of MCP-1 gene expression is due to the infiltration of macrophages, and the local increase in gene expression may be caused by immune cells (14). The induction of MCP-1 gene expression during acute exercise may be due to the repair and adaptation of damaged tissue (1,12). In this study, the training protocol was not very intense; but probably, the activity of MCP-1 gene expression increased in the ischemia endurance training group compared to the ischemia control group. The contradictory findings with the results of this research may be due to the decreasing effects of chemokines following exercise, which will prevent the increase in MCP-1 gene expression in the brain (15,16). Also, endurance training with increased hyperoxia and metabolites has increased endogenous adenosine and MCP-1 gene expression. The type of training protocol, previous training history, and history of using supplements or drugs similar to adenosine, and age and gender of the research sample can also be influential factors in the research findings (17). Of course, preconditioning with endurance training is also effective on the expression of the MCP-1 gene in the CA1 region of the hippocampus after ischemic stroke and cerebral reperfusion. The evidence has shown that sports activity, in addition to modulating the risk factors of ischemic stroke, also has neurological rehabilitation effects and can act as a factor for neuro-protection and preserving the life of neurons before ischemia and in conditions of ischemic damage (18).

Regarding the effects of adenosine on MCP-1 gene expression and the significant increase in gene expression in the ischemic adenosine drug group, the type of adenosine receptor is effective in creating the role of adenosine (6). MCP-1 will increase when the A3 adenosine receptor is activated and MCP-1 expression will be suppressed when A2A and A2, and A2B receptors are activated. It may be possible to explain the effect of adenosine through the A3 receptor, which works against other receptors. Adenosine A3 receptors mainly bind to Gi proteins. Since Gi proteins are mediators for the release of MCP-1, this mediator is the reason for the different effects of adenosine on MCP-1 (19).

Another possibility is that adenosine production depends on the amount of ATP used (20). In stressful conditions such as sports, increasing the amount of ATP used per unit of time will increase adenosine levels (8). In cerebral ischemia, the demand for energy and cellular oxygen increases. As a result of this increase in demand, the level of adenosine will increase for supporting roles (21). Adenosine exerts its neuroprotective properties by regulating endogenous neurons, and with its regulatory effects on cell proliferation, survival, and death, it will probably improve apoptosis by inhibiting chemokine inflammatory responses (22). Also, endogenous adenosine suppresses inflammation and cellular and molecular mechanisms of inflammation through its receptors. It protects damaged tissues against acute and chronic inflammation by suppressing the glomerular expression of the chemokine MCP-1. The exact mechanism of neuroprotection caused by pretraining with exercise activity has not yet been fully elucidated. Still, the conducted studies have suggested several mechanisms that can be mentioned to strengthen the blood-brain barrier, expand the capillary and arterial network of the brain, improve brain metabolism and reduce metabolic disorders, increase the expression of neurotrophins, reduce inflammation, oxidative stress, and apoptosis (8).

Absence of significant change in the adenosine ischemia endurance training group, it can be said that CA1 neurons of the hippocampus are the most sensitive area to hypoxic ischemia damage, which will cause complete ischemia in the CA1 hippocampus 5 minutes after occlusion of the common carotid (21). Endurance training and adenosine injection separately increase adenosine and produce MCP-1 gene expression (23). In the endurance exercise group, adenosine ischemia may have been increased by exercise; however, by injecting exogenous adenosine, its endogenous production has been inhibited, and the inhibitory effect of using adenosine has caused a lower increase in MCP-1 gene expression in this group (21). Following an ischemic stroke, a cascade of initial molecular events begins, which will aggravate the damage caused by ischemia. The release of glutamate and using glutamate antagonists also have a neuroprotective effect and potentially increase resistance to ischemia. Adenosine can probably be one of these antagonists (19). Also, among chemokines, MCP-1 is a neuroinflammatory mediator and can play an essential role in the ischemia-induced inflammatory response (23). The involvement of MCP-1 in inflammatory responses prevents the spread of ischemic brain damage. The expression of the MCP-1 gene in some types of neurons and astrocytes may be an effective treatment for cerebral ischemia (8).

Determining the essential physiological capacities of the heart rate, the effect of stress on the impact of electric shock, the movement of animals, and the development of controlling the amount of blood flow during blocking and self-establishment in the carotid arteries are factors contributing to the mismatch of the results with the findings of other researchers (24). The effect of signaling through adenosine receptors, inflammatory factors such as tumor necrosis factor (CRPILS/TNF, C-reactive protein factor, Interleukins), and periodic and strength training methods on CCL2 gene expression should be investigated (25). The present study showed that preconditioning with endurance exercise and adenosine drugs before ischemic stroke will significantly regulate chemokinetic factors. When exercise and adenosine are used as preventive stimulants of ischemic stroke, they have synergistic protective effects after ischemia. They are of interest as an effective method of reducing brain complications caused by ischemia.

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