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Paroxetine Attenuates Cerebral Ischemia/Reperfusion Injury in Rat *Via* Its Anti-Inflammatory and Antioxidant Effects

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Review Article

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ABSTRACT

Cerebral ischemia is often associated with sensory-motor impairment and memory deficit. Previous studies have demonstrated that paroxetine, the antidepressant drug, exhibits anti-inflammatory and antioxidant effects. In this study, we investigated the protective effects of paroxetine on neuronal damage and activation of microglial cells induced by cerebral ischemia/reperfusion in the rat. Cerebral ischemia/reperfusion injury was induced by transient bilateral common carotid artery occlusion. The Wistar rats were assigned to sham, ischemia, and paroxetine-treated groups. Paroxetine (10 mg/kg) was administered intraperitoneally once daily for 7 days subsequent to surgery. In order to evaluate spatial memory in rats, Morris water maze test (MWM) was conducted. The viability of pyramidal neurons in the hippocampus was assessed by Nissl staining method. Microglial activation and production of pro-inflammatory cytokines (IL-1β, TNF-α) were examined using Iba1-immunostaining and ELISA methods, respectively. Oxidative stress was evaluated by measuring the levels of malondialdehyde (MDA) in homogenates of hippocampal tissue. In MWM test, paroxetine significantly enhanced learning performance in rats subjected to cerebral ischemia/reperfusion. Our findings indicated that paroxetine significantly suppressed ischemia-induced microglial activation and decreased the IL-1β and TNF-α level in the hippocampus. In addition, paroxetine inhibited lipid peroxidation and decreased MDA levels in homogenates of hippocampal tissue. These results establish that paroxetine exert a protective effect against cerebral ischemia/reperfusion-induced damage to hippocampal neurons and memory impairment in rats through anti-inflammatory and antioxidant effects.

INTRODUCTION

Cerebral ischemia is an important and debilitating neurologic insult which is caused by a reduction in the cerebral blood flow. When blood flow to the brain is interrupted, neural cells are subjected to deprivation of oxygen and glucose which in turn induces oxidative stress and consequent neuronal damage in vulnerable regions of the brain. It has been shown that hippocampus is among the most vulnerable region of the brain to cerebral ischemia/reperfusion injury. Cerebral ischemia-induced damage to the hippocampal neurons causes impairment of memory after cerebral ischemia/reperfusion. These observations reinforce the clinical evidences describing cerebral ischemia as one of the most common causes of cognitive disorders. Memory impairment has been reported as the prominent sign among various cognitive problems when cerebral ischemia is diagnosed.

Inflammation and oxidative stress are among the most important mechanisms of neuronal damage as a result of ischemia/reperfusion. There are several evidences indicating that microglial cells are activated following cerebral ischemia/reperfusion, releasing large amounts of oxygen free radicals and inflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α). Moreover, the blood brain barrier is compromised following microglial activation owing to its macrophage-like capabilities such as releasing matrix metalloproteinases and inflammatory cytokines. This may result in the infiltration of peripheral leukocytes into the brain which in turn exacerbate the inflammatory processes and worsen neuronal damage [1]. On the contrary, during cerebral ischemia, the oxidative respiratory chain in mitochondria is compromised and cytoplasmic oxidases are activated. Under these circumstances, large amounts of reactive oxygen species (ROS) are generated within the cells which initiate pathways toward the neuronal necrosis and apoptosis. Collectively, these pathological processes progressively destruct neural cells particularly in the hippocampus and cause the impairment of learning and memory. Given that microglial activation and resultant overproduction of inflammatory cytokines have a critical role in neuronal damage and memory impairment following cerebral ischemia/reperfusion injury, the use of therapeutic strategies which focused on these underlying mechanisms have been shown to be effective against neuronal damage and memory loss. Recent studies have shown that anti-inflammatory and antioxidant agents prevent damage to neurons and thus can be considered as part of treatment of cognitive disorders following cerebral ischemia.

Paroxetine, a common selectiveF serotonin reuptake inhibitor (SSRI), is extensively prescribed as an antidepressant drug. Nevertheless, some other pharmacological properties have been described for paroxetine rather than its antidepressant effect. For instance, it has been demonstrated that paroxetine exert anti-inflammatory effects *via* the suppression of activated microglia, thus resulting in the inhibition of nitric oxide synthesis and reduction in the expression of inflammatory cytokines [2]. In addition, paroxetine suppresses the expression of astroglial myeloperoxidase and decreases the production of NADPH oxidase-derived ROS indicating its potent antioxidant effect. There is also evidence showing that selective interaction of paroxetine with mitochondrial proteins in the brain underlies its neuroprotective effects.

Considering the anti-inflammatory and antioxidant effects of paroxetine, it can be hypothesized that paroxetine may be effective in the prevention of neuronal damage and memory loss following cerebral ischemia. Therefore, this current study aims to evaluate the neuroprotective effect of paroxetine against memory impairment and neuronal damage following cerebral ischemia/reperfusion using a transient bilateral common carotid artery occlusion method in rats.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 250-300 g were used in this study. Animals were kept in separate cages under conditions of 12/12 hours, light/dark cycle and 20 ± 2 ºC temperature with free access to food and water. All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and were conducted according to the protocols approved by the ethics committee on the use of animals in medical research, the Shahid Beheshti University of Medical Sciences.

Chemicals

Paroxetine hydrochloride, ketamin hydrochloride, xylazine hydrochloride and cresyl violet were purchased from Sigma-Aldrich Co., USA. Thiobarbituric acid, 1, 1, 3, 3-tetramethoxypropane and n-butanol were obtained from Merck Co., Germany. Diaminobenzidin and Iba1-antibody were purchased from Abcam Co., UK.

Surgical procedure and experimental groups

Cerebral ischemia/reperfusion was induced by transient bilateral common carotid artery occlusion method. Concisely, rats were anesthetized with intraperitoneal (i.p.) injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). A 2 cm longitudinal incision was made on the ventral neck. The common carotid arteries were exposed on both sides of the neck and occluded for 20 minutes using micro-vascular clamps. After 20 minutes of ischemia, micro-vascular clamps were detached and reperfusion through common carotid arteries was re-established. Afterwards, the neck incision was sutured. Body temperature of the anesthetized animals during surgery was maintained at 37°C by a heating lamp.
A total of 42 rats were randomly divided into three groups (n=14). In each group, 7 rats were considered to prepare fresh or freeze hippocampal tissues to determine malondialdehyde (MDA) or cytokines. The other 7 rats were used for preparation of fixed hippocampal tissues to perform immunohistochemistry experiments.

Morris water maze

In order to evaluate learning and memory, MWM test was performed seven days after reperfusion according to the previously described method. In MWM test, each animal had four trials per day (once from each starting point) for four consecutive days. Escape latency time (the swimming time to locate the hidden platform), distance travelled and swimming speed were measured during each trial days. Probe test was carried out on the fifth day. In this test, the platform was removed and animals were allowed to swim in the pool for 60 seconds. The time spent in the target quadrant was measured for each animal in the probe test.

Measurement of IL-1β and TNF-α level in the rat hippocampus using ELISA method

The inflammatory cytokines in the rat hippocampus were measured using rat ELISA kits for IL-1β (Abcam, ab-100767, UK) and TNF-α (Abcam, ab-100785, UK). Animals were decapitated by guillotine under deep anesthesia with high doses of ketamine, one day after the end of MWM test. The brain was instantly removed from the skull and the hippocampus was dissected out and kept at -80°C until tested. Hippocampal samples were prepared according to the method previously described. The samples and standard solution were then added to each well of rat IL-1β or TNF-α ELISA kits. Biotinylated TNF-α or IL-1β antibodies, HRP-streptavidin and TMB one step substrate were added to each well base on the manufacturer's instructions.

Lipid peroxidation measurement by MDA assay in the rat hippocampus

In this study, MDA was measured to evaluate lipid peroxidation in the rat hippocampus using the thiobarbituric acid (TBA) method. In this method MDA reacted with TBA to produce a pink-colored complex which has peak absorbance at 532 nm. For MDA assay, hippocampal samples were prepared similar to sample preparation for ELISA method. The tissue sample was homogenized in KCL solution (1.5%) so that a homogenous suspension (10%) was obtained. 0.5 ml of suspension was transferred to a centrifuge tube and 3 ml of phosphoric acid 3% and 1 ml of TBA 0.6% were added to it. The mixture was then incubated in boiling water bath for 45 min. After cooling, 5 ml of n-butanol was added to the contents of the tubes. The tubes were centrifuged at 20000 rpm for 20 min. Finally, a spectrophotometer was used to record the absorption of the organic layer (n-butanol) at 535 nm. 1, 1, 3, 3-tetramethoxypropane was used as a standard to make a standard curve. The results were expressed as nanomoles of MDA per gram of hippocampal tissue.

Formalin-fixed and paraffin-embedded brain tissue preparation for histopathological tests

Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) a day after the end of Morris water maze test and first transcardially perfused with 0.9% normal saline and then with 4% paraformaldehyde for 30 minutes. The animals were then decapitated and the fixed brains were removed from the skull and post-fixed in 4% paraformaldehyde at 4°C for 24 hours. The fixed brains were placed in tissue molds and embedded in paraffin in order to carry out histological tests.

STATISTICAL ANALYSIS

Data were reported as mean ± SEM. One-way analysis of variance (ANOVA) followed by *Tukey's* test One-way ANOVA was used for data analysis. A value of $P < 0.05$ was considered as the level of statistical significance.

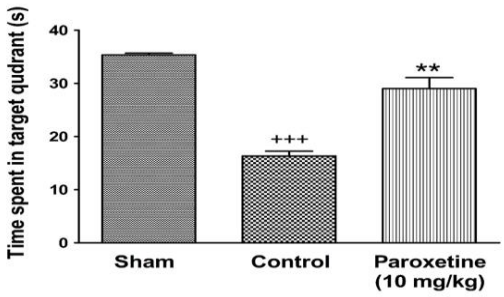
RESULTS

Effect of paroxetine on learning and memory in Morris water maze test

Cerebral ischemia/reperfusion injury induced by transient occlusion of common carotid artery evidently impaired spatial memory performance in ischemic rats. Repeated measures two-way ANOVA of escape latency time showed significant effects of day ($F(3,24)=16.48$, $P < 0.001$) and group ($F(3,8)=28.42$, $P < 0.001$) but no significant day×group interaction. The results revealed that escape latency in the control group was longer than that of the sham group on days 3 and 4 ($P < 0.01$). Nevertheless, treatment with paroxetine (10 mg/kg) significantly prevented ischemia-induced prolongation of escape latency on day 3($P < 0.001$) and day 4($P < 0.001$) in comparison with the control group. Similarly, for distance travelled ANOVA showed significant effects of day ($F(3,24)=73.27$, $P < 0.001$) and group ($F(3,8)=71$, $P < 0.001$) and a significant day×group interaction ($F(9,24)=4.26$, $P < 0.001$). The results obtained from the analysis of distance revealed that the travelled distance in the control group was significantly longer than that of the sham group on day 1 ($P < 0.01$), day 2 ($P < 0.05$), day 3 ($P < 0.001$) and day 4 ($P < 0.001$), however in paroxetine-treated group, distance travelled was significantly less than that of the control group on day 2 ($P < 0.01$), days 3 and 4 ($P < 0.001$). Analysis of swimming speed showed a significant effect of day ($F(3,36)=5.10$, $P < 0.01$), but no significant effects of group ($F(3,12)=0.45$, $P > 0.05$) and day×group interaction ($F(9.36)=0.22$, $P > 0.05$). There was no significant difference with respect to swimming speed between the sham, control, and paroxetine groups during training trials on days 1 to 4 ($P > 0.05$) (Figure1).

The probe trial results on the day 5 indicated that the time spent in the target quadrant in the control group was significantly further than that of the sham group ($P < 0.001$). Furthermore, the time spent in the target quadrant in the paroxetine-treated group (10 mg/kg) was significantly less than that of the control group ($F(3,8)=24.96$, $P < 0.01$).

Figure 1. The effects of cerebral ischemia/reperfusion and treatment with paroxetine (10 mg/kg) on the time spent in the target quadrant in the probe trial test on day 5.



Effect of paroxetine on hippocampal pyramidal neuron survival following cerebral ischemia

Micrograph of Nissl-stained sections revealed that most of CA1 pyramidal cells in the sham group were intact. On the contrary, the number of damaged neurons with pyknotic nuclei was more in the control group than that of the sham group. Treatment with paroxetine (10 mg/kg) significantly increased the number of surviving pyramidal neurons with palely stained nuclei and Nissl substance, when compared with the control group. The quantitative results demonstrated that pyramidal cell death in the CA1 region of the hippocampus following ischemia/reperfusion was significantly increased in the control group in comparison with the sham group ($P < 0.001$). Nevertheless, treatment with paroxetine significantly improved the percentage of viable pyramidal cells in the CA1 region of the hippocampus when compared to the control group ($F(2,12) = 119.1$, $P < 0.001$).

Effect of paroxetine on microglial activation in the hippocampus following cerebral ischemia

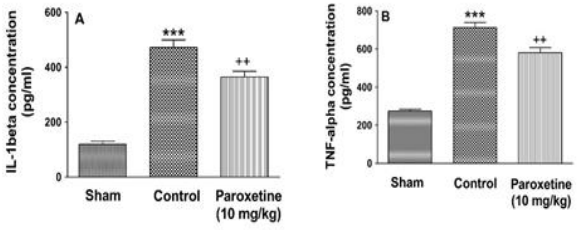
The effect of paroxetine on the activation of microglia following ischemia/reperfusion in the CA1 region of the hippocampus was evaluated using Iba1 immunostaining method. The results showed that the number of Iba1-immunopositive cells and the intensity of staining were higher in the control group than in the sham group. A decrease in the number of Iba1-positive cells and the intensity of staining was observed after treatment with paroxetine (10 mg/kg).

Effect of paroxetine on the levels of IL-1β and TNF-α in the hippocampus following cerebral ischemia

The levels of IL-1β and TNF-α (pg/ml) in the rat hippocampus were measured using ELISA method. The results revealed that the levels of IL-1β and TNF-α increased in the hippocampus after the induction of cerebral ischemia/reperfusion. As it is presented in, the levels of IL-1β and TNF-α in the control group were significantly higher than that of the sham group ($P < 0.001$) (Figure 2). Administration of paroxetine (10 mg/kg, i.p.) significantly

decreased the levels of IL-1β ($F(2,12) = 74.23$, $P < 0.001$) and TNF-α ($F(2,12) = 109$, $P < 0.001$) in the rat hippocampus when compared with the control group.

Figure 2. The effect of treatment with paroxetine (10 mg/kg) on the concentration (pg/ml) of IL-1β (A) and TNF-α (B) in the rat hippocampus following cerebral ischemia/reperfusion (I/R).



Effect of paroxetine on the MDA level in the rat hippocampus following cerebral ischemia

Cerebral ischemia/reperfusion injury (induced by bilateral common carotid occlusion) significantly enhanced the degree of lipid peroxidation in the hippocampus so that the level of MDA in the rat hippocampus was significantly higher in the control group in comparison with the sham group ($P < 0.001$). However, the administration of paroxetine (10 mg/kg), significantly decreased MDA level in the hippocampus of rats subjected to cerebral ischemia ($F(2,12) = 36.75$, $P < 0.001$).

DISCUSSION

In the current study, paroxetine attenuated learning and memory impairment in rats subjected to cerebral ischemia/reperfusion injury and improved the survival of pyramidal neurons in the hippocampus. Anti-inflammatory and antioxidant mechanisms seem to mediate the neuroprotective effect of paroxetine. In this study, we provided evidence demonstrating that paroxetine prevents cerebral ischemia-induced microglial activation and subsequent release of inflammatory cytokines

Microglial cells, which serve as the resident macrophages in the central nervous system (CNS), act as inflammatory cells in the brain. Previous studies have established that microglial cells are activated in the CA1 region of the hippocampus following cerebral ischemia/reperfusion. When cerebral ischemia occurs, the resting microglia are activated in the brain and transformed to amoeboid morphology with enlarged somata and multiple short and thick processes [3]. In addition, activation of microglia is connected with overexpression of Iba1 (the marker of microglial activation) in the ischemic area. Activated microglia produces ROS and inflammatory cytokines (IL-1β, IL-6, TNF-α), which promote cellular damage and lead to the progressive loss of neuron. In the present study, the effect of paroxetine on cerebral ischemia/reperfusion -induced microglial activation was studied by Iba1 immunostaining. The results of immunohistochemistry revealed that treatment with paroxetine significantly suppressed the cerebral ischemia/reperfusion-induced microglial activation in CA1 region of the hippocampus. We also provided evidence demonstrating that paroxetine prevents cerebral ischemia-induced release of inflammatory cytokines including IL-1β and TNF-α. These findings are in agreement with previous studies demonstrating that paroxetine exhibits anti-inflammatory effects and suppresses LPS-induced microglial activation. The results of several studies have indicated that microglial activation after cerebral ischemia/reperfusion result in the release of IL-1β, IL-6, and TNF-α which in turn promote the inflammatory reactions and worsen neuronal injury. The neuroprotective effect of paroxetine in cerebral ischemia/reperfusion -induced injury may be connected with its inhibitory effect on the activation of microglia following cerebral ischemia.

Neuro-inflammation mechanism prompted by cerebral ischemia/reperfusion, have a critical role in structural and functional damage to neurons in the susceptible regions such as the cerebral cortex and hippocampus. The hippocampus plays a significant role in memory acquisition and retrieval and is widely susceptible to damage following cerebral ischemia. Neuronal injury induced by cerebral ischemia in the hippocampus, leads to impaired learning performance in the Morris water maze test. This significantly reflects deficits in hippocampal-dependent spatial memory. Consistent with these evidences, the current study revealed that the spatial learning and memory were impaired after the induction of cerebral ischemia/reperfusion injury in rats. Seven days subsequent to

surgery, Morris water maze test was carried out to evaluate spatial learning and memory in rats. The results indicated that the escape latency time and the distance travelled in the control group were significantly more prolonged than those in the sham group [4]. Conversely, treatment with paroxetine during a 7-day administration schedule significantly enhanced spatial learning and prevented ischemia-related memory loss in rats. These results demonstrate the neuroprotective effect of paroxetine against memory impairment due to cerebral ischemia/reperfusion injury in rat.

We also provided cellular evidence indicating the protective effect of paroxetine on hippocampal neurons in rats subjected to brain ischemia. The results from Nissl staining revealed that post-ischemic administration of paroxetine significantly reduced pyramidal cell death in the hippocampus, so that the percentage of viable pyramidal cells in the CA1 region of the hippocampus was comparable to that of the sham-operated group.

Numerous studies have demonstrated that oxidative stress has a pivotal role in neuronal death following cerebral ischemia/reperfusion injury. Overproduction of ROS and progression of oxidative stress following cerebral ischemia/reperfusion injury promote oxidation of macromolecules such as DNA, lipids, and proteins which may lead to neuronal death. Several studies have shown a link between lipid peroxidation and neuronal damage following cerebral ischemia/reperfusion. It has been well established that after cerebral ischemia/reperfusion in several parts of the brain, the level of MDA was raised [5]. MDA is one of the end products of membrane lipid peroxidation and is considered a marker of oxidative stress. We measured MDA in the hippocampus in the control and treatment groups to assess lipid peroxidation level in rats subjected to cerebral ischemia. Our findings indicated that treatment of rats with paroxetine significantly reduced MDA level in the hippocampus. The attenuating effect of paroxetine on lipid peroxidation was comparable to that of the sham-operated group. These findings reflect the protective effect of paroxetine against lipid peroxidation in the neural cells in the hippocampus and show the attenuating effect of paroxetine on oxidative stress.

CONCLUSION

Collectively, our findings establish that paroxetine prevents memory impairment and hippocampal neuronal damage in rats subjected to cerebral ischemia/reperfusion. The neuroprotective effects of paroxetine appeared to be mediated through its anti-inflammatory and antioxidant effects. Our results are in agreement with previous studies indicating the protective effects of antioxidant agents including paroxetine against neuronal damage following oxidative stress. In this regard, there are several evidence which indicate that paroxetine act as a radical scavenge and suppresses oxidative stress in the brain. Our results are also in good agreement with previous reports indicating that SSRIs like fluoxetine have neuroprotective effect after cerebral ischemia and enhance spatial memory following cerebral ischemia/reperfusion as a result of their anti-inflammatory and antioxidant effects.

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