Protective Effects of Deprenyl in Transient Cerebral Ischemia in Rats

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Abstract

Cerebral ischemia leads to neuronal damage in the hippocampus and cognitive decline. Reactive oxygen species play an important role in the neuronal loss after cerebral ischemia and reperfusion injury. Deprenyl, an irreversible monoamine-oxidase B inhibitor, has antioxidant and neuroprotective effects against reactive oxygen species. In the present study, the effect of deprenyl on spatial memory impairment, oxidative stress and apoptotic neuronal cell death following transient cerebral ischemia in rats was investigated. Transient ischemia was induced by occlusion of left common carotid artery of rats for 30 min and reperfusion for 24 h or 1 week. Rats received intraperitoneal injection of 1 mg/kg deprenyl (n = 24) or equal volume of saline (n = 24) for 14 days before the experiment. Deprenyl treatment attenuated spatial memory deficits following ischemia-reperfusion as measured by the Morris water maze task. Deprenyl treatment elicited a significant decrease in lipid peroxidation and increase in superoxide dismutase activities in ischemic rat brains. The number of TUNEL-positive cells decreased significantly in deprenyl-treated group when compared with the control group. The results show that deprenyl reduces the ischemia-induced oxidative stress and thus prevents spatial memory deficits and apoptotic neuronal cell death when it is administered before ischemia-reperfusion.

Key Words: deprenyl, ischemia-reperfusion, spatial memory, oxidative stress, apoptosis

Introduction

Ischemia resulting from stroke or cardiac arrest leads to functional and structural damage in various regions of the brain, but this situation is further aggravated following reperfusion. A number of mechanisms have been invoked to explain this phenomenon, all of which involve a critical participation of reactive oxygen species (ROS) and oxidative stress (9). The intracellular enzymatic defense against ROS involves superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Administration of antioxidants that inhibit lipid peroxidation, or other natural or synthetic agents known to have antioxidant activity, has been reported to protect brain cells against the effects of ischemia-reperfusion. Therefore, the primary aim of any therapeutic intervention is to reduce the volume of brain damage and thus to minimize neurological impairment (15, 30).

The brain reperfusion after ischemia results in neuronal loss and cell necrosis or apoptosis. The consequence of cerebral ischemia is selective loss of vulnerable neurons by apoptosis in specific brain regions, such as hippocampal CA1 area (34, 35). Oxidative stress resulting from ROS production is also implicated in apoptosis; therefore, administration of antioxidants could alleviate apoptosis and protect nerve cells against the ischemic injury (1).

Spatial memory in rats and humans is largely dependent on the hippocampal formation (10).

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Hippocampal CA1 neurons are highly vulnerable to cerebral ischemia; therefore the ischemic neuronal damage may lead to spatial memory impairments. Cerebral ischemia leads to behavioral consequences which can be tested by a variety of tasks. A frequently used task in behavioral neuroscience, the Morris water maze task, has been adopted for use with rodents in stroke research. It has been suggested that there might be a correlation between hippocampal neuronal damage and deficits in spatial memory (4-6).

Deprenyl (selegiline) is an irreversible monoamine-oxidase B (MAO-B) inhibitor which has antioxidant and neuroprotective effects (18, 24, 32). Deprenyl is known to upregulate activities of antioxidant enzymes in rat brain. However, there is no direct data that address the effect of deprenyl on antioxidant enzyme activities under ischemic rat brains. Deprenyl is used for treatment of Parkinson's disease because of these effects and can attenuate the progressive degeneration of nigro-striatal dopaminergic neurons during aging and neurodegenerative disorders (13, 25). Deprenyl may also improve age-related cognitive deficits in aged rats (3, 17). In this study, we aimed to examine the effects of deprenyl treatment on spatial memory, oxidant stress and apoptosis in an animal model of cerebral ischemia. We evaluated parameters associated with oxidative stress, SOD enzyme activities in the prefrontal cortex, striatum and hippocampus regions of rats that form interconnected neural circuits for spatial memory, and cellular damage as indicated by lipid peroxidation.

Materials and Methods

Animals

Male Wistar rats (200-250 g) were used in all experiments. The rats were housed under a 12 h light/dark cycle with free access to food and water. All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine.

Animals were divided into three groups; the sham-operation group (sham, n = 14), the ischemia-induction group (control, n = 24) and ischemia-induction and deprenyl group (deprenyl, n = 24). In the deprenyl group, the rats were injected *i.p.* with 1 mg/kg/day of deprenyl dissolved in saline solution every other day for 14 days. Control rats received *i.p.* injections of physiological saline solution on an identical schedule. The treatments were continued for 14 days before, but not on the day of ischemia. In the sham group, the rats were subjected to sham operation and received no treatment. The rats in the control and deprenyl groups were subjected to 30 min of cerebral ischemia and perfused at 24 h or 7 days. The rats perfused at 7 days after surgery were given behavioral training in Morris water maze.

To induce transient cerebral ischemia, rats received sodium pentobarbital anesthesia (50 mg/kg, *i.p.*). Body temperature was maintained at normothermia using heating lamps. The left common carotid arteries were exposed through a midline cervical incision under a dissection microscope and occluded for 30 min. Subsequently, the carotid arteries were released and inspected for immediate reperfusion. Sham-operated rats received anesthesia and initial surgical procedures but did not receive occlusion. Half of the rats in each group was sacrificed by cervical dislocation and used for estimation of oxidant stress parameters, and the other half was perfused with saline followed by 10% formalin under ether anesthesia and used for histological evaluation. The rats perfused at 7 days after surgery were given behavioral training in Morris water maze on days 1-5.

Morris Water Maze Testing

To assess spatial learning, the Morris water maze task was used. The maze was 2 m in diameter and 75 cm in height. The water level in the maze was 50 cm, which was 1.5 cm above the height of the escape platform. The pool was filled with opaque water to prevent visibility of the platform in the pool. The escape platform was placed in the middle of one of the random quadrants of the pool and was kept in the constant position throughout the experiments (north-east for this study). On each test day, rats were placed in the water (22 ± 1°C) and trained until they found the hidden platform within 60 s, using extra maze cues. On successive days the start position was randomly altered. The swimming was monitored by a video camera, which was positioned directly above the center of the pool. If the rat failed to locate the platform in 60 s, the experimenter would place the rat on the platform and leave it there for 30 s. Each rat was tested for five swimming trials daily for 4 consecutive days, with an inter-trial interval of 60 s (a total of 20 trials). On the fifth day, a probe trial was run in the pool. The platform was removed, and the rats were placed into the pool and swam for 60 s. Time in seconds spent in the correct quadrant was recorded. The data were analyzed for latency to find the platform and time spent in the correct quadrant.

Biochemical Estimations

Half of the rats in each group was sacrificed by cervical dislocation under ether anesthesia. The brains were removed and divided into two hemispheres.

Hippocampus, striatum and prefrontal cortex tissues were dissected on an ice-cold surface. Tissue homogenates were prepared as described by Carrillo *et al*. (8). An aliquot of the homogenate and supernatant was stored at -70°C until thiobarbituric acid reactive substances (TBARS) levels, an indicator of lipid peroxidation, and SOD enzyme activities were determined. Determination of TBARS levels and antioxidant enzyme activities were performed spectrophotometrically.

Determination of TBARS was performed on homogenate according to the method of Rehncrona *et al*. and expressed as nmol/mg protein (29). SOD activities were measured on supernatant by using RANSOD kit (Randox Labs., Crumlin, UK) and expressed as units/mg protein. The protein contents of supernatant and homogenate were determined using a total protein kit.

TUNEL Staining

Half of the rats in each group was anesthetized *via* inhalation of ether. The rats were perfused intracardiacally by using isotonic sodium chloride followed by 10% formalin in phosphate buffer. Following the perfusion process, brains were removed and stored in the same solution for 24 h. Brain tissues were processed by routine histological methods and embedded in paraffin blocks. Paraffin blocks were placed in a rotary microtome and cut into 6 µm thickness coronal sections at multiple levels. Three coronal sections were taken through the hippocampus that corresponded approximately to Plates 21, 23, 25 in accordance with the rat atlas of Paxinos and Watson. To detect apoptotic cells, TUNEL staining was performed using an *In Situ* Cell Death Detection Kit® (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, the sections were deparaffinized, hydrated by successive series of alcohol, washed in distilled water followed by phosphate-buffered saline (PBS) and deproteinized by proteinase K (20 µg/ml) for 30 min at 37°C. Then the sections were rinsed and incubated in the TUNEL reaction mixture. The sections were rinsed and visualized using converter-POD with 0.02% 3,3′-diaminobenzidine (DAB). The sections were counter-stained with hematoxylin. For quantitative analysis of TUNEL-positive cells in hippocampus, cells exhibiting apoptotic features (condensed cytoplasm and chromatin, intense TUNEL reactivity, and a rounded cell body) were counted in hippocampal CA1 region using a computer assisted image analyzer system consisting of a microscope (Olympus BX-50 Tokyo, Japan) and a high-resolution video camera (JVC TK-890E, Yokohama, Japan). For establishing apoptotic index, 1000 cells were counted randomly in this area of ipsilateral hemisphere and calculated the percentage of the apoptotic cells. All counting procedures were performed blindly.

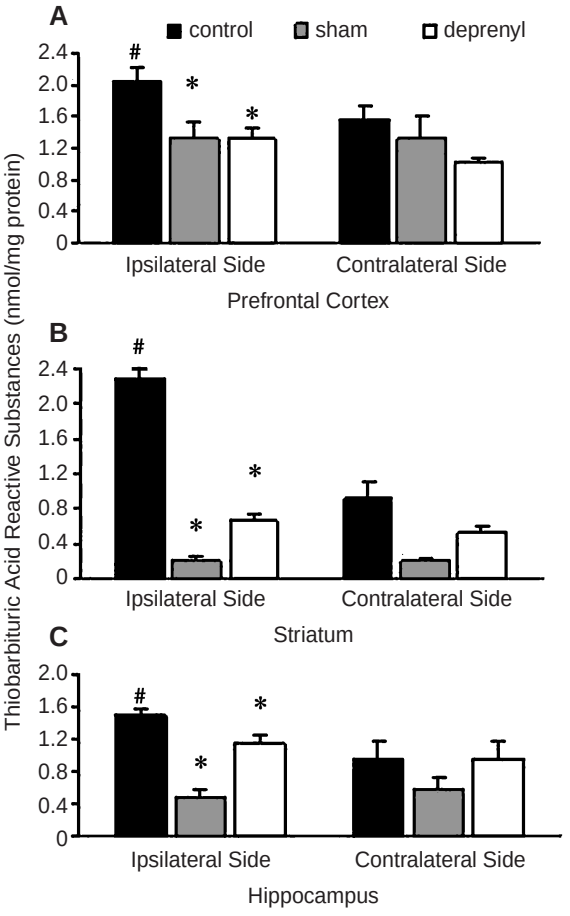

Fig. 1. TBARS levels in different brain regions from ipsilateral and contralateral hemispheres 24 h after ischemia in rats. Data are means ± SEM. $^{*}P < 0.05$ compared to control group. $^{\#}P < 0.05$ compared to contralateral side control group.

Statistical Analysis

Results are presented as means ± SEM. All data were analyzed by one-way analysis of variance followed by the Tukey test. *P* smaller than 0.05 was considered to be significant.

Results

To examine the protective effect of deprenyl, TBARS levels of ipsilateral and contralateral hemisphere were determined. Fig. 1A, B and C present TBARS levels in prefrontal cortex, striatum and hippocampus regions, respectively. After 24 h of reperfusion, TBARS levels in ipsilateral hemisphere of control rats were significantly increased, as compared to those in sham-operated rats ($P < 0.05$).

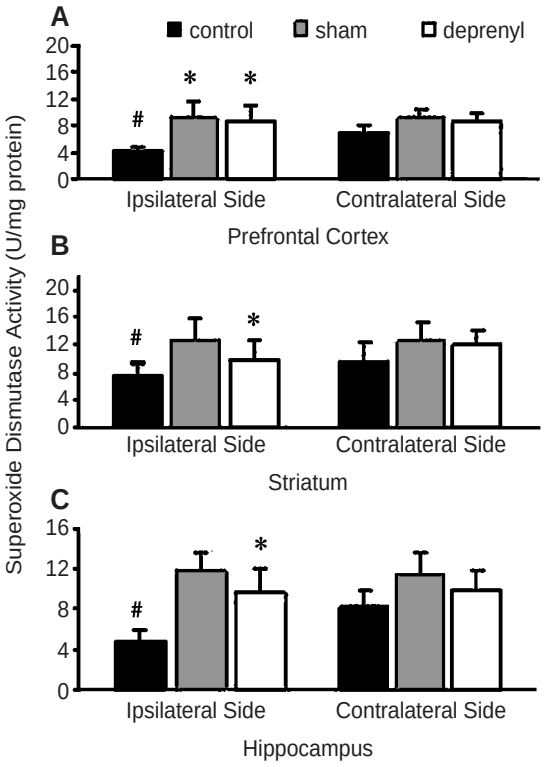

Fig. 2. Effects of deprenyl on SOD activity in different brain regions from ipsilateral and contralateral hemispheres 24 h after ischemia in rats. Data are means ± SEM. *$P < 0.05$, as compared to control group.

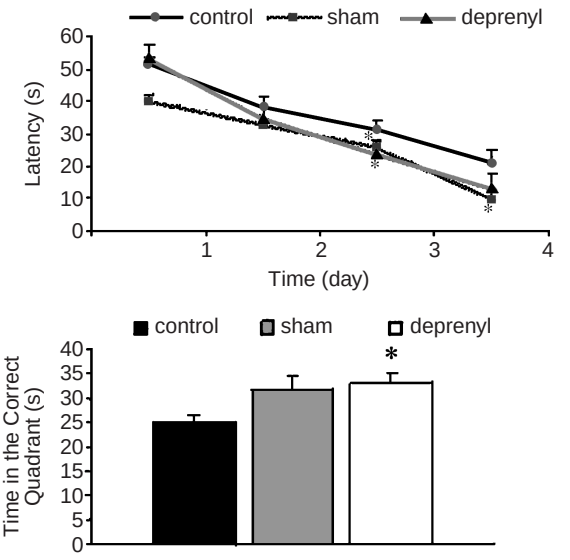

Fig. 3. Escape latency for escape trials (A) and time spent in the correct quadrant during the probe trial (B). Data are means ± SEM. *$P < 0.05$ compared to control group.

Deprenyl treatment caused a significant decrease in TBARS levels of these brain regions when compared to the control group ($P < 0.05$). No significant increase in lipid peroxidation was found in any brain region after 7 days of reperfusion when compared with that of the control group (data not shown).

Fig. 2A, B and C represent the prefrontal cortex, striatum and hippocampus SOD activities, respectively. The 30-min cerebral ischemia and 24 h of reperfusion caused a decrease in SOD activity of ipsilateral hemisphere, as compared with contralateral control group. Deprenyl-treated rats showed a significant increase in SOD activities, as compared to the control group ($P < 0.05$). No significant change in SOD activity was found in brain regions after 7 days of reperfusion, when compared with control group (data not shown).

The effects of deprenyl on latency times in the Morris water maze are presented in Fig. 3A. The latency to reach the platform declined progressively throughout four days in all groups. Deprenyl had a significant effect on escape latency during the escape trials. In the probe trial, control rats spent significantly less time in the correct quadrant than did sham-operated rats. The reduction in time spent in the correct quadrant by ischemic control rats was altered by treatment with deprenyl (Fig. 3B).

Representative photographs of TUNEL staining in the ipsilateral hippocampus CA1 area after 24 h reperfusion of ischemia are shown in Fig. 4. Sham-operated rats showed fewer TUNEL-positive cells in all brain areas studied. In the control group, the number of TUNEL-positive cells was increased significantly in CA1 region (13.4 ± 0.8%). In the deprenyl group the number of TUNEL-positive cells was reduced to 9.0 ± 0.7% (Fig. 4D). The difference of the number of TUNEL-positive cells between the control and deprenyl group was statistically significant ($P < 0.05$).

Discussion

The present study revealed that deprenyl treatment showed a protective effect against ischemia-induced oxidative stress, spatial memory impairment and neuronal apoptosis. The brain tissue is highly vulnerable to oxidative stress because of its oxidative damage potential (12). ROS are known to be excessively induced during ischemia and reperfusion. Cerebral ischemia reperfusion results in neuronal death associated with increased production of ROS which occurs preferentially in some brain regions. Lipid peroxidation is one of the determinants of ROS induced oxidative damage. Our study showed that the TBARS levels increased in the left hemisphere of the saline treated group 24 h after ischemia. The findings of the study reported here support those of other investigators who demonstrated that increased lipid peroxidation occurs at 24 h of reperfusion in brain ischemia. Candelario-

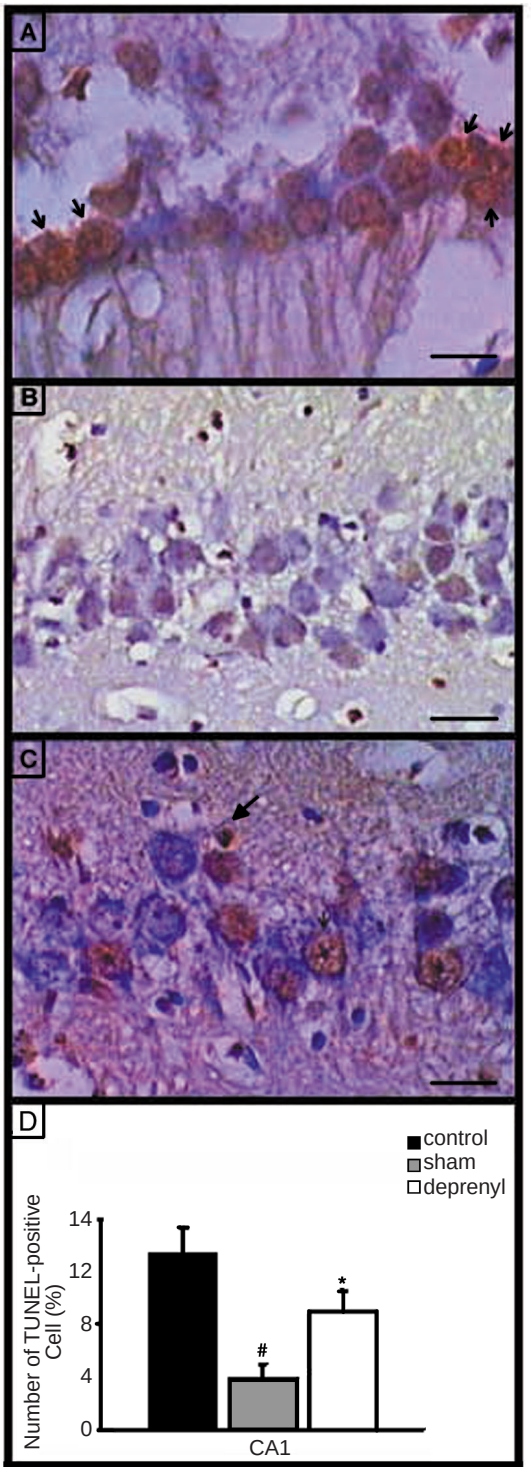

Fig. 4. Representative photomicrographs of 6 μm thick coronal sections of the rat hippocampal CA1 region after ischemia-reperfusion. A, B and C show the sections of control, sham and deprenyl groups, respectively. The arrows show dark-brown colored TUNEL-positive cells stained with TUNEL (scale bar = 20 μm). D: Effect of deprenyl on the number of TUNEL-positive cells in the hippocampus after ischemia-reperfusion. Data are means ± SEM. *$P < 0.05$ compared to control group. CA1: Hippocampal CA1 region

Jalil *et al*. reported that cerebral ischemia results in marked increase in lipid peroxidation at 24 h of reperfusion and no significant increase after 7 days of reperfusion in brain regions (7). Age-related increase in lipid peroxidation decreases with deprenyl treatment in aged rats (16, 17). In global brain ischemia models, it has been demonstrated that deprenyl can prevent higher lipid peroxidation in the rat hippocampus and the synaptosomes of the gerbil hippocampus (19, 23). In this study deprenyl treatment significantly decreased lipid peroxidation in all the three brain regions.

The organism has some protective defense enzymes and repair systems against oxidative damage. The activities of antioxidant enzymes decrease in brain regions of rats under ischemia (14). Our results have shown a significant decrease in prefrontal cortex, striatum and hippocampus SOD activities 24 h after ischemia. Deprenyl treatment elicited a significant increase in SOD activities in these brain regions. Deprenyl is known to upregulate activities of antioxidant enzymes such as SOD and CAT in specific brain regions. However, there is no direct data addressing the effect of deprenyl on antioxidant enzyme activities under ischemic rat brains (2, 18). In this study we showed that deprenyl treatment was effective in free radical damage under ischemia-reperfusion. Deprenyl treatment did not change SOD activities in these brain regions after 7 days of reperfusion. The reason behind this could be a long-term compensatory mechanism of antioxidant enzyme activity modulation (7).

Cerebral ischemic injury is known to induce memory impairment. Global ischemia in rodents leads to deficits in spatial learning and memory in the water maze. Selective cell death of the CA1 hippocampal neurons due to ischemia lead to an impairment of spatial learning (4, 6). In the present study, the effect of deprenyl on the ischemia-induced spatial memory impairment was evaluated using a Morris water maze task. The latency was shortened by ischemia-reperfusion, and significantly improved by deprenyl treatment. This means that the deficit in spatial memory induced by ischemia-reperfusion was reduced by deprenyl. Cerebral ischemia leads to an increase in reactive species production. Therefore oxidative stress might play a role in neuronal damage and contributes to cognitive deficits in ischemia- reperfusion injury. In the present study, lipid peroxidation levels were significantly increased whereas superoxide dismutase activities were markedly reduced in the ischemic rat brains. Deprenyl treatment returned the TBARS levels and reduced superoxide dismutase activity towards to control values. These protective effects of deprenyl against oxidative stress may be related to the increasing ischemia-induced spatial memory impairment. Several studies have shown that selegiline improves learning and memory impairment induced by pharmacological

treatments or age (20, 21, 31). Deprenyl reduces behavioral and cognitive deficits produced by cerebral ischemia when combined with housing in enriched environment or administered alone (23, 28).

Cerebral ischemia is caused by deprivation of oxygen and glucose, resulting in neuronal cell death. Apoptosis is a process of self-destructive cell death and is involved in ischemia-reperfusion injury and neurodegenerative disorders. The morphological characteristics of neuronal apoptosis are nuclear DNA fragmentation, membrane blebbing, cell shrinkage and condensation of nucleus (22, 26). In the present study, the number of TUNEL-positive cells in the ipsilateral ischemic hemispheres of control rats was significantly increased compared to sham rats. This indicates that ischemia induces apoptotic neuronal cell death in the hippocampus. Deprenyl treatment significantly suppressed the ischemia-induced apoptosis in this brain region. Neuroprotective effects of deprenyl have been reported using a variety of experimental ischemia models (11, 27, 33).

In conclusion, the results show that prophylactic treatment of deprenyl can improve spatial memory impairment and alleviate apoptotic neuronal death in a transient cerebral ischemia model in rats. Deprenyl treatment before cerebral ischemia shows a potent protection against oxidative stress through increasing SOD activity parallel to reducing lipid peroxidation. Deprenyl thus prevents spatial memory deficits and neuronal loss. Further studies are needed to demonstrate clinical implications of deprenyl and therapeutic potential of the drug in the treatment of cerebral ischemia.

Acknowledgments

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