

# Effect of L-Deprenyl on the Putrescine Level and Neuronal Damage after Transient Global Cerebral Ischemia in Gerbils

Hyung Lee<sup>1</sup>, Yeun-Kyung Chu<sup>2</sup>, Joon-Ho Shon<sup>2</sup>, Kyung-Hee Chun<sup>3</sup>, Jee-In Kim<sup>4</sup>, Seong-Ryong Lee<sup>5\*</sup>

<sup>1</sup>Department of Neurology, School of Medicine and Brain Research Institute, Keimyung University, Daegu, Korea

<sup>2</sup>School of Kinesiology, Yeungnam University, Gyeongsan, Korea

<sup>3</sup>Department of Medical Education, School of Medicine, Yeungnam University, Daegu, Korea

<sup>4</sup>Department of Molecular Medicine, Obesity-Mediated Disease Research Center, School of Medicine, Keimyung University, Daegu, Korea

<sup>5</sup>Department of Pharmacology, Obesity-Mediated Disease Research Center, School of Medicine, Keimyung University, Daegu, Korea

Email: \*srlee@dsmc.or.kr

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## Abstract

L-Deprenyl is selective and irreversible monoamine oxidase B inhibitor, known to have neuroprotective properties. Putrescine, one of polyamine, is thought to be important in the neuronal cell damage associated with various type of excitatory neurotoxicity. We examined the effects of L-deprenyl on the changes in putrescine level and neuronal damage after transient global ischemia in gerbils. Male Mongolian gerbils weighing 65 - 75 g were used in the experiment. Global ischemia was induced by occlusion of common carotid arteries for 3 min to observe neuronal injury in hippocampal pyramidal cells. L-Deprenyl group was given 10 mg/kg of L-deprenyl intraperitoneally immediately after, 3 h and 6 h after global ischemia. Treated animals were processed in parallel with ischemic animals receiving saline as a vehicle and with sham-operated controls. Hippocampal putrescine level was increased by global ischemia and inhibited by L-deprenyl treatment. In histological findings, counts of viable neurons were made in the pyramidal cell layer of the hippocampal CA1 area 3 days after ischemic insult. The number of viable neurons in the pyramidal cell layer of CA1 area was significantly increased in animals treated with L-deprenyl compared to vehicle-treated ischemic animals ( $p < 0.05$ ). In terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay, semiquantitative analysis of dark-brown neuronal cells was made in the hippocampal CA1 area. There was also a significant difference in the degree of TUNEL staining in the hippocampal CA1 area between vehicle-treated and L-deprenyl-treated animals ( $p < 0.05$ ). These data show L-deprenyl is effective as a prophylactic treatment for neuronal injury

when it is administrated before ischemia but a further study need to know the effects of administration of L-deprenyl after ischemia and at given times after reperfusion.

## Keywords

L-Deprenyl, Polyamine, Global Ischemia, Hippocampus, Gerbil, Neuroprotection

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## 1. Introduction

The naturally occurring polyamines in mammalian cells are putrescine (PU), spermidine (SD), and spermine (SM) that play an essential role in the process of cellular growth, development, and differentiation [1] [2]. Endogenous polyamines have multiple effects in the central nervous system and have been suggested to be neurotransmitters or neuromodulators [3]. Various kinds of stressful stimuli including stresses, seizures, excitotoxic conditions, and traumatic brain injuries increase the polyamines responses [4]-[10]. The changes in brain polyamine levels after brain ischemia have been studied [11] [12] [13] and polyamines, especially putrescine is thought to be important in the generation of brain edema, blood-brain barrier breakdown and neuronal cell damage associated with various type of brain injury including brain ischemia and trauma [4] [5] [6] [11] [12] [14]. Strategies including the inhibition of polyamine metabolism have been reported to have neuroprotective effect against ischemic neuronal injury [6] [11] [15].

L-Deprenyl (Selegiline) is a selective an irreversible inhibitor of monoamine oxidase-B (MAO-B) [16] [17] and a useful form of adjunct therapeutic drug to levodopa in the symptomatic treatment of Parkinson's disease [18]. And it has several other characteristics, which are independent of its action on MAO-B, include antioxidant action, induction of scavenger enzyme activity, and this may partially explain the described neuroprotection of L-deprenyl [19] [20] [21] [22]. L-Deprenyl is known to reduce the neurodegeneration in nigrostriatal dopamine system after chronic administration and recover the neurological symptoms of Alzheimer's disease [23] [24]. In addition, L-deprenyl showed anticonvulsive effect against various seizure models in mice [25]. However, the protective effect of L-deprenyl on the ischemic neuronal damage is controversial. This study was conducted to investigate whether L-deprenyl can attenuate the changes in PU level and neuronal damage following transient global ischemia in gerbils.

## 2. Materials and Methods

### 2.1. Animals and Drug Administration

Male Mongolian gerbils (*Meriones unguiculatus*) weighing 65 - 75 g (10-week old) were used in this study. These animals were housed in laboratory cages and maintained on a 12-h light-dark cycle, with *ad libitum* access to food and water

throughout the study period. The gerbils were treated with L-deprenyl (10 mg/kg, i.p., purchased from RBI Laboratories, Natick, MA, USA). L-Deprenyl group was given 10 mg/kg of L-deprenyl intraperitoneally immediately after, 3 h and 6 h after global ischemia. L-Deprenyl was dissolved in normal saline. In the ischemic control groups, the vehicle (normal saline, i.p.) was administered to gerbils according to the same schedule of L-deprenyl.

## 2.2. Surgery for Transient Global Ischemia

Gerbils were sacrificed 3 days after global ischemia. Gerbils were anesthetized with isoflurane (3% for induction and 1.5% for maintenance) with N<sub>2</sub>O (70%) and oxygen (30%). In the supine position, a midline ventral incision of 2 cm was made in the neck. Both common carotid arteries were exposed, separated carefully from the vagus nerve and vein and occluded for 3 min with micro-clips [26]. Blood flow during the occlusion and reperfusion after removal of the clips was confirmed visually and the incision was closed. The rectal temperature was monitored and maintained at 37°C ± 0.5°C with a feedback-controlled thermostat (CMA, Stockholm, Sweden) and an incandescent light was placed over the head from the induction of anesthesia until 3 h after ischemia. In the sham-operated group, the neck incision was made only to expose both common carotid arteries without occlusion. Other procedures were identical to those of other groups.

## 2.3. Polyamine Extraction and High Performance Liquid Chromatography (HPLC) Analysis

The animals were sacrificed 6, 12, 24, or 72 h after ischemia for polyamine extraction. The brains were removed rapidly from the skull and dissected for separation of the hippocampus. The extraction procedure was carried out in ice-chilled conditions. Derivation and HPLC analysis of polyamines were based upon the previous method [9]. Each brain sample was homogenized with a glass tissue homogenizer in 10 volumes of ice-chilled 0.4 M perchloric acid containing 2 mM disodium EDTA and 1,8-diaminooctane  $4 \times 10^{-5}$  M as an internal standard. The homogenate was centrifuged at 12,000 g for 10 min, at 4°C and 100 µl of the supernatant was evaporated by a vacuum drier. The dried tissue was dissolved in 100 µl of 1 M sodium bicarbonate then derivated with 300 µl of 4-fluoro-3-nitrobenzo-trifluoride (FNBT) reagent (a mixture of 10 µl of FNBT and one ml of dimethyl sulfoxide) at 60°C for 20 min. At the end of derivation, 40 µl of 1 M histidine in 1 M sodium bicarbonate was added to the reaction mixture then the derivation continued for another 5 min to scavenge excess FNBT. After cooling the mixture in an ice basket, the N-2'-nitro-4'-trifluoromethylphenyl derivatives of polyamines were extracted twice with 2 ml of 2-methylbutane. After centrifugation (3000 g for 10 min), the organic phase was evaporated and the residue was reconstituted with 1.0 ml of methanol. The 20 µl of the methanol solution was applied to the isocratic reversed phase HPLC system (Gilson Medical Electronics, France), then the separation of NTP-polyamines was accomplished

by elution of acetonitrile-water (80:10, v:v) mobile phase at the flow rate of 1.0 ml/min within 30 min. The eluent was monitored by UV/VIS detector set at 242 nm and a Microsorb™ C18 column (5 μM, 4.6 mm × 25 cm, Rainin instrument Co., Woburn, USA) was used.

## 2.4. Histology

The gerbils were sacrificed 72 h after ischemic insult. They were deeply anesthetized with ether and perfused transcardially with cold heparinized phosphate-buffered saline (PBS, pH 7.2) and 10% formalin in PBS. The brains were removed from the skull and fixed in the same fixative for 24 h. Thereafter the brains were embedded in paraffin and representative coronal sections (6-μm thick), which included the dorsal hippocampus, were obtained using a rotary microtome. Tissue sections were stained with hematoxylin and eosin. The hippocampal CA1 damage was determined by counting the surviving pyramidal neurons. The mean number of CA1 pyramidal neurons per millimeter for both hemispheres in a section of dorsal hippocampus was calculated for each group of the gerbils.

## 2.5. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Staining

Histochemical staining for TUNEL was performed with a kit (Roche Diagnostic Co., USA). Tissue sections were deparaffinized in xylene and hydrated in a sequence of ethanol washes followed by a final wash in phosphate-buffered saline (PBS). Nuclei of tissue sections were stripped of proteins by incubation with proteinase K (20 μg/ml in 10 mM Tris/HCl, at 37°C) for 15 minutes. The slices were then washed in distilled water and PBS and incubated in 0.3% hydrogen peroxide to remove endogenous peroxidases. After equilibration, each section was incubated with 50 μl of TUNEL mixture (5 μl of terminal deoxynucleotidyl transferase (TdT) and 45 μl of fluorescence-labeled nucleotide) for 60 min at 37°C. The sections were treated with horseradish peroxidase conjugated anti-fluorescence antibodies. After a detection of double strand breaks in genomic DNA with 2,3'-diaminobenzidine tetrahydrochloride (DAB) (0.5 mg/ml in 50 mmol/L Tris-HCl buffer, pH 7.4) as a substrate for the peroxidase.

## 2.6. Statistics

Statistical analysis was performed using ANOVA followed by Scheffe's *post-hoc* test and significance refers to results where  $p < 0.05$  was obtained.

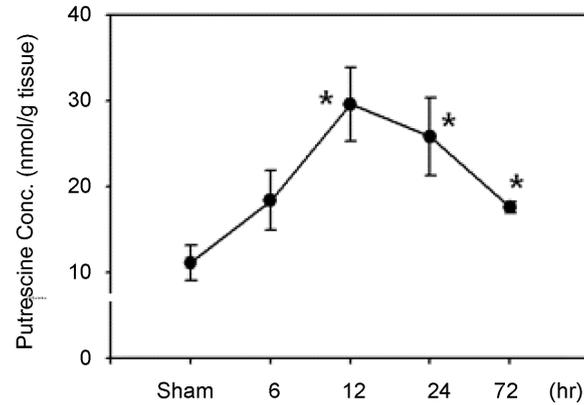
## 3. Results

### 3.1. Effect of Forebrain Ischemia on Polyamine Levels in the Hippocampus

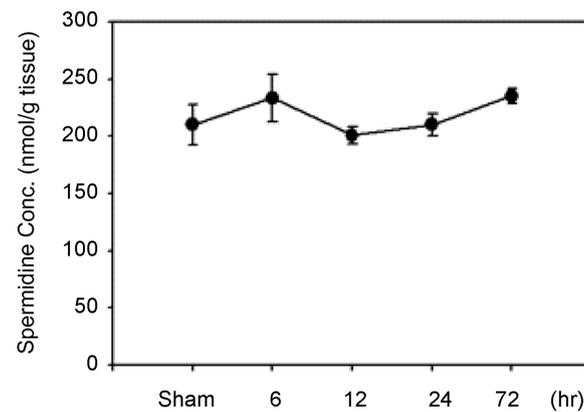
The changes in the hippocampal polyamine levels were examined 6 h, 12 h, 24 h or 72 h after ischemia. The PU level was significantly increased at 12 h, 24 h, or 72 h after ischemia (respectively,  $p < 0.05$ ). The PU level was highest 12 h after

ischemia compared with the sham-operated group (**Figure 1(a)**).

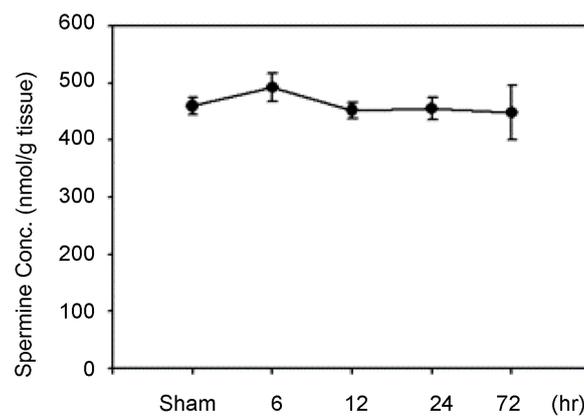
The hippocampal SD level also did not show significant changes compared with the sham-operated group (**Figure 1(b)**). The hippocampal SM levels were not also significantly changed after ischemia (**Figure 1(c)**).



(a)



(b)

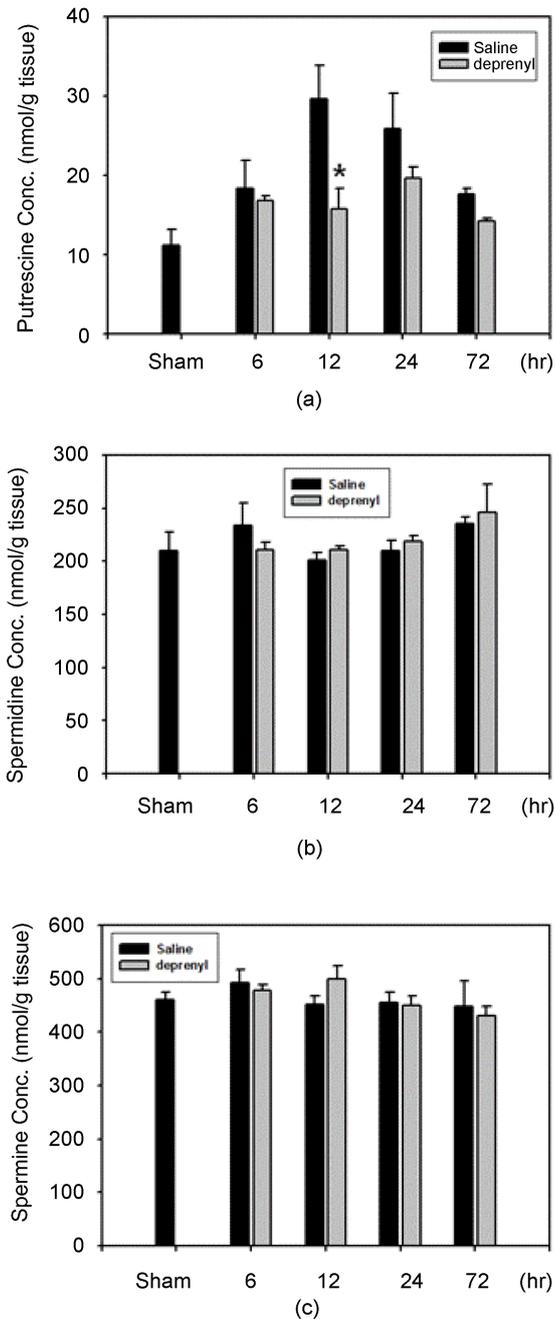


(c)

**Figure 1.** Polyamine (a) Putrescine; (b) Spermidine; and (c) Spermine) profiles of gerbil hippocampus in global ischemia (6 h, 12 h, 24 h, or 72 h after reperfusion). Polyamine levels are given in nmol/g wet tissue. N = 7, respectively. Statistically significant differences compared to sham-operated group are indicated by \* $p < 0.05$ , \*\* $p < 0.01$ . Conc: concentration.

### 3.2. Effect of Administration of L-Deprenyl in the Changes of Polyamine Levels

Administration of L-deprenyl attenuated the increases of the hippocampal PU levels at 12 h after ischemia ( $p < 0.05$ , **Figure 2(a)**). Administration of L-deprenyl did not change the SD or SM levels in the hippocampus following ischemia (**Figure 2(b)** and **Figure 2(c)**).



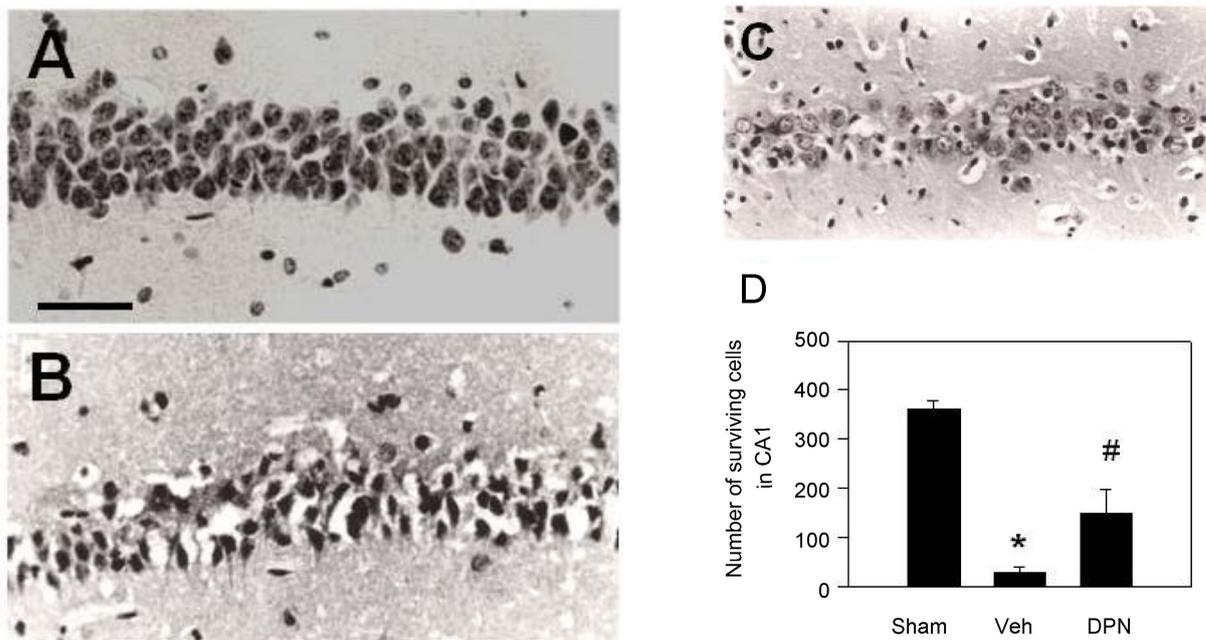
**Figure 2.** Changes of putrescine (a); spermidine (b) or spermine (c) levels in gerbil hippocampus after global ischemia and effect of L-deprenyl administration. Polyamine levels are given in nmol/g wet tissue.  $N = 7$ , respectively. Statistically significant differences compared to saline-treated group are indicated by \* $p < 0.05$ . Conc: concentration.

### 3.3. Histological Changes

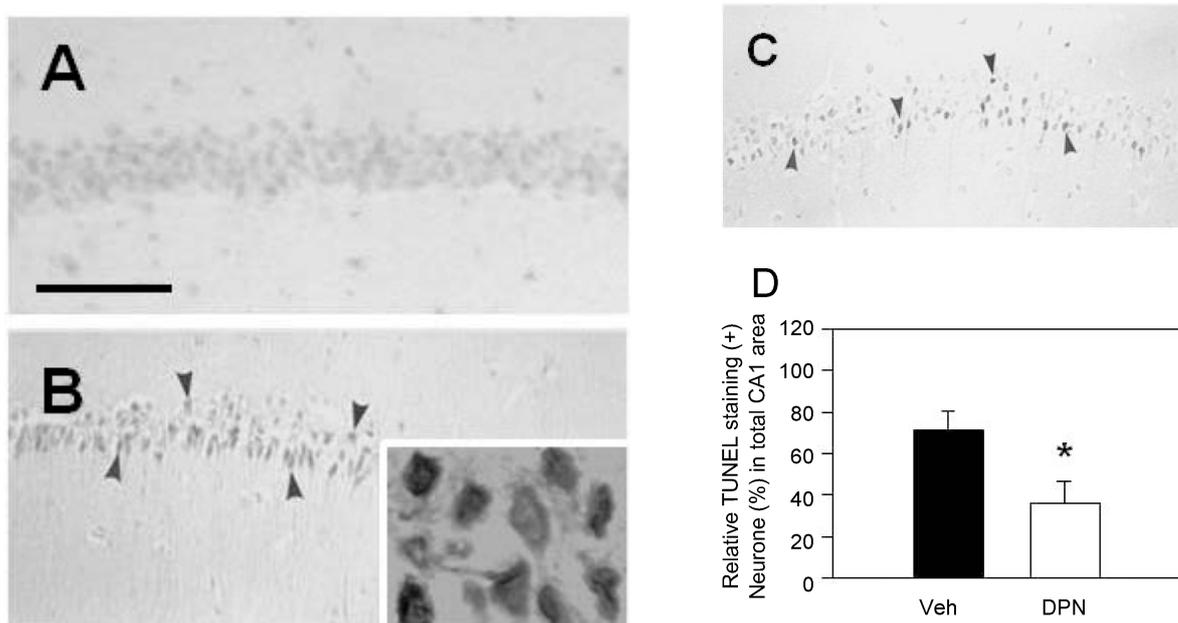
Histological examination of the nervous system demonstrated marked cell damage in the hippocampal CA1 region in the gerbils treated with a vehicle when compared with the sham-operated group (**Figure 3(a)** and **Figure 3(b)**). CA1 pyramidal neurons showed pyknosis, eosinophilia, karyorrhexia, and chromosome condensation in the vehicle-treated group (**Figure 3(b)**). This neuronal cell damage was inhibited by L-deprenyl administration. Administration of L-deprenyl administered after ischemic insult significantly reduced neuronal damage ( $p < 0.05$ , **Figure 3(c)** and **Figure 3(d)**).

### 3.4. TUNEL Staining

In sham-operated group, there were no TUNEL staining positive cells in the hippocampal CA1 area (**Figure 4(a)**). Numerous cells in the hippocampal CA1 area were strongly positive for TUNEL staining after global ischemia and vehicle-treated group (**Figure 4(b)**). The number of normal viable cell in the global ischemia and L-deprenyl-treated group was more than that in the global ischemia and vehicle-treated group (**Figure 4(c)**). For semi-quantification, L-deprenyl administration significantly decrease the TUNEL staining-positive cells in compared with vehicle-treated group (**Figure 4(d)**,  $*P < 0.05$ ).



**Figure 3.** Protective effect of L-deprenyl on global ischemia-induced neuronal damage in hippocampal CA1 area of gerbil. Hippocampal CA1 pyramidal neurons in sham-operated group (n = 7) (a); Hippocampal CA1 pyramidal neurons in global ischemia and vehicle administration group (n = 10) (b); Hippocampal CA1 pyramidal neurons in global ischemia and L-deprenyl administration (n = 10) (c); Remarkable reduction in the number of viable neurons at the 3 days after the ischemic episode when compared with sham-operated controls. Administration of L-deprenyl attenuated the ischemic neuronal injuries (d); After transient global ischemia, there are remarkable reduction of surviving neurons in gerbil hippocampus when compared with sham-operated controls ( $*P < 0.05$ ). Significant increase of surviving neurons in CA1 area is observed in DPN animals when compared with Veh ( $\#P < 0.05$ ). Data are presented as the mean  $\pm$  S.E.M. Sham: Sham-operated animals, Veh: vehicle-treated animals, DPN: L-deprenyl treated animals. Scale bar = 50  $\mu$ m.



**Figure 4.** Effect of L-deprenyl on TUNEL staining in the hippocampal CA1 subsector 3 days after transient global ischemia in gerbil. Hippocampal CA1 pyramidal neurons in sham-operated group (n = 7) (a); Hippocampal CA1 pyramidal neurons in global ischemia and vehicle administration group (n = 10) (b); Hippocampal CA1 pyramidal neurons in global ischemia and L-deprenyl administration (n = 10) (c); Remarkable increase in the number of TUNEL staining (+)-cells at the 3 days after the ischemic episode when compared with sham-operated controls. Administration of L-deprenyl attenuated the TUNEL staining (+)-cells (D). Significant decrease of TUNEL staining (+)-cells in CA1 area is observed in L-deprenyl-treated animals when compared with vehicle-treated animals (\*P < 0.05). Data are presented as the mean  $\pm$  S.E.M. Veh: vehicle-treated animals, DPN: L-deprenyl-treated animals. Scale bar = 50  $\mu$ m.

## 4. Discussion

L-Deprenyl, also named selegiline, is selective and irreversible inhibitor of monoamine oxidase B (MAO-B) widely used in the management of Parkinson's disease (PD). L-Deprenyl is known to have neuroprotective properties, including antioxidant, anticonvulsant actions.

Degeneration of nigrostriatal dopaminergic neurons is the major pathogenic substrate of PD. Inhibitors of monoamine oxidase B (MAO-B) have been used in the treatment of PD and at least one of them, *i.e.*, L-deprenyl, also displays antioxidant activity. Dopamine (DA) autoxidation produces reactive oxygen species implicated in the loss of dopaminergic neurons in the nigrostriatal pathway. But there is little report that L-deprenyl has neuroprotective effect against delayed neuronal injury in global ischemia.

These results show L-deprenyl is effective as a prophylactic treatment for neuronal injury when it is administered before ischemia but a further study need to know the effects of administration of L-deprenyl after ischemia and at given times after reperfusion.

### 4.1. Effects of Administration of L-Deprenyl on the Changes of Polyamine Level in Gerbil Brain Regions after Transient Global Ischemia

It is suggested that polyamines released from necrotic neurons into the extracel-

lular compartment bind to the NMDA receptor of cells located in close vicinity and thus render neurons vulnerable to subtoxic levels of excitotoxins. Several researchers examined the changes in brain polyamine levels after focal or global ischemia [6] [11] [13]. Various kinds of stimuli or stresses such as seizures, excitotoxicity, and traumatic brain injury modify the ornithine decarboxylase (ODC), the regulatory enzyme in the polyamine biosynthesis [4] [5] [10] [27]. These changes may be related to modifications of intracellular calcium ion fluxes because polyamines increase the cytosolic amino acids. Some authors have shown discrepancies between ODC activity and the concentration of polyamine [28], a finding suggesting that the latter might be more useful than the former.

In this study, PU levels in cortex and hippocampus increased after transient global ischemia. These changes in PU levels bear a strong similarity to those described by Paschen *et al.* [13]. The diamine precursor of polyamines, PU is normally in low level and long lasting accumulation of PU may be harmful [13]. An association between brain damage and high PU levels in the ischemic brain has also been found previously suggesting a role for PU in mediating the ischemic damage. ODC and polyamines are thought to be important in the generation of edema and neuronal cell loss associated with cerebral ischemia [13]. Baskaya *et al.* [5] suggested that polyamines may play a role in posttraumatic brain edema formation particularly in brain regions.

Polyamines are known to increase cytosolic calcium ion concentration [29] [30] and induce the release of excitatory amino acid [27]. A remarkable increase of the extracellular concentration of excitatory amino acids including glutamate, induced by cerebral ischemia leading to a large amount of calcium ion influx through glutamate receptor in neurons and neuronal injury [31]. PU levels particularly correlate with the density of cell necrosis [13]. PU might be a reliable marker for acute pathology in brain tissue injury [32]. Tissue PU increased in the penumbra region that developed brain edema in permanent focal cerebral ischemia [6]. In addition, the blockade of ODC resulted in a protective effect against focal or global ischemic brain damage [15] and partially antagonized the convulsant activity [33] suggesting that polyamine metabolism plays a role in the development of neuronal injuries following brain ischemia or epileptic seizure. In regarding the effect of L-deprenyl on the PU level, although there is no definite evidences, we can suggest two possibilities. First, L-deprenyl attenuates the harmful accumulation of PU by influence on the polyamine metabolism. Second, L-deprenyl-induced neuroprotection due to antioxidant effect or anti-apoptotic effect may decrease the PU response to excitotoxicity.

In this study, SD and SM levels in the cortex and hippocampus showed no significant changes after ischemia. These are in agreement with results of de Vera *et al.* [33] and Paschen *et al.* [12]. In addition, L-deprenyl did not show any influences on the SD and SM levels. Activation of interconversion pathway enzymes, SD/SM N<sup>1</sup>-acetyltransferase [34] and PA oxidase [35] which convert SM to SD and SD to PU, is a probable major factor in PU accumulation [36]. In addition, SD has been shown to bind to NMDA-gated calcium ionophores in-

creasing also the affinity of [<sup>3</sup>H]-MK-801 for NMDA receptor [3] [37]. Therefore, the reduction of SD level may be a compensatory mechanism unrelated to the ODC activation against ischemic insult. Tissue PU levels change to a remarkable degree than those of SD and SM after various pathological conditions [4] [5] [6] [9] [12] [14] [30].

The results of this study suggest that administration of L-deprenyl early after ischemia is effective to attenuate the increase of PU levels because polyamine biosynthesis increased rapidly after ischemia. However, the role of polyamines, especially PU, in the pathogenesis of brain ischemia is not clear and needs to be further studied.

#### **4.2. Effect of Administration of L-Deprenyl against Neuronal Damage after Transient Global Ischemia**

The present study shows the neuroprotective effect of administration of L-deprenyl against ischemic neuronal damage. Many researchers have described the effects of L-deprenyl in inhibition of free radical-induced lipid peroxidation and apoptosis in neural tissue. Recently, the antioxidant effects of L-deprenyl were extensively studied. L-Deprenyl protects lipid peroxidation [22] and increases the scavenging effect of anti-oxidant enzymes [38]. Wu *et al.* [39] reported L-deprenyl-induced facilitation of nigral neuron recovery by its anti-oxidant effect. In addition, L-deprenyl has been shown to protect the neuronal injury and function after exposure to beta-amyloid protein [40].

In transient global ischemia, neuronal damage shows mainly apoptotic pathway in hippocampal pyramidal cell layer [41] [42]. In previous studies, L-deprenyl shows anti-apoptotic effect in various neuronal damage models [43]. In this study animals that received L-deprenyl treatment displayed a significant decrease in the number of TUNEL staining positive neurons in the hippocampal CA1 region.

It has been well known that oxygen radical-induced lipid peroxidation has been strongly suggested to play a role in ischemic neuronal damage [44] [45]. Recently, a variety of studies have examined the neuroprotective properties of antioxidants in brain ischemia [46] [47]. These results demonstrated that administration of L-deprenyl has neuroprotective effect against transient global ischemia-induced neuronal injury in gerbils. L-Deprenyl attenuated the increase of putrescine level in the cerebral cortex and hippocampus and the neuronal damages in the hippocampal CA1 region after ischemia. Administration of L-deprenyl did not show complete neuroprotection, it seems to be a promising strategy for attenuation of global ischemia-induced neuronal injury.

The present data show that the administration of L-deprenyl attenuates the ischemia-induced increases in PU levels and has a neuroprotective effect against hippocampal neuronal damage in a gerbil model of global ischemia. L-Deprenyl is neuroprotective against neuronal damage after transient global ischemia. These findings suggest that L-deprenyl may have a promise in the acute treatment of stroke.

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