의학박사 학위논문

Post-ischemic administration of pravastatin reduces neuronal injury by inhibiting Bax protein expression after transient forebrain ischemia in rats

백서의 일과성 전뇌허혈 모델에서 허혈 후 프라바스타틴 투여로 인한 Bax 단백질 발현 억제에 의한 신경 세포 손상 감소

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Abstract

Introduction: This study investigated the neuroprotective effect of pravastatin administration after forebrain ischemia in rats.

Materials and Methods: Forebrain ischemia was induced by bilateral common carotid artery occlusion and systemic hypotension for 8 min. Pravastatin at 1 mg/kg (pravastatin group, n = 10), or an identical volume of normal saline (control group, n = 10), was injected 10 min, and 1−4 days after reperfusion. Arterial blood gas was analyzed 10 min before ischemia onset and 10 min after ischemia completion. Viable and apoptotic neuronal cells were evaluated 7 days after ischemia by hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase (TdT)−mediated deoxyuracil triphosphate biotin *in situ* nick−end labeling (TUNEL) staining of the hippocampal Cornu Ammonis area (CA1). Expression of Bcl−2 and Bax proteins was quantified by Western blot analysis.

Results: The proportion of viable neuronal cells after ischemia was greater in the pravastatin vs. control group ($p < 0.01$), with greater expression of apoptotic cells in the control vs. pravastatin group ($p < 0.05$). Bax protein expression was significantly decreased in the pravastatin group ($p < 0.05$), whereas Bcl−2 expression was increased, but not significantly ($p > 0.05$).

Conclusions: Our findings suggest that pravastatin administration after forebrain ischemia confers neuroprotection in rats by inhibiting Bax protein expression.

Keywords: apoptosis, Bax protein, brain ischemia, pravastatin

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Introduction

Statins have generated interest for their potential to promote pharmacologic neuroprotection. As well as lowering low-density lipoprotein (LDL) cholesterol levels, statins also play a pleiotropic role by promoting endothelial nitric oxide synthase (eNOS) and scavenging reactive oxygen species (ROS) expression, preventing inflammation and promoting antithrombosis [14, 28]. Recent studies have demonstrated that prophylactic statins can inhibit the apoptotic pathway following brain injury [4, 7]. Sugiura et al. [28] demonstrated that post-ischemic administration of statins inhibits infarct expansion after focal brain ischemia in rats. In a rat forebrain ischemia model, preemptive pravastatin treatment for 14 days significantly reduced delayed neuronal death in the hippocampus [5].

Global cerebral ischemia typically occurs following cardiac arrest, shock, asphyxia and complex cardiac surgery [9]. Its clinical course depends on the severity of the insult and the effectiveness of immediate resuscitation and post-resuscitation management; however, prognosis is generally poor [10]. Global cerebral ischemia after cardiocirculatory arrest is a major cause of death following cardiopulmonary resuscitation [25]. If a pharmacologic agent could reduce the ischemic territory or improve neurological outcome following global cerebral ischemia, it would be useful for patients at

risk of cerebral ischemia–reperfusion injury. However, no drug has been proven to reduce neuronal injury after global cerebral ischemia in humans, *in vivo* [1, 15]. Predicting global cerebral ischemia in clinical practice is problematic, because it occurs in medical emergencies such as cardiac arrest or hypotensive shock. Therefore, the optimum time at which to treat global cerebral ischemia would be following ischemic insult. In our current study, forebrain ischemia was induced in rats by clamping bilateral carotid arteries for modeling of global cerebral ischemia.

Pravastatin is suitable for intravenous administration due to its hydrophilic nature, with a more rapid onset compared with other orally ingested statins. Many studies have assessed the neuroprotective effects of pravastatin treatment before ischemic stroke or following focal cerebral ischemia. Pravastatin treatment reduces brain infarction and improves neurological scores following temporary middle cerebral artery occlusion in rats [2, 28]. Pravastatin treatment in rats before transient forebrain ischemia significantly reduced delayed neuronal death [5]. However, there has been no report on the effects of pravastatin therapy after forebrain ischemia. We herein investigate the effect of post-ischemic pravastatin treatment (1 mg/kg) on neuronal injury in rats following transient forebrain ischemia.

Material and Methods

This experiment was approved by the Seoul National University Hospital Institutional Animal Care and Use Committee and performed according to the Guide for the Care and Use of Laboratory Animals.

1. Surgical Preparation

A total of 24 male Sprague-Dawley rats, weighing between 300-320 g and with a gestational age between 9-11 weeks, were used. Rats were housed under a 12-h day-night schedule with a constant temperature maintained, and were fasted for 12-16 h before the experimental procedure began, with free access to water. Anesthesia was induced using oxygen and 5% sevoflurane, and maintained by intraperitoneal Zoletil injection. A 22-gauge needle thermistor was inserted subcutaneously under the temporalis muscle, and the pericranial temperature was monitored and servo-regulated (model TCAT-2 Temperature Controller; Harvard Apparatus, Holliston, MA) at 37° C by heating or cooling. The tail artery was cannulated with a PE-50 catheter for continuous blood pressure monitoring and serial blood testing during the experiment. A silicone catheter was inserted into the right internal jugular vein

for drug injection and exsanguination. Bilateral carotid arteries were exposed with strings applied for ischemia.

2. Transient Global Cerebral Ischemia

After stabilizing for 30 min, heparinization (50U) was performed, and the rats were then exsanguinated to reduce the mean arterial blood pressure to 30 mmHg. Forebrain ischemia was induced for 8 min by clamping both carotid arteries with surgical clips [12, 26]. After ischemia, the clips were carefully removed and blood was re-infused slowly. Ten minutes before and after ischemia, arterial blood gas and hemoglobin were measured.

3. Group Assignment

The two groups scheduled for ischemia (n = 20) were randomized into five blocks of four rats, with the sham procedure (n = 4) performed at the interval of each block (Fig. 1). The sham group underwent identical anesthesia and surgical preparation but ischemia was not induced. In the pravastatin group (n = 10), 1 mg/kg of pravastatin was dissolved in 1 ml of normal saline and injected five times: intravenously at 10 min, and intraperitoneally at 1-4 days after reperfusion [2]. In the control group (n = 10), 1 ml of vehicle solution (normal saline) was injected via the same route during the same time period following reperfusion.

4. Histopathological Analyses

After a 7-day post-ischemia recovery period, rats were anesthetized and brains were fixed in situ by intra-aortic perfusion of normal saline (100 ml) and 10% formalin (200 ml). Heads were removed, with brains separated from the skull and sagitally cut into two parts using the rat brain slice matrix. The right side of the brain was fixed in buffered 10% formalin for histopathological staining. The hippocampus was extracted from the left side of the brains, frozen in liquid nitrogen and stored at −80° C for Western blot analysis of Bax and Bcl-2 proteins.

The right side of the brain was embedded in paraffin and serially cut into 5 μm-thick sections. Cornu Ammonis area 1 (CA1) of the hippocampus, which is the most vulnerable area to ischemic insult, was evaluated. Brain sections were stained with hematoxylin and eosin (H&E) for evaluation of the necrotic and viable neuronal cells. For each animal, three optical fields were examined under a microscope (400×). To detect DNA fragmentation, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil triphosphate biotin *in situ* nick-end labeling method (TUNEL) of staining was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100; Millipore Corp., Billerica, MA) according to the manufacturer's instructions. For each animal, three optical fields were again examined under a microscope (400×). An investigator blinded to the assigned group examined four brain

tissue slides (two H&E, two TUNEL staining) per animal using light microscopy. The total number of neurons was counted for each animal. Cell necrosis was defined as pyknosis, karyolysis, or eosinophilic cytoplasm on H-E staining. The proportion of necrotic CA1 neurons was calculated as necrotic (pyknotic or karyolitic or eosinophilic) neurons × 100/total neurons. Apoptotic cells were identified as TUNEL-positive cells with brown-stained nuclei. The percentage of apoptotic CA1 neurons was calculated as apoptotic neurons × 100/ total neurons.

5. Western blot Analysis

Western blotting was performed with monoclonal anti-rabbit antibody against Bcl-2 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse antibody to Bax (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's instructions. The blots were processed with anti-rabbit IgG (whole molecule) - peroxidase antibody produced in goat (1:2000 dilution, A0545, Sigma-Aldrich, St.Louis, MO) and anti-mouse IgG (whole molecule) - peroxidase antibody produced in goat (1:2000, A4416, Sigma-Aldrich, St.Louis, MO). Forty microgram of each protein was electrophoresed. Proteins were detected using Amersham ECL Kit (Amersham, UK). The optical intensity of the band was quantified by densitometric analysis. β-

actin intensity levels, representing the control values, were arbitrarily set at 1.

6. Statistical Analyses

All data were expressed as means ± *SD*. All physiological variables were analyzed by repeated-measures analysis of variance. Group differences were assessed using the Kruskal-Wallis tests for comparison of three groups, and Mann-Whitney U tests for comparison of two groups. A value of $p < 0.05$ was taken to indicate statistical significance.

Results

Physiological variables

Hemoglobin, PaO2, PaCO2, blood glucose, and pH did not differ among the groups before and after ischemia (Table 1).

Proportion of viable and apoptotic cells

Percentage of viable neuronal cells was significantly lower in the control vs. sham group ($p < 0.01$, Fig.2), and in the control (25 ± 9%) vs. pravastatin (47 ± 19 %, $p < 0.01$, Fig. 2) group. Proportion of apoptotic cells was significantly higher in the control vs. sham group ($p < 0.01$, Fig.3), and in the control (64 ± 9%) vs. pravastatin group (39 ± 25%, $p < 0.05$, Fig. 3).

Expression of apoptosis-related proteins (Bax, Bcl-2)

Bax protein expression was significantly higher in the control vs. sham group ($p < 0.05$, Fig.4A), whereas the Bcl-2 protein expression was increased without significance in the control vs. sham group ($p > 0.05$, Fig. 4B). When comparing the pravastatin group and the control group, Bax protein expression was significantly decreased in the pravastatin group (0.15 ± 0.08, vs. 0.92 ± 0.17 in the control group, $p < 0.05$, Fig. 4A), whereas Bcl-2 protein expression was increased in the pravastatin group without significance (0.25 ± 0.19 in the control group, vs. 0.37 ± 0.15 in

the pravastatin group, $p > 0.05$, Fig. 4B). The overall Bax/Bcl-2 ratio was 7.44 ± 5.29 in the control group, and 0.98 ± 0.68 in the pravastatin group ($p < 0.01$, Fig. 4C).

Discussion

This study provides novel evidence that pravastatin treatment after transient forebrain ischemia confers neuroprotection via an anti-apoptotic effect in rats. Administration of pravastatin after transient forebrain ischemia enhanced the percentage of viable neuronal cells and blocked the apoptotic cascade. The expression of Bax, and the Bax/Bcl-2 ratio, was decreased following pravastatin administration.

Previous studies demonstrated that statins modulate the apoptosis-related protein expression [7, 13]. Bax and its related protein Bcl-2, act as regulators of apoptosis at the mitochondrial level [7, 8, 16]. Overexpression of Bax protein following cerebral ischemia plays an important role in DNA fragmentation and delayed neuronal death [8]. Bax heterodimerizes at the Bcl-2-interacting domain of the mitochondrial membrane and induces the release of apoptogenic factors, whereas the anti-apoptotic protein Bcl-2 neutralizes Bax by interacting with it and inhibiting activation of the apoptosis signaling cascade [3]. Increased Bcl-2, and decreased Bax protein expression, have been observed in the brain cells of guinea pigs treated with simvastatin [7]. In our study, Bcl-2 expression increased non-significantly; the overall Bax/Bcl-2 ratio, which plays a pivotal role in cellular apoptosis, was significantly decreased after pravastatin treatment. Therefore, we suggest that

pravastatin administration following forebrain ischemia mediates neuroprotection by inhibiting Bax protein expression in rats.

Neuroprotection provided by statins in cerebral ischemia is a complex process in which many different pathways work closely with each other. Upregulation of eNOS activity as well as modulation of apoptosis plays a fundamental role in this process. Reduced infarction size and enhanced eNOS expression were shown after focal cerebral ischemia in simvastatin-treated rats [27]. Laufs et al. investigated the effect of rosuvastatin treatment before focal cerebral ischemia in mice; the pre-treated animals showed smaller infarct size, upregulation of eNOS mRNA level and activity [18]. Upregulation of eNOS system by statin therapy and the resultant nitric oxide (NO) production facilitate anti-inflammatory, vasodilatory and antithrombotic effects [29]. Enhanced eNOS expression can protect certain cells from apoptosis by altering the gene expression of apoptosis-related proteins [24]. Enhanced eNOS expression might have also affected the apoptosis-related protein expression in our current study, but additional studies are required for further clarification.

In our study, apoptosis and expression of Bax, Bcl-2 were evaluated 7 days after forebrain ischemia to assess the delayed neuronal death. In a previous study by Hara et al., expression of Bax peaked at 72 hours and disappeared at 96 hours following forebrain ischemia in gerbils [8], which is inconsistent with our results that showed elevated Bax level especially in the control

group 7 days after forebrain ischemia. Hara et al. measured the expression of apoptosis-related proteins by comparing the immunoreactivity of the hippocampus with the choroid plexus, which was set as the internal positive control. Since another experiment using gerbils demonstrated elevated Bax level 7 days after forebrain ischemia [20], we carefully concluded that this particular method might have underestimated the actual expression of the apoptosis-related protein in the late phase. Other experiments using rats demonstrated increased Bax expression 7 days after forebrain ischemia [6, 22] so differences among animal models might have affected the result.

Pravastatin is a hydrophilic statin that weakly penetrates into the blood-brain barrier. However, when used at the correct dosage, pravastatin therapy has a potent neuroprotective effect [2, 5]. Lipophilic statins, which induce neuronal apoptosis and delayed neuronal death, are markedly more neurotoxic compared with hydrophilic statins [5]. Due to its hydrophilic nature, pravastatin was injected intravenously in the current study.

The clinical benefits of statin therapy surpass the effect that would be expected to result from changes in serum lipid levels [7, 11, 17, 19]. Statins are effective in terms of prophylaxis [18], as well in reducing infarct volume and neurological damage following focal cerebral ischemia [2, 21, 23, 27, 28] in various animal models. Prophylactic statin treatment, before forebrain ischemia onset, reduces delayed neuronal death [5]. The present study indicates

that statin treatment after forebrain ischemia can also reduce neuronal cell death.

We repeatedly injected 1 mg/kg of pravastatin after forebrain ischemia. The precise dose of pravastatin that promotes neuroprotection *in vivo* has not been determined. In terms of prophylaxis, a higher dose of pravastatin is required. Previous studies using oral pravastatin at 20 mg/kg demonstrated significantly reduced delayed neuronal death in the hippocampal CA1 subfield of rats. Low dose pravastatin (2 mg/kg) was ineffective [5]. In a dose-finding study, various doses of pravastatin were repeatedly injected in rats after onset of focal cerebral ischemia; higher doses of pravastatin (> 0.1 mg/kg) significantly reduced infarct size, with the greatest reduction obtained at 1 mg/kg [2]. Therefore, we also used 1 mg/kg of pravastatin in the present study.

Our study has several limitations. First, serum cytokine, glutamate levels and eNOS activity were not measured. Pro-inflammatory cytokines such as IL-6 induce neuronal damage by producing neuronal NO and promoting glutamate-mediated neurotoxicity. Because statins are known to modulate cytokine production in the CNS, measuring and comparing the serum cytokine and glutamate levels of the groups would have provided additional information on the mechanism underlying the neuroprotection conferred by pravastatin. Assessment of eNOS activity would have provided more direct evidence for the relationship between eNOS activity

and apoptosis. However, the altered expression of Bax/Bcl-2 proteins and resultant inhibition of apoptosis that we observed represents a powerful explanation of the mechanism underlying pravastatin-induced neuroprotection. Because the rats were decapitated 7 days after the procedure, long-term neurological effects could not be evaluated. Further studies are required to evaluate the long-term effects of pravastatin administration following forebrain ischemia. Finally, we did not evaluate the dose-response relationship. Previous experiments have used various doses of pravastatin to elucidate the optimal dose for neuroprotection. In our study, 1 mg/kg of pravastatin reduced neuronal damage, but neuroprotection after forebrain ischemia might vary in extent if other doses are applied; this should be a target for further study.

Conclusions

Pravastatin administration following transient forebrain ischemia reduced neuronal injury in rats, probably by inhibiting the expression of the pro-apoptotic Bax protein. Our results provide new insight into the potential use of pravastatin as a pharmacologic neuroprotective agent following forebrain ischemia.

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초 록

본 연구는 백서의 전뇌허혈 모델에서 허혈 후 프라바스타틴의 투여가 신경보호적 효과가 있는지 알아보고자 실시하였다. 백서의 경동맥을 겸자하고 8 분 동안 뇌허혈을 유발한다. 뇌허혈 후 실험군(n = 10)에서는 프라바스타틴을 1 mg/kg 로, 대조군(n = 10)에서는 동량의 생리식염수를 재관류 후 10 분, 1 일, 2 일, 3 일, 4 일 5 번에 걸쳐 복강 내 투여한다. 뇌허혈 유도 10 분 전과 뇌허혈 완료 10 분 후에 동맥혈 가스 분석을 시행한다. 허혈 후 7 일째 백서의 해마 조직에서 세포괴사와 세포자멸사를 각각 hematoxylin and eosin (H&E) 염색과 terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil triphosphate biotin *in situ* nick-end labeling (TUNEL) 염색으로 분석하였다. Bcl-2 와 Bax 단백질 발현은 Western blot 으로 분석하였다. 신경 세포가 생존한 분율은 대조군에 비해 프라바스타틴을 투여한 군에서 높았으며 ($p < 0.01$), 세포자멸사는 대조군에서 프라바스타틴을 투여한 군에 비해 많이 관찰되었다 ($p < 0.05$). Bax 단백질 발현은 프라바스타틴 군에서 통계적으로 유의하게 감소되었으며 ($p < 0.05$), Bcl-2 발현은 프라바스타틴을 투여한 군에서 증가되었으나 통계적 유의성을 보이지 않았다 ($p > 0.05$). 본 연구 결과에 따라, 백서의 일과성 전뇌허혈 모델에서 허혈 후 프라바스타틴 투여는 Bax 단백질 발현 억제에 의한 기전으로 신경 세포의 손상을 감소시킨다는 것을 알 수 있다.

주요어: 뇌허혈, 세포자멸사, 프라바스타틴, Bax protein

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Table 1. Physiological Variables 10 min Before and After Ischemia.

Data are expressed as means ± *SD*.

		pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	Hemoglobin (g/dl)	Glucose (mg/dl)
Sham	before	7.38 ± .02	46 ± 2	252 ± 12	10.5 ± 0.4	116 ± 20
	after	7.35 ± .03	48 ± 4	208 ± 16	10.5 ± 0.7	
Control	before	7.36 ± .04	48 ± 5	225 ± 30	11.2 ± 1.1	110 ± 14
	after	7.30 ± .01	52 ± 4	185 ± 40	11.2 ± 1.1	
Pravastatin	before	7.37 ± .03	47 ± 5	245 ± 40	10.6 ± 0.5	113 ± 19
	after	7.34 ± .04	49 ± 6	177 ± 38	10.4 ± 0.8	

Group	Surgical preparation	Ischemia	Reperfusion and administration of drugs				
Sham (n=4)	Surgical preparation						
Control (n=10)	Surgical preparation	Occlusion	N/S	N/S	N/S	N/S	N/S
Pravastatin (n=10)	Surgical preparation	Occlusion	P	P	P	P	P
Time	30 min	8 min	10 min	Day 1	Day 2	Day 3	Day 4

Figure 1. Illustration of the experimental protocol. P = pravastatin 1mg/kg, N/S= same volume of normal saline

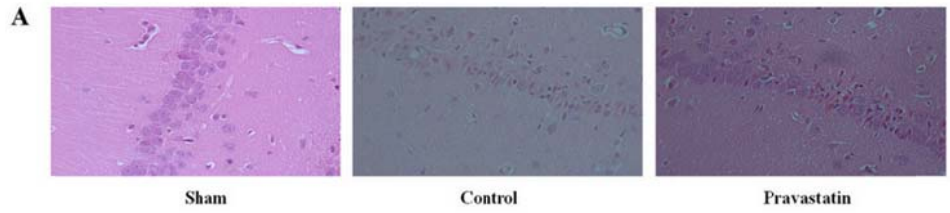

Figure 2A. Representative photomicrographs of H&E stained hippocampus CA1 area 7 days after forebrain ischemia.

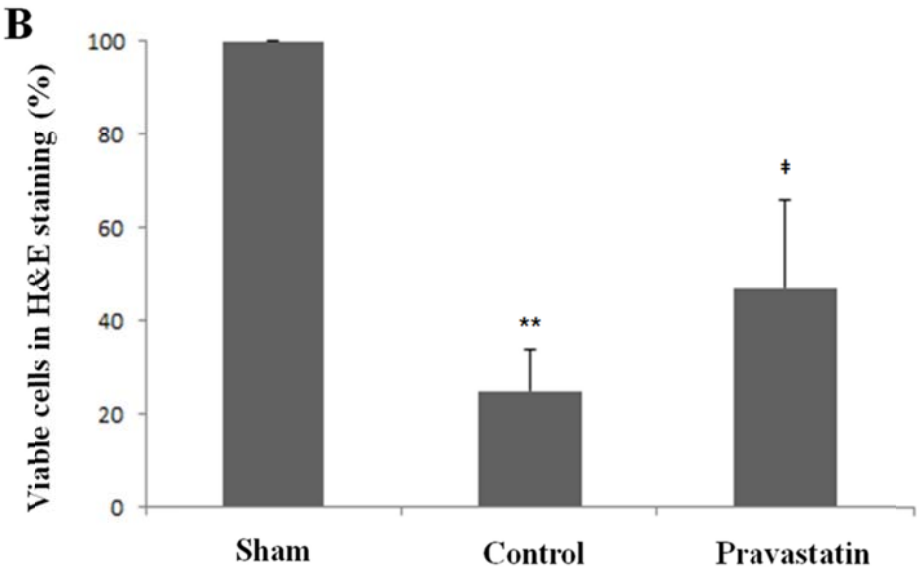

Figure 2B. Percentage of viable cells on the hippocampus CA1 7 days after forebrain ischemia. The values are expressed as means ± *SD*, ** $p < 0.01$ vs. sham group; ‡ $p < 0.01$ vs. control group.

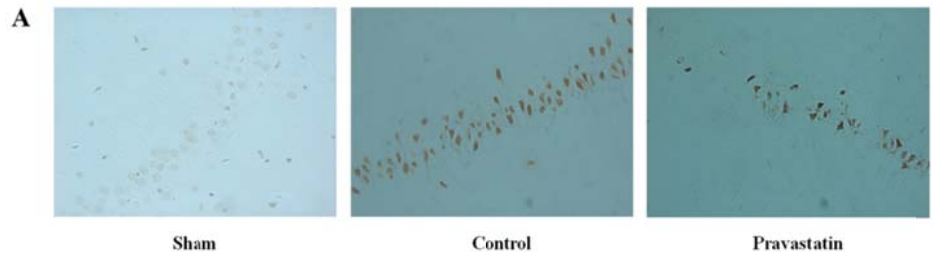

Figure 3A. Representative photomicrographs of TUNEL assay in the hippocampus CA1 area 7 days after forebrain ischemia.

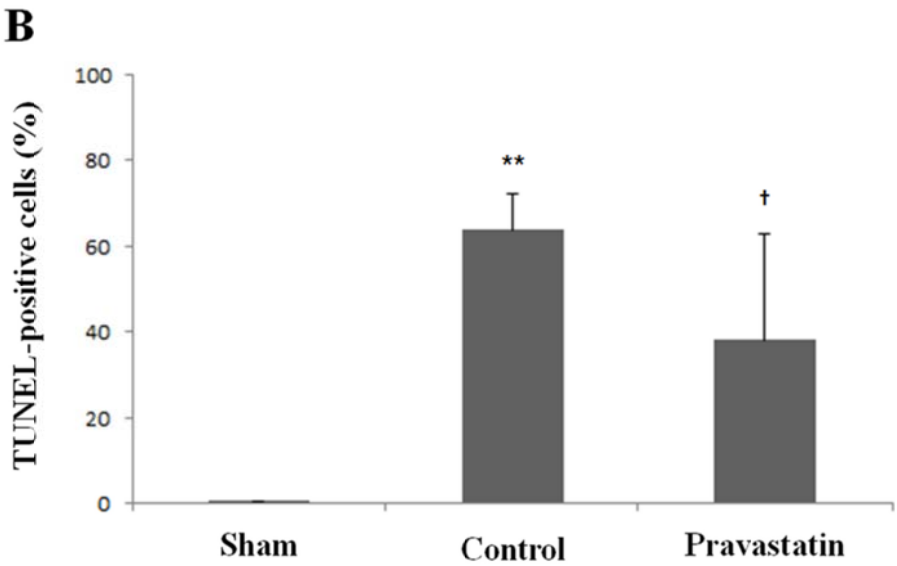

Figure 3B. Percentage of TUNEL-positive cells in the hippocampus CA1 7 days after forebrain ischemia. The values are expressed as means ± *SD*, ** $p < 0.01$ vs. sham group; † $p < 0.05$ vs. control group.

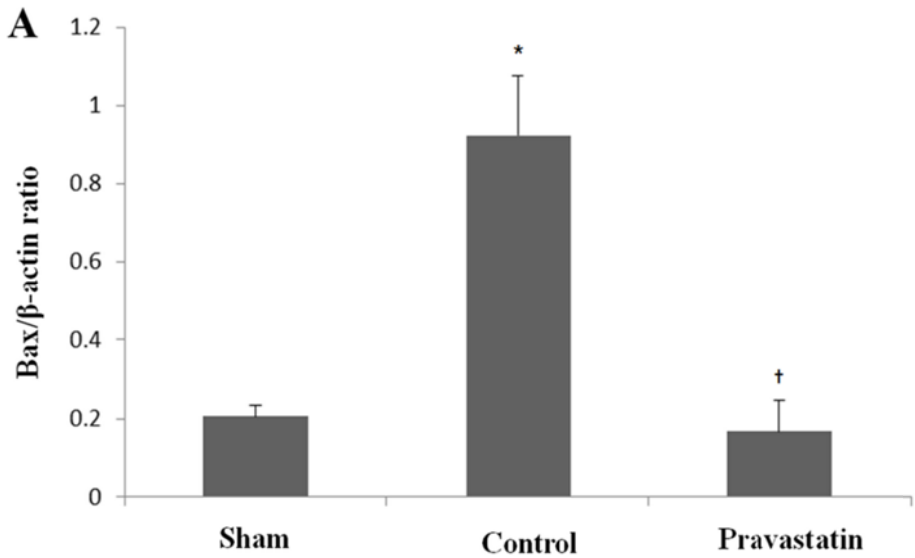

Figure 4A. Expression of Bax/β−actin ratio in the hippocampus of sham, control and pravastatin group 7 days after forebrain ischemia. The values are expressed as means ± *SD.* * $p < 0.05$ vs. sham group; † $p < 0.05$ vs. control group

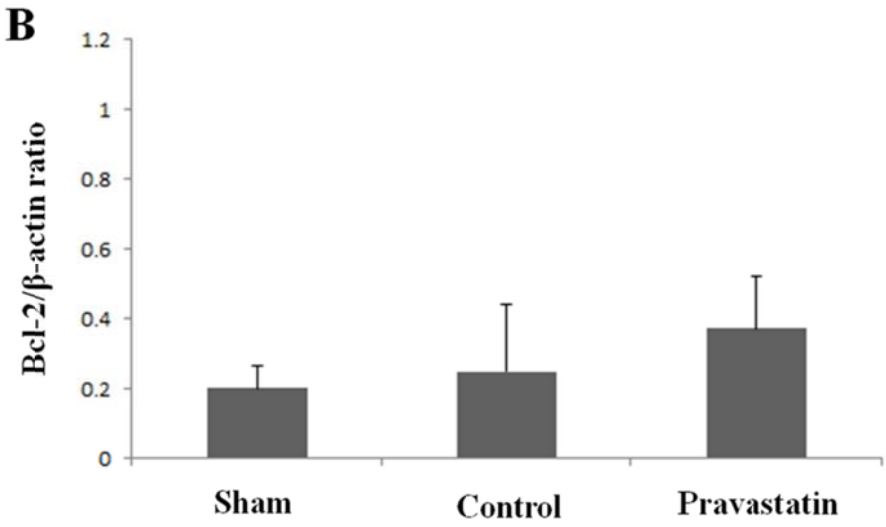

Figure 4B. Expression of Bcl-2/β-actin ratio in the hippocampus of sham, control and pravastatin group 7 days after forebrain ischemia. The values are expressed as means ± *SD.*

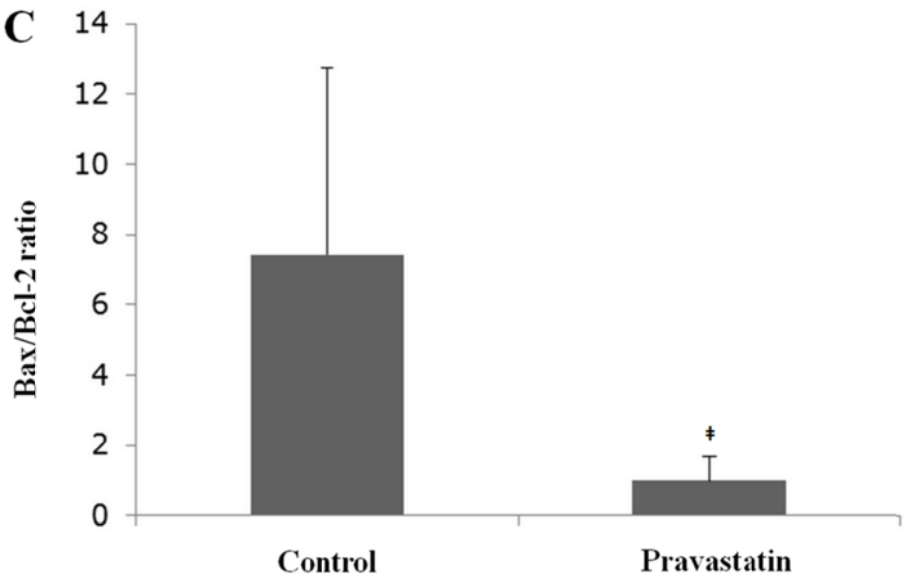

Figure 4C. Expression of Bax/Bcl-2 ratio in the hippocampus of sham, control and pravastatin group 7 days after forebrain ischemia. The values are expressed as means ± *SD.* † $p < 0.01$ vs. control group