Early Human Development (2008) **84**, 243–247

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/earlhumdev

The role of trapidil on neuronal apoptosis in neonatal rat model of hypoxic ischemic brain injury

Aytug Atici[a,*], Gulcin Bozlu[b], Ali Haydar Turhan[a], Ayse Polat[c], Ali Nayci[d], Cetin Okuyaz[e], Hakan Taskinlar[d]

[a] *Department of Pediatrics, Division of Neonatology, University of Mersin School of Medicine, Mersin, 33079, Turkey*
[b] *Department of Pediatrics, University of Mersin School of Medicine, Mersin, 33079, Turkey*
[c] *Department of Pathology, University of Mersin School of Medicine, Mersin, 33079, Turkey*
[d] *Department of Pediatric Surgery, University of Mersin School of Medicine, Mersin, 33079, Turkey*
[e] *Department of Pediatrics, Division of Pediatric Neurology, University of Mersin School of Medicine, Mersin, 33079, Turkey*

Accepted 26 June 2007

KEYWORDS
Trapidil;
Apoptosis;
Brain injury;
Hypoxia;
Ischemia;

Abstract

Background: Hypoxic ischemic brain injury (HIBI) is a common cause of neonatal mortality and morbidity. Trapidil is an antiplatelet agent and several studies demonstrate the beneficial effect of trapidil in various forms of tissue injury. The effects of trapidil on neuronal apoptosis in HIBI have not been reported previously.
Aims: The aim of this study is to evaluate the effect of trapidil on neuronal apoptosis in neonatal rat model of HIBI.
Study design: Seven-day-old Wistar rat pups were subjected to right common carotid artery ligation and hypoxia (92% nitrogen and 8% oxygen) for 2h. They were treated with trapidil or saline either immediately before or after hypoxia. In sham group animals, neither ligation, nor hypoxia were performed. Neuronal apoptosis was evaluated by the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 staining methods.
Results: Trapidil treatment either before or after hypoxia results in significant reduction of the numbers of apoptotic cells in both hemispheres, when it is compared with saline treatment group. The numbers of apoptotic cells in right hemispheres in all groups are significantly higher than that in the left hemispheres.
Conclusions: These results show that trapidil administration either before or after hypoxia reduces neuronal apoptosis and we propose that trapidil may be a novel approach for the therapy of HIBI.

1. Introduction

Hypoxic ischemic brain injury (HIBI) is a major cause of perinatal mortality and it *may lead to* neurological sequelae in survivors [1,2]. Although the mechanism of neuronal damage

* Corresponding author. Department of Pediatrics, Division of Neonatology, Mersin, 33079, Turkey. Tel.: +90 324 337 4300; fax: +90 324 337 4305.
E-mail address: aatici@superonline.com (A. Atici).

0378-3782/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.earlhumdev.2007.06.005

has not been exactly understood in neonatal HIBI, hypoxic ischemia triggers a cascade of biochemical and molecular events including activation of neutrophils, membrane depolarization, increased intracellular calcium, production of lipid peroxidation and generation of oxygen-derived free radicals [3]. These events induce DNA damage, neuronal injury, neurodegeneration, and cell death [4].

Trapidil [5-methyl-7-diethylamino-s-triazolo(1,5-α)pyrimidine], is an antiplatelet agent that acts in part as a phosphodiesterase inhibitor and a competitive inhibitor of the platelet-derived growth factor (PDGF) receptor. Trapidil also has nitroglycerine-like vasodilating action and it was originally developed as an antianginal drug [5]. Trapidil has been reported to stimulate the production of prostacyclin (prostaglandin I2) and selectively inhibit the synthesis of tromboxane A2 (TxA2) and reduce lipid peroxidation [6,7]. Additionally, trapidil reduces the production of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-12 (IL-12) and procoagulant activity by inhibition of the CD40 pathway of monocytes and macrophages [8].

Over the past decade, several studies *have* demonstrated the beneficial effect of trapidil in various forms of tissue injury including spinal cord [7], kidney [9], peripheral nerves [10], small intestine [11], heart [12] and lung [13]. However, no study has investigated the role of trapidil in neonatal rat model of HIBI. The aim of this study was to evaluate the effect of trapidil on neuronal apoptosis in neonatal rat model of HIBI.

2. Materials and methods

Seven-day-old Wistar rat pups (n = 100) of either sex, delivered spontaneously, were used in this experimental study. The brain of the rat at this stage is histologically similar to that of a 32–34-week gestation human fetus or newborn infant and this model has proved to be useful in many studies [14]. All animal experiments follow a protocol approved by the ethical committee on animal research at our institution.

2.1. Animal preparation and surgical procedure

Rat pups were anaesthetized by halothane inhalation and duration of anaesthesia was less than 5min. Hypoxic ischemia was induced according to the Levine–Rice model [15]. A median incision was made in the neck. Under the microscopic magnification, the right common carotid artery was dissected and ligated with a 6-zero silk suture. After the wound was sutured, the animals were allowed to have 3h recovery and feeding period. Except for the sham group, rats were then placed in a plastic chamber and exposed to a continuous flow of 8% oxygen–92% nitrogen for 2h. After the hypoxic period, the rats were allowed to have a 2h recovery period in an open chamber without any supplemental oxygen. The animals in the sham group were placed in an open chamber for the same intervals. The chambers were partially submerged in a water bath at 37°C to maintain a constant thermal environment. After these procedures, all the pups were euthanized by decapitation and brains were removed for pathological evaluation.

Saline treatment group: Saline (0.5ml) was injected intraperitoneally either immediately before (n = 20) or after (n = 20) hypoxia. Fourteen rats (6 before hypoxia, 8 after hypoxia) in this group died during the procedure.

Trapidil treatment group: The rat pups were administered intraperitoneally 20mg/kg trapidil which was dissolved in saline, either immediately before (n = 20) or after (n = 20) hypoxia. Seven rats (4 before hypoxia, 3 after hypoxia) in this group died during the procedure.

Sham group (n = 20): After median neck incision was made, neither ligation, nor hypoxia was performed. Four rats in this group died during the procedure.

2.2. Histopathological evaluation

2.2.1. Caspase-3 method

Serial sections from paraffin-embedded coronal brain sections were deparaffinized in xylene and dehydrated through graded concentrations of ethanol. After the blocking of endogenous peroxidase activity with hydrogen peroxide, the sections were heated in 0.01mol/L citrate buffer in a microwave cooker for 20min. Sections were incubated using caspase-3 polyclonal antibody (dilution 1/100, Neomarkers, RB-1197-B0, USA) for one hour at room temperature in a humidified chamber, and were then stained using the avidin–biotin complex (ABC; Labvision, Fremont, USA) immunoperoxidase technique with a commercially available reagent. The sections were counterstained with Mayer's haematoxylin and mounting media (Labvision, Fremont, USA).

2.2.2. TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) method

To investigate DNA fragmantation at neurons, TUNEL method (in Situ Apoptosis Detection Kit, Biogen, USA) was selected. After deparaffinized and rehydrated, sections were pretreated with proteinase K for 15min at room temperature, then endogen peroxidase activity was quenched with 2% H_2O_2. Slices were then incubated at 37°C for 60min in moist chamber with 50μl of TdT buffer. Finally, the reaction was visualized by streptavidin–biotin–peroxydase complex and diaminobenzidine. TUNEL labeled slides were counterstained with 1% methyl green.

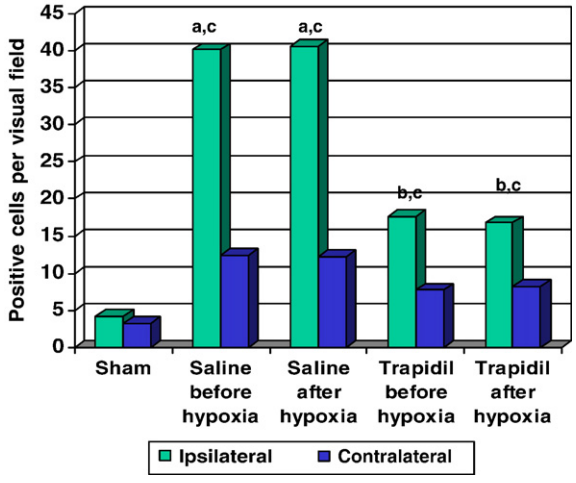

Figure 1 Caspase-3 positive apoptotic cells in the ipsilateral and contralateral hemisphere of the sham, saline and trapidil treated animals. [a]$p<0.0001$ vs. sham animals, [b]$p<0.0001$ vs. saline treated animals, [c]$p<0.0001$ vs. contralateral hemisphere.

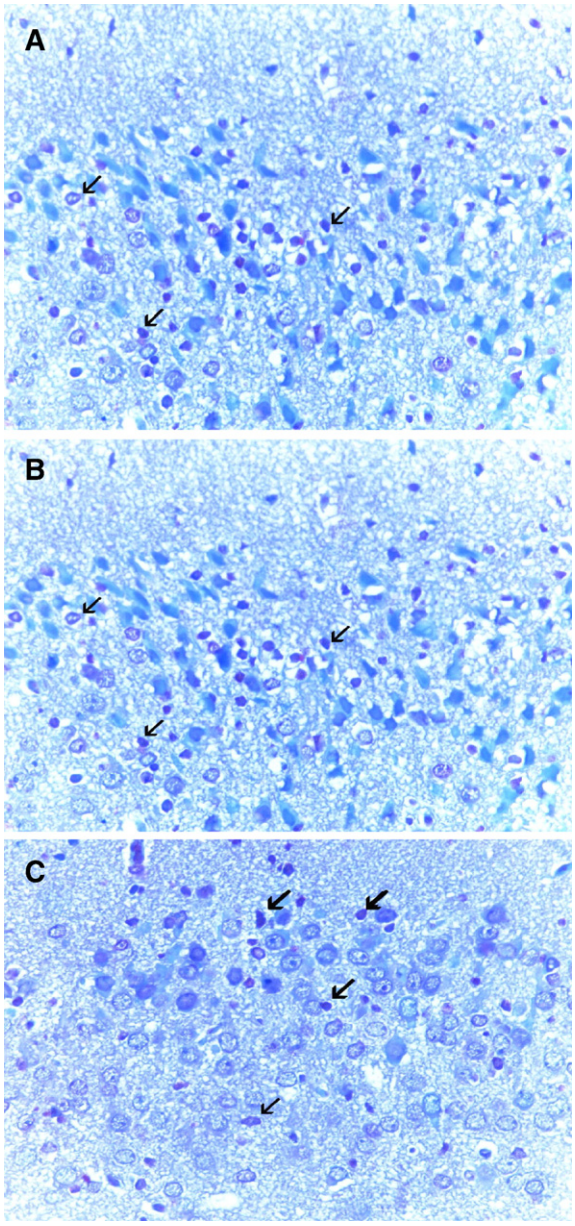

Figure 2 Caspase-3 positive apoptotic cells in hippocampus in the (A) sham group, (B) saline and (C) trapidil treatment (after hypoxia) groups. Original magnification, X400.

Apoptotic Cell Counting: Apoptotic cell counting was performed in subtalamic nuclei, hippocampus and pariatal cortex of both right and left hemispheres (2, 3). In evaluating numeric density, total TUNEL and caspase-3 positive stained neurons were calculated in 5 high power fields (5X400) under the light microscope [16]. Microscopic examinations were made by a single pathologist who was unaware of the characteristics or treatment of the animals.

2.3. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Animal mortality rate was evaluated by chi-square testing using Yates correction whenever necessary. Unpaired *t* test and ANOVA was used for statistical analysis. Multiple comparisons were made using Tukey's procedure with $p < 0.05$ considered statistically significant.

3. Results

Caspase-3 positive apoptotic cells of each groups are shown in Figs. 1 and 2. The numbers of caspase-3 positive apoptotic cells in both hemispheres were significantly higher in the saline treatment group than in the sham group ($p < 0.0001$). The numbers of caspase-3 positive apoptotic cells decreased significantly in trapidil treated group compared with the saline group ($p < 0.0001$). There was no significant difference in the number of caspase-3 positive apoptotic cells between the animals treated with trapidil before or after hypoxia ($p > 0.05$).

TUNEL-positive apoptotic cells of each group are shown in Figs. 3 and 4. The numbers of apoptotic cells in both hemispheres were significantly higher in the saline treatment group than in the sham group ($p < 0.0001$). The animals treated with trapidil either before or after hypoxia had a significant decrease in the values of TUNEL-positive apoptotic cells compared with the saline group ($p < 0.0001$). The number of apoptotic cells in the animals treated with trapidil before hypoxia were not statistically significantly different than in the animals treated with trapidil after hypoxia ($p > 0.05$).

Except the sham group, the numbers of apoptotic cells in right hemispheres were significantly higher than that in the left hemispheres in all of the groups (Figs. 1 and 3) ($p < 0.0001$). Mortality rates did not differ among the groups ($p > 0.05$).

4. Discussion

Our study demonstrates that trapidil administration either before or after hypoxic ischemia results in a significant decrease in the numbers of apoptotic cells in the neonatal rat brain. There is good evidence that hypoxic ischemia causes neuronal injury and neuronal cell death after hypoxic ischemia.

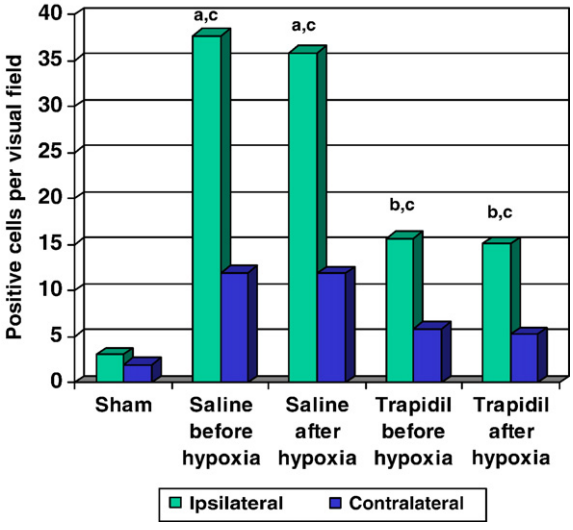

Figure 3 TUNEL-positive apoptotic cells in the ipsilateral and contralateral hemisphere of the sham, saline and trapidil treated animals. [a]$p < 0.0001$ vs. sham animals, [b]$p < 0.0001$ vs. saline treated animals, [c]$p < 0.0001$ vs. contralateral hemisphere.

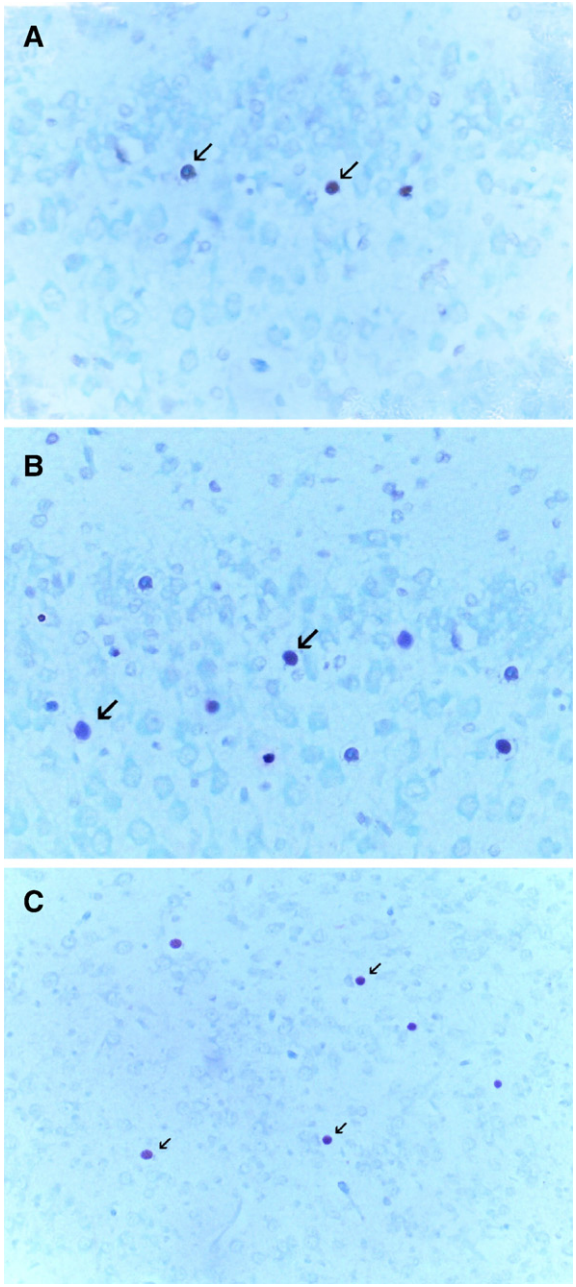

Figure 4 TUNEL-positive apoptotic cells in hippocampus in the (A) sham group, (B) saline and (C) trapidil treatment (after hypoxia) groups. Original magnification, X400.

Although there is no doubt that necrosis plays major role in the neuronal cell death, accumulating data suggests that apoptosis plays an important role in the evolution of hypoxic ischemic injury in the neonatal brain [3,17].

Trapidil has been used as a coronary agent in clinical practice for many years [5]. On the other hand, since trapidil has a broad spectrum of biological activities that protect tissue injury, several investigators have used this drug in various forms of tissue injury in animal models. It has been demonstrated that treatment with trapidil leads to a reduction of the ischemia-reperfusion injury to the small intestine, heart and lung [11–13]. Protective effects of trapidil on gentamicin-induced nephrotoxicity [9] and oxidative organ damage in burn injury were also documented [18].

Over the past decade, some research studies have been done to investigate the role of trapidil in nervous system. It has been demonstrated that trapidil is very effective in the treatment of the cerebral vasospasm [19]. Tokiyoshi et al. reported that trapidil is not only effective in decreasing the occurrence of symptomatic vasospasm, but also reducing the neurological deterioration due to vasospasm after subarachnoid hemorrhage in rabbits [20]. Effects of trapidil on ATPase, lipid peroxidation and ultrastructure in experimental spinal cord injury were evaluated in a recent study [7]. The authors found that trapidil attenuated Na^+–K^+/Mg ATPase inactivation, significantly reduced the lipid peroxidation and their microscopic findings supported the biochemical results in rat model of spinal cord injury. Administration of trapidil successfully reduced myelin damage and axonal swelling in rat peripheral nerve injury model as well as serum malondialdehyde level [10].

Although the protective effect of trapidil in neuronal injury has been reported in a few studies, no study has investigated the role of trapidil in neonatal rat model of HIBI. Successful results of previous studies have led us to evaluate the effect of trapidil on neuronal apoptosis in neonatal rat model of HIBI. To our knowledge, the exact role of trapidil on neonatal rat model of HIBI is unknown. In the present study, we found that treatment with trapidil reduced neuronal apoptosis in the neonatal rats subjected to hypoxic ischemic insult. Our study is the first report in the literature to show that trapidil has beneficial effect in neonatal rat model of HIBI. The exact mechanism of the antiapoptotic function of trapidil is still unclear. However, it has been demonstrated that TNF receptors induce apoptosis [21] and trapidil reduces the production of TNF-α [8,19]. Prevention of lipid peroxidation by trapidil may also reduce apoptosis [6,7]. The mechanism of antiapoptotic effect of trapidil may be explained by reduction of TNF-α production in combination with prevention of lipid peroxidation.

The relationship between antiplatelet action of trapidil and neuronal apoptosis has not been specifically tested in this study. The results of our previous study showing the beneficial effect of platelet activating factor antagonist (ABT-491) in HIBI [22] should encourage the investigators to plan further studies to test the possible role of platelets in HIBI of the newborn. Although the results of the current study indicate that trapidil is neuroprotective immediately after hypoxic insult, long-term effects of this drug should also be investigated.

Acknowledgements

This work was supported by Mersin University Research Foundation (BAP-TF DTB, AA 2004-3).

References

[1] Ferriero DM. Neonatal brain injury. N Engl J Med 2004;351: 1985–95.
[2] Vannucci RC. Hypoxic–ischemic encephalopathy. Am J Perinatol 2000;17:113–20.
[3] Johnston MV, Trescher WH, Ishida A, Nakajima W. Neurobiology of hypoxic–ischemic injury in the developing brain. Pediatr Res 2001;49: 735–41.

[4] Blomgren K, Hagberg H. Free radicals, mitochondria, and hypoxia–ischemia in developing brain. Free Radic Biol Med 2006;40: 388–97.
[5] Maresta A, Balducelli M, Cantini L, Casari A, Chioin R, Fabbri M, et al. Trapidil (triazolopyrimidine), platelet-derived growth factor antagonist, reduces restenosis after percutaneous transluminal coronary angioplasty: results of the randomized, double-blind STARC study. Circulation 1994;90:2710–5.
[6] Nieder J, Claus P, Auguntin W. Effect of trapidil on the PGI_2 and TxA_2 synthesis in human umbilical veins perfused in vitro. Prostaglandins 1995;49:311–8.
[7] Gocer AI, Ildan F, Tuna M, Polat S, Tamer L, Erman T, et al. Effects of trapidil on ATPase, lipid peroxidation, and correlation with ultrastructure in experimental spinal cord injury. Neurosurg Rev 2001;24:136–42.
[8] Zhou L, Ismaili J, Stordeur P, Thielemans K, Goldman M, Pradier O. Inhibition of the CD40 pathway of monocyte activation by triazolopyrimidine. Clin Immunol 1999;93:232–8.
[9] Buyukafsar K, Yazar A, Dusmez D, Ozturk H, Polat G, Levent A. Effect of trapidil, an antiplatelet and vasodilator agent on gentamicin-induced nephrotoxicity in rats. Pharmacol Res 2001;44:321–8.
[10] Bagdatoglu C, Saray A, Surucu HS, Ozturk H, Tamer L. Effect of trapidil in ischemia/reperfusion injury of peripheral nerves. Neurosurgery 2002;51:212–20.
[11] Colak T, Polat A, Bagdatoglu O, Kanik A, Turkmenoglu O, Aydin S. Effect of trapidil in ischemia/reperfusion injury on rat small intestine. J Invest Surg 2003;16:167–76.
[12] Sichelschmidt OJ, Hahnefeld C, Hohfeld T, Herberg FW, Schror K. Trapidil protects ischemic hearts from reperfusion injury by stimulating PKAII activity. Cardiovasc Res 2003;58:602–10.
[13] Somuncu S, Cakmak M, Erdogan S, Caglayan O, Caglayan F, Akman H, et al. Protective effects of trapidil in lung after abdominal aorta induced ischemia–reperfusion injury: an experimental study. Pediatr Surg Int 2005;21:983–8.
[14] Northington FJ. Brief update on animal models of hypoxic–ischemic encephalopathy and neonatal stroke. ILAR J 2006;47:32–8.
[15] Rice JE, Vanucci RC, Brierley JB. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann Neurol 1981;9: 131–41.
[16] Zhu C, Wang X, Cheng X, Qiu L, Xu F, Simbruner G, et al. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia–ischemia. Brain Res 2004;996:67–75.
[17] Northington FJ, Graham EM, Martin LJ. Apoptosis in perinatal hypoxic–ischemic brain injury: how important is it and should it be inhibited? Brain Res Brain Res Rev 2005;50:244–57.
[18] Avlan D, Taskinlar H, Tamer L, Camdeviren H, Ozturhan H, Ozturk C, et al. Protective effect of trapidil against oxidative organ damage in burn injury. Burns 2005;31:859–65.
[19] Bagdatoglu C, Guleryuz A, Unlu A, Kanik A, Berk C, Ozdemir C, et al. Resolution of cerebral vasospasm with trapidil; an animal model. J Clin Neurosci 2001;9:429–32.
[20] Tokiyoshi K, Ohnishi T, Nii Y. Efficacy and toxicity of tromboxane synthetase inhibitor for cerebral vasospasm after subarachnoid hemorrhage. Surg Neurol 1991;36:112–8.
[21] Niemann-Jonsson A, Ares MP, Yan ZQ, Bu DX, Fredrikson GN, Branen L, et al. Increased rate of apoptosis in intimal arterial smooth muscle cells through endogenous activation of TNF receptors. Arterioscler Thromb Vasc Biol 2001;21:1909–14.
[22] Bozlu G, Atici A, Turhan AH, Polat A, Nayci A, Okuyaz C, et al. Platelet-activating factor antagonist (ABT-491) decreases neuronal apoptosis in neonatal rat model of hypoxic ischemic brain injury. Brain Res 2007;1143:193–8.