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Research Report

Effects of activated protein C on neonatal hypoxic ischemic brain injury

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ABSTRACT

Perinatal hypoxia–ischemia remains the single most important cause of brain injury in the newborn, leading to death or lifelong sequelae. White matter injuries in newborn infants have long-term effects on physical, visual, motor, sensory, cognitive and social development in human infants. There is no known cure for neonatal hypoxic ischemic encephalopathy (NHIE). Activated protein C has potent anticoagulant activity due to its ability to inactivate factor Va and VIIIa. APC is the first effective biological therapy approved for the treatment of severe sepsis. Although APC is well defined as a physiological anticoagulant, emerging data suggest that it also has cytoprotective, anti-inflammatory and antiapoptotic properties. APC has been shown to provide neuroprotection in ischemic brain and spinal cord injury. Here, we propose that APC, which modulates many of these processes, may represent a promising therapeutic agent for NHIE. Seven days old Wistar Albino rat pups have been used in the study ($n=42$). Experimental groups in the study were: sham-operated group, APC treated group, and vehicle treated group. In hypoxia–ischemia groups, the left common carotid artery was ligated permanently on the seventh postnatal day. Two hours after the procedure, hypoxia (92% nitrogen and 8% oxygen) was applied for 2.5 h. APC were injected (intraperitoneally; i.p.) as a single dose immediately after the hypoxia period. Brain nitrite levels, neuronal cell death, and apoptosis were evaluated in both hemispheres 72 h after the hypoxic–ischemic insult. Histopathological evaluation demonstrated that APC significantly diminished the number of "apoptotic cells" in the hippocampal CA1, CA2, CA3 and gyrus dentatus regions in both hemispheres. APC treatment significantly reduced "apoptotic cell death" in both hemispheres, when compared with vehicle treated group. APC significantly preserved the number of neurons CA1, CA3 regions of the hippocampus, when compared with vehicle treated group. Our results showed that hypoxic–ischemic injury caused a significant increase in NO production. The APC-treated animals were reduced brain nitrite levels in carotid ligated hemispheres. To our knowledge, this is the first study that demonstrates a protective effect of the APC against hypoxia–ischemia in the developing brain.

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1. Introduction

Preterm infants and ill infants are at risk for brain injury, subsequent neurodevelopmental delay, and cerebral palsy caused by hypoxia, ischemia, acidosis, hypoglycemia, and infections, among other insults. Perinatal hypoxia–ischemia remains the single most important cause of brain injury in the newborn, leading to death or lifelong sequelae. White matter injuries in newborn infants have long-term effects on physical, visual, motor, sensory, cognitive and social development in human infants. Many of these insults may activate similar cell death pathways and lead to necrotic and apoptotic neuronal death (Hagberg et al., 2002; Vannucci, 2000). Neonatal hypoxic isachemic encephalopathy (NHIE) is obvious that destructive processes such as glutamate and nitric oxide (NO) neurotoxicity, free radical formation, intracellular calcium accumulation and immune/inflammatory activation continue to damage the brain for many hours after oxygenation and circulation have been restorated (Fritz and Papadopoulos, 2006). The complexities of NHIE pathophysiology suggest that successful neuroprotection could be achieved only with a multitherapeutic approach (Hagberg et al., 2002).

A rodent model of neonatal HI injury was developed which employs unilateral carotid artery ligation combined with inhalation of a hypoxic gas mixture to produce a reproducible and survivale brain injury (Rice et al., 1981). Unilateral carotid ligation followed by hypoxia in the neonatal rat and mouse has become an accepted model for hypoxic–ischemic (HI) brain injury (Vannucci and Vannucci, 2005). However, in vivo models of NHIE have a number of disadvantages which are firstly related to the different neural maturation timing observed in several species; thus, in the widely used immature rat model, different studies lead to the vague conclusion that 7- to 14-day-old rats are comparable to 34- to 38-week-old foetuses (Ferrer et al., 1997; Hagberg et al., 1997). In addition, resistance to hypoxia may change depending on the background strain of the animals as well as on the environmental circumstances, so that the hypoxic period necessary to induce neuronal injury fluctuates among 30 min and 3 h (David Lopez et al., 2005). These white matter changes were similar to injuries found in infant brains with periventricular leukomalacia.

Protein C is a vitamin K-dependent plasma glycoprotein that circulates as an inactive zymogen. It is activated by thrombin in complex with thrombomodulin (Esmon and Fukudome, 1995) and this reaction is enhanced by the endothelial protein C receptor (Taylor et al., 2001). The activated enzyme, activated protein C (APC) has potent anticoagulant activity due to its ability to inactivate factor Va and VIIIa (Fulcher et al., 1984). APC is the first effective biological therapy approved for the treatment of severe sepsis (O' Brien et al., 2006). Although APC is well defined as a physiological anticoagulant, emerging data suggest that it also has cytoprotective, anti-inflammatory and antiapoptotic properties (Esmon, 2001; Mosnier and Griffin, 2006; Okajima, 2004). APC has been shown to provide neuroprotection in ischemic brain and spinal cord injury (Cheng et al., 2003; Griffin et al., 2006). The anti-inflammatory, cytoprotective, and anti-apoptotic effects of APC mediate its neuroprotective action. It has been shown that APC protects neurons against *N*-methyl-D-aspartate (NMDA) toxicity both in vitro and in vivo (Guo et al., 2004). The cytoprotective effects of APC has also been reported for other type of cells including endothelial cells, keratinocytes, monocytes, pancreatic beta cells and gastric epithelial cells (Hirose et al., 2000; Mizutani et al., 2000; Schoots et al., 2004; Shibata et al., 2001). APC attenuates ischemia/reperfusion injury in various organs such as brain, kidneys and skeletal muscle. In recent years, interest in the neuroprotective possibilities of activated protein C has grown (Aoki et al., 2000).

There is no known cure for NHIE. Here, we propose that APC, which modulates many of these processes, may represent a promising therapeutic agent for NHIE. Therefore, the aim of this study was to investigate the possible neuroprotective and ameliorating effect of activated protein C treatment immediately after hypoxic–ischemic injury induced neuronal cell death, apoptosis and NO formation in a neonatal rat model.

2. Results

2.1. *Effects of activated protein C treatment on neuronal density*

Seventy-two hours after hypoxic–ischemic brain injury, treatment with APC significantly preserved the number of neurons CA1 and CA3 regions of hippocampus, CA2 and dentate gyrus in the right and left hemispheres when compared with vehicle-treated group ($p<0.05$) (Tables 1 and 2) The neuronal densities of CA1, CA2, CA3 and gyrus dentatus were significantly higher than vehicle-treated groups in the right hemispheres in the brains of these animals (Table 1). The densities of the CA1 and CA3 neurons were significantly higher in the APC treatment

Table 1 – The effect of APC treatment on neuronal density of the hippocampus, and apoptotic cell and nitrite levels of newborn rats with hypoxic–ischemic brain injury in the right hemispheres

	Neuronal density of right hemispheres				*Nitrite values (μM)
	*CA1	*CA2	*CA3	*GD	
1-Sham ($n=7$)	57.48±2,22	38.21±0.59	26.10±2.62	68.09±1.90	7.54±1.03
2-APC ($n=7$)	29.89±2.01	27.87±2.09	19.85±2.59	53.89±3.11	10.5±3.28
3-Vehicle ($n=7$)	26.27±0.41	26.53±0.45	15.25±1.02	47.33±7.00	12.71±4.3
p values					
1 vs 3	0.002	0.003	0.02	0.002	n.s.
2 vs 3	0.002	n.s.	0.002	n.s.	n.s.

Table 2 – The effect of APC treatment on neuronal density of the hippocampus, and apoptotic cell and nitrite levels of newborn rats with hypoxic–ischemic brain injury in the left hemispheres

	Neuronal density of left hemispheres				*Nitrite values (μM)
	*CA1	*CA2	*CA3	*GD	
1-Sham ($n=7$)	58.37±1.35	38.74±0.38	25.43±2.75	67.25±1.17	7.91±0.99
2-APC ($n=7$)	26.08±0.71	23.82±3.95	16.57±1.05	45.52±5.21	7.78±0.77
3-Vehicle ($n=7$)	23.73±1.56	23.1±1.08	13.25±1.75	40.30±3.18	12.94±4.09
p values					
1 vs 3	0.002	0.002	0.002	0.002	0.001
2 vs 3	0.006	n.s.	0.006	n.s.	0.002

group when compared with vehicle -treated group ($p<0.05$) (Table 2) in the left hemispheres.

2.2. *Effects of APC treatment on apoptosis*

TUNEL positive cells showed the typical morphological features of apoptosis such as the chromatin condensation, cytoplasmic budding and apoptotic bodies. Seventy -two hours after hypoxic–ischemic brain injury increased cells with fragmented DNA labeled by TUNEL assay were found in the hippocampus (CA1, CA2, CA3, and dentate gyrus) of the vehicle-treated groups, when compared with sham-operated group ($p<0.05$). APC treatments significantly reduced the number of TUNEL positive neurons in the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus, as compared with the vehicle treated groups in the both hemispheres ($p<0.05$) (Tables 1 and 2). For quantitative measurement of the number of cells that underwent apoptosis, 100 cells were randomly counted in these different areas and the percentage of the apoptotic cells were calculated (Tables 1 and 2).

2.3. *Effects of APC treatment on nitrite levels*

In sham operated groups, as expected, the mean nitrite levels were not significantly different between the right and left hemispheres in the brains of these animals. APC treatment significantly decreased the mean nitrite levels on the ligated side when compared ($p<0.05$) with the mean value of vehicle e-treated animals, whereas no significant NO change was recorded in the nonligated hemisphere (Tables 1 and 2), as consistent with our previous reports (Kumral et al., 2004a).

3. Discussion

The present data indicate that APC is an effective neuroprotective agent in this particular animal model when administrated consequently after exposure to hypoxic–ischemic insult. This neuroprotection indicates possible human therapeutic implications. Drugs known to have potential neuroprotective properties that can cross the blood–brain-barrier and modulate multiple pathogenetic mechanisms, and that have been proven to be safe in children would be a reasonable initial therapeutic choices (Gressens and Spedding, 2004).

As a model of human brain development, the post-natal day 7 (P7) mouse, or rat, approximates a 34–36 week premature human (Dobbing and Sands, 1979; Romijn and Hofman, 1991). This developmental age was used because, for either rats or mice, this age falls in the middle of the brain growth spurt period. HI injury in the P7 rat (Almli and Levy, 2000; Jansen and Low, 1996) and P7 mouse produces cognitive deficits, defined as performance deficits on spatial memory tasks, and impaired motor performance, defined as decreased ability to run on a rotating rod and circling following apomorphine, but not abnormal locomotor activity in adult animals. The present study has implications for further studies and some limitations. Although functional parameters were not measured, our data corroborate reports showing that APC has a strong neuroprotective effect and, therefore, may improve functional outcomes.

NHIE is obvious that destructive processes such as glutamate and NO neurotoxicity, free radical formation, intracellular calcium accumulation and immune/inflammatory activation continue to damage the brain for many hours after oxygenation and circulation have been restorated (David Lopez et al., 2005). The exact mechanisms responsible for the in vivo neuroprotective effects of APC remain to be defined. Numerous mechanisms such as reducing NO overproduction that mediates glutamate neurotoxicity and preventing free radical formation potentially exist whereby APC protects the immature brain from hypoxic–ischemic damage. Recent studies suggest that excitatory amino acids (EAAs) such as glutamate and aspartate may be important for the development of hypoxic–ischemic brain injury in the newborn (Delivoria-Papadopoulos and Mishra, 1998; Fellman and Raivio, 1997). Suppression of NO toxicity, known to be involved in the neuropathological mechanisms triggered by EAAs, appears to be involved in the neuroprotective action of APC in vitro against glutamate neurotoxicity. Free radical injury is also implicated in hypoxic–ischemic brain injury in neonates (Fellman and Raivio, 1997). This is the first in vivo study that has investigated the effects of APC using this model. We found that APC decreases neuronal damage and that the protective effects of APC are associated with its ability to reduce the NO formation and reduced apoptotic cell death in the neonatal rat brain. Apoptosis contributes significantly to cerebral damage in the perinatal period. Infants who die after intrauterine insults have a significant number of cells in the brain with the morphologic characteristics of apoptosis (Edwards et al., 1997). In the neonatal hypoxic–ischemic model, there is evidence that cerebral ischemia leads to delayed cell death with DNA damage (Pulera et al., 1998). Recent studies both in vitro and in vivo have shown that APC counteracts endotoxin-induced systemic changes, such as hemodynamic, inflammatory, and

coagulative effects, as well as cell death and tissue damage (Chamnanvanakakij et al., 2002; Folkerth, 2005). In our study, we observed a significant increase in apoptotic cells in the CA1 and CA3. APC reversed this increase. APC inhibits apoptosis in a variety of injury models and cells, including keratinocytes, pancreatic beta cells, monocytes, neurons, and endothelial cells (Aoki et al., 2000). The antiapoptotic effect of APC is dependent on EPCR and PAR-1 (Guo et al., 2004; Mosnier and Griffin, 2003). Although glial cells express PARs, (Balcaitis et al., 2003; Suo et al., 2002), the role of these receptors in the apoptotic effect of APC in the NHIE model is not clear. The possible mechanisms of the anti-apoptotic effect of APC in neuronal and endothelial cells include the inhibition of caspase activation, blockage of the nuclear translocation of apoptosis- inducing factor (AIF), modulation of Bcl-2 antiapoptotic protein, transcriptionally dependent inhibition of tumor suppressor protein p53, the normalization of the pro-apoptotic Bax/Bcl-2 ratio, and reduction of caspase-3 signaling (Cheng et al., 2003; Guo et al., 2004). The contribution of these mechanisms to the anti-apoptotic action of APC in the HIE model is not known and needs further investigation. In the present study, histopathological evaluation using the TUNEL method, as well as quantitative measurement of apoptotic cells, confirm this hypothesis and suggests that apoptosis is one of the modes of cell death upon which APC has its neuroprotective effect in hypoxic–ischemic brain injury.

The direct cytoprotective effect of APC is not cell-type specific and includes a variety of cells such as neuronal, endothelial, epithelial, and mononuclear cells (Cheng et al., 2003; Haynes et al., 2003). Cytoprotective effects of APC have also been demonstrated in ischemia/reperfusion injury models of various organs including the heart, brain, spinal cord, pancreas, small intestine, and skeletal muscle (Hirose et al., 2000; Mizutani et al., 2000; Schoots et al., 2004; Shibata et al., 2001). The protective effects of APC may be mediated by distinct mechanisms involving several processes. Although the exact mechanisms of the cytoprotective action of APC are not completely known, APC plays a role in the promotion of cell survival signaling cascades, the upregulation of the expression of antiapoptotic proteins, the modulation of intracellular calcium metabolism, and the attenuation of NO production, in addition to having antiapoptotic, antioxidative, and anti-inflammatory actions.

Thus, in addition to the anti-apoptotic and cytoprotective effects that were found in the present study, other effects of APC, including anti-oxidant, angiogenic, hemodynamic, intracellular calcium modulating, and anticoagulant may also contribute to the cytoprotective action of APC in endotoxin-induced brain injury (Aoki et al., 2000).

These results suggest that APC, via its intrinsic anti-oxidant properties, may, in settings of oxidant stress, exert important cytoprotective and anti-apoptotic effects that are distinct from its anticoagulant activity as an antioxidant protein (Shibata et al., 2001). Activated microglias are a major source of iNOS and other reactive nitrogen species. (Haynes et al., 2003). Further investigation is needed to study the effect of APC on oxidative stress in NHIE.

In summary, APC treatment attenuates hypoxia–ischemia induced with neuronal density of hippocampus, decreased apoptotic cell index in this animal model. Activated protein C that is systemically administered immediately after hypoxic–ischemic insult selectively inhibits NO formation in the "hypoxic–ischemic" hemisphere of the brain in neonatal rats. This is the first in vivo demonstration of the selective inhibitory effect of APC on NO overproduction in this model. To our knowledge, this is the first in vivo study that has shown such an effect. Given our results, it is possible that a single dose of APC is sufficient to reduce brain injury and may possess clinical relevance for treating NHIE.

4. Experimental procedures

4.1. *Animals*

This study was 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. Wistar rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow. Forty-two offspring delivered spontaneously were reared with their dams until the time of initial experimentation at 7 days of postnatal age (P7; day of birth is day 1).

4.2. *Induction of hypoxic–ischemic brain injury and treatment groups*

A modification of Levine preparation was used as a model for perinatal hypoxic–ischemic brain injury as previously described (Vannucci and Vannucci, 2005). Twenty-eight pups underwent permanent unilateral carotid ligation. The midline of the neck was incised at the longitudinal plane under halothane anesthesia. The left carotid artery was permanently ligated with 5-0 surgical silk. Total time of surgery never exceeded 5 min. Animals were excluded from the study if there was bleeding during ligation or respiratory arrest resulting from anesthesia. Following a 2 h period of recovery and feeding, the animals were exposed to a 2.5 h period of hypoxia (92% N_2, 8% O_2) by placing them in airtight containers partially submerged in a 37 °C water bath to maintain a constant thermal environment.

APC was dissolved in 10% dimethyl sulfoxide in phosphate-buffered saline (PBS; 0.13 M NaCl, 0.003 M KCI, 0.01 M Na_2HPO_4, and 0.002 M KH_2PO_4) and stored as 1 mM aliquots. APC treatment group received an intraperitoneal (i.p.) injection of APC at a dose of 0.2 mg/kg (Guo et al., 2004) in 0.4 ml of vehicle solution after the hypoxic episode. After this procedure, the pups were returned to their dams until scarification (Mosnier et al., 2004). Sterile PBS solution was given to control group after the hypoxic episode.

4.3. *Histopathological evaluation*

All histopathological analyses described below were performed by an investigator blind to rat's treatment. To histological examination, seventy-two hours after hypoxia the animals in each group were perfused by 10% formalin. Brain tissues were removed and fixed in 10% formalin in phosphate buffer for 3 days. The brains were sectioned coronally into sequential 6 μm slices using a rat brain slicer. Each sample was subjected to the estimation of neuron number by taking three coronal

sections through the hippocampus that corresponded approximately to plates 21, 23, 25 in the rat atlas of Paxinos and Watson (Paxinos and Watson, 2002). All sections were stained by Cresyl violet. The images were analyzed by using a computer assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with a high-resolution video camera (JVC TK-890E, Japan). The numbers of neurons in hippocampal CA1, CA2, CA3 and gyrus dentatus regions were counted by help of a 15,000 μm^2 counting frame viewed through a 20× Nikon lens at the monitor (Fig. 1). The counting frame was placed randomly five times on the image analyzer system monitor and the numbers of neurons were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was taken. Hippocampal neuron density was calculated.

4.4. *Estimation of hippocampus neuron density*

Each sample was subjected to the estimation of hippocampus CA1, CA2, CA3 and dentate gyrus neuron density. The boundary of hippocampus, and dentate gyrus was defined in the rat atlas of Paxinos and Watson (Taylor et al., 2001). Three coronal sections were taken through the hippocampus that correspond approximately sections 19, 20, 21 and CA1, CA2, CA3, and dentate gyrus neuron densities of hippocampus were estimated. The images were analyzed by using a computer assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with high-resolution video camera (JVC TK 890E, Japan). The numbers of CA1, CA2, CA3, dentate gyrus were counted by the help of a 15000 μm2 counting frame viewed through a 20× Nikon lens at the monitor. The counting frame was placed randomly five times on the image analyzer system monitor and the neuron numbers of CA1, CA2, CA3, dentate gyrus regions of hippocampus were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was taken. All counting and measurement procedures were performed blindly. The neuron numbers of CA1, CA2, and CA3, gyrus dentatus regions of the hippocampus were calculated sepa-

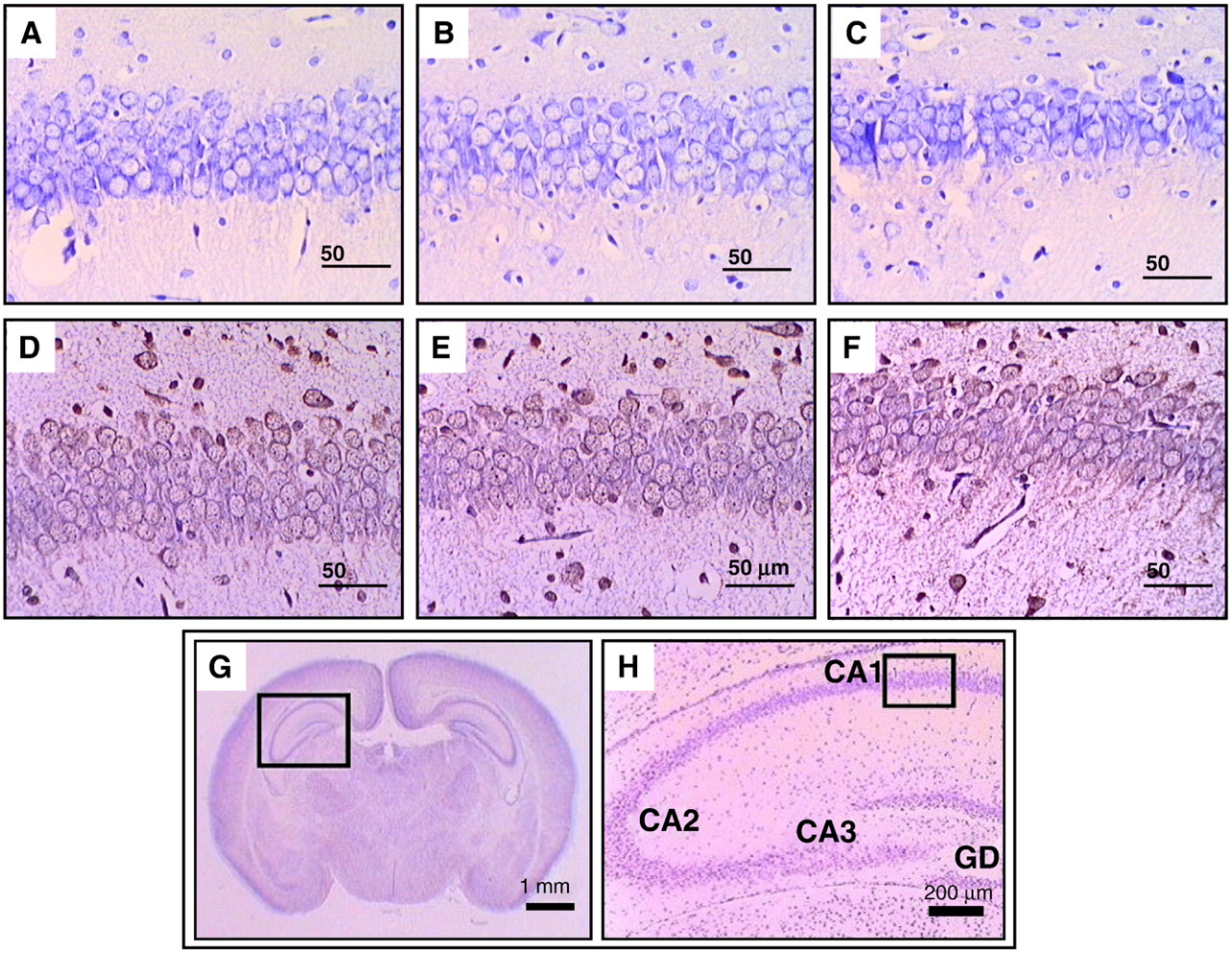

Fig. 1 – Effect of APC treatment on the neuron density in CA1 region of hippocampus (A, B, and C). Representative pictures obtained by Cresyl-violet staining. Lower (G) and higher magnifications (H) of coronal section from CA1 region of hippocampus of neonatal rat brain tissues from sham-operated group. APC groups (B) significantly preserved number of neurons of hippocampal CA1 region as compared with vehicle treated rat pups (C). Effect of APC treatment on the TUNEL immunoreactivity in hippocampus (D, E, and F). The panel D represent nuclear fragmentation which is characteristic for apoptosis from control group. TUNEL positive cells were significantly decreased treatment with APC (E) when compared with control groups (F).

rately for the right and left hemispheres (Kumral et al., 2004b).

4.5. *In situ cell death detection*

To detect DNA fragmentation in cell nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction was applied to the paraffin sections by using a commercial kit (Promega, USA). After deparaffinization, the sections were treated with 20 g/ml proteinase K for 10 min, with 0.3% H_2O_2 in methanol for 10 min and 0. 1 % Triton X-100 in 0.1% sodium citrate for 2 min on ice. Then the sections were incubated with TUNEL reaction mixture for 60 min at 37 °C. Further incubation with peroxidase-conjugated antibody was performed for 30 min at 37 °C. The sections were stained with diaminobenzidine solution for 10 min at room temperature and then counterstained with mayers 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 CA1, CA2, CA3 and dentate gyrus regions using a Olympus BH-2 Tokyo microscope at ×200 magnification connected to a 14-in. monitor. For establishing apoptotic index, 1000 cells were counted in CA1, CA2, CA3 and dentate gyrus regions of hippocampus. Cells showing apoptotic morphology were given as percentile.

4.6. *Measurement of nitrite*

The nitrite levels of the brain samples were measured by previously described methods (O'Brien et al., 2006). Briefly, following sacrification of the animals, the brains were dissected into right and left hemispheres and transported to the laboratory on an ice-cooled plate. After addition of buffer (0.1 M, pH 7.5, potassium phosphate buffer (PBS), 20 mM EDTA (1:10; w/v)), the brain hemispheres were homogenized by an ultrasound homogenizer (Bandelin Electronics, Berlin, Germany) on ice. Then left and right hemisphere supernatants were obtained through centrifugation (14,000 rpm for 10 min, then 20 min at +4 °C). Following centrifugation the supernatants were assayed for nitrite contents. Nitrite is generated by the rapid oxidation of NO. To assay nitrite, we used a modification of a previously published method (Kumral et al., 2004a). Right (hypoxic-nonligated; $n=7$) and left (hypoxic-ligated; $n=7$) hemisphere samples and control samples ($n=7$) were analyzed. Aliquots of 100 mL were mixed with 100 mL of equal volumes of a Griess reagent (A: naphthylethylenediamine dihydrochloride; B: sulfanilamide in *O*-phosphoric acid) mixture in a 96-well microtiter plate (Maxisorb Immunoplate, NUNC). After 10 min of incubation at room temperature, the absorbance at a wavelength of 540 nm was measured in a microplate reader (Model 230S; Organon Technica). A range of 2-fold dilutions of sodium nitrite (0–128 μM) in PBS was run in each assay to generate a standard curve.

4.7. *Statistical analysis*

All data regarding the brain infarct were expressed as the mean ± standard deviation (SD). Statistical comparison between treated, sham and vehicle groups (neuron density, mean NO levels and apoptotic index from the right and left hemispheres of the animals in all experimental groups) were performed by Anova testing using a computer software (SPSS 11.0, Chicago, IL, USA). Comparisons between groups were done using a t test. A probability level (p) of 0.05 or less was chosen to represent statistical significance.

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