Effects of Hypoxic Preconditioning in Antioxidant Enzyme Activities in Hypoxic-Ischemic Brain Damage in Immature Rats

Hipoksik ön Koşullamanın Neonatal Hipoksik-İskemik Beyin Hasarında Antioksidan Enzim Aktivitelerine Etkisi

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ABSTRACT

AIM: HI (hypoxic–ischemic) brain injury is a major cause of neonatal mortality and long-term neurological morbidity. The aim of the present study was to investigate the effects of HPC (hypoxic preconditioning) on the oxidative-antioxidative status in the neonatal HI brain model.

MATERIAL and METHODS: Fifty five 7-day-old rats were placed into; Control, HPC, HPC+HI insult, and HI insult groups. HPC, The HPC+HI insult groups were subjected to hypoxia (37°C, 8%O2) and the control group to normoxia for 2.5 hrs. Twenty-four hours later, the rats in the HPC+HI insult and HI insult groups were exposed to cerebral HI produced by unilateral right common carotid artery (CCA) occlusion combined with 90 min hypoxia. Four hours after recovery, the malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), and glutathione peroxidase (GPx) were determined in the brain tissues of the rats.

RESULTS: The findings of the present study suggest increased lipid peroxidation and/or decreased antioxidant activity in the brain of the HI rats.

CONCLUSION: The beneficial effects of HPC might not be related to the alterations in the antioxidative activity.

KEY WORDS: Antioxidant enzymes, Hypoxic ischemic brain damage, Lipid peroxidation, Neuroprotection, Preconditioning

ÖZ

AMAÇ: Yenidoğanda hipoksik iskemi, santral sinir sistemi hücrelerinde kalıcı hasara yol açan önemli sebeplerden biridir. Çalışmanın amacı hipoksik önkoşullamanın yenidoğan hipoksik iskemik beyin harabiyetinde oksidatif/antioksidatif süreçlere olan etkisini incelemektir.

YÖNTEM ve GEREÇ: Çalışmamızda, 7 günlük, Spraque-Dawley (n:55) sıçanlar kullanıldı. Gruplar: Kontrol, hipoksik önkoşullama, hipoksik önkoşullama + hipoksik iskemik hasar, hipoksik iskemik hasar olmak üzere planlandı. Hipoksik önkoşullama 150 dak süre ile 6 günlük sıçanlara uygulandı. Kontrol grubu hariç tüm gruplar, 7. günde sağ arteria karotis kommunis koterizasyonunu takiben, 90 dakikalık hipoksiye (37°C, 8%O2) maruz bırakıldılar. İyileşmenin 4. saatinde MDA, SOD ve GPx düzeyleri araştırılmak üzere dekapite edildiler.

BULGULAR: Hipoksik iskemik neonatal sıçanlarda artmış lipid peroksidasyonu ve/veya azalmış antioksidan aktivite saptandı.

SONUÇ: Hipoksik önkoşullamanın öngörülen yararlı etkileri antioksidan aktivite ile ilişkili olmayacağı sonucuna varıldı.

ANAHTAR SÖZCÜKLER: Antioksidan enzim, Hipoksik iskemik beyin hasarı, Lipid peroksidasyonu, Nöronal korunma, Önkoşullama

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INTRODUCTION

The concept of ischemic preconditioning was introduced in the late 1980s. The concept was that a brief subcritical ischemic challenge could mobilize intrinsic protective mechanisms that increased tolerance against subsequent critical ischemia. Preconditioning describes a variety of treatments that induce neurons to become more resistant to a subsequent ischemic insult. How preconditioned neurons adapt to subsequent ischemic stress is not fully understood, but is likely to involve multiple protective mechanisms. There have been many studies investigating the effects of hypoxic ischemia on the neonatal rat brain and various results have provided information on the possible mechanisms involved. Sarco et al. states that the neonatal brain appears to be selectively vulnerable to oxidative stress (25). Several potential mechanisms associated with altered reactive oxygen species metabolism would explain the increased susceptibility. They include increased accumulation of hydrogen peroxide with subsequent neurotoxicity. This enhanced neurotoxicity from H_2O_2 accumulation may be related to the inadequate scavenging abilities of the immature nervous system, such as lower glutathione peroxidase activity. Contributing to the immaturity of the scavenging enzymes is the inability of the developing nervous system to maintain glutathione stores. The immature nervous system is rich in iron, and has more free iron than the mature nervous system. As H_2O_2 accumulates because of these defective mechanisms, it is exposed to this free iron. This exposure results in the generation of OH radical (Fenton reaction), a more potent free radical that can cause severe damage. The rapid conversion of H_2O_2 to OH in the setting of free iron sets up the immature nervous system for increased cytotoxicity (8;25;26).

Mishra et al, claimed that the role of oxygen free radicals (OFR) in ischemia-reperfusion-induced brain damage during the perinatal period is well known (18). These authors showed that the generation of OFR results in lipid peroxidation of cell membrane, damage to DNA and other subcellular organelles, and subsequently cell death. The neonatal brain appears to be highly vulnerable to oxidative damage because of its high concentrations of unsaturated fatty acids, low concentrations of antioxidants, high rate of oxygen consumption and availability of redox-active iron (5;8;13).

Hypoxic events are common in newborns but their consequences on brain development have not been demonstrated. Short-term hypoxia before the insult has been reported to completely prevent brain damage in newborn animal models of cerebral hypoxic-ischemic insult. The mechanisms of this brain tolerance are not yet fully understood. Preconditioning by a sublethal stimulus induces tolerance to a subsequent, and otherwise lethal insult (11). The underlying mechanism(s) for the neuroprotection of hypoxic preconditioning (HPC) in the immature rat has not been elucidated but probably involves the induction of genes or proteins that favorably influence oxidative and energy metabolic events known to occur during hypoxia-ischemia, culminating in brain damage (11;26;29).

The aim of the present study was to investigate the effects of HPC on the oxidative-antioxidative status in the neonatal hypoxic ischemic (HI) brain model and to try to establish a relationship between the up-regulated antioxidant system and enhanced neuroprotection.

MATERIALS and METHODS

The study protocol was approved by the Ethics Committee of Uludag University and all animal use procedures were performed accordingly. Seven-day-old Sprague Dawley rat pups of both genders weighing more than 12g were used in the experiment. The pups were housed with the dam under a 12:12 hrs light-dark cycle, with food and water available ad libitum throughout the study.

The animals were divided into the following 4 groups that are also presented at Table I.

• **Group 1, control** (n:13)   No   hypoxic preconditioning or hypoxic ischemic insult were used. Control animals were maintained at 37°C under normoxic conditions.

• **Group 2, HPC** (n:14) On the post-natal 6th day (P6), 150 min hypoxic preconditioning was produced by the method described below. A hypoxic ischemic insult was not used.

• **Group 3, HPC+HI insult** (n:14) On P6, 150 min hypoxic preconditioning was produced by the method described below. A hypoxic ischemic insult was used on P7.

• **Group 4, HI insult** (n:14) A hypoxic ischemic insult was used on P7.

Table I: Neonatal rat pups brain tissue SOD, GPx and MDA levels in the 4 groups

Group I - control, Group II - HPC only, Group III - HPC+HI, Group IV - HI only. The groups' brain content is expressed.

SOD Umg/protein

	Left	Right
Group I	0.69489±0.19114	0.93521±0.17203*
Group II	0.85061±0.15531	0.91668±0.15058**
Group III	0.52591±0.16076	0.55957±0.25680
Group IV	0.50777±0.095904	0.64215±0.11186

GPx Umg/protein

	Left	Right
Group I	0.00259±0.00148	0.00451±0.00182
Group II	0.00278±0.00137	0.00395±0.00188
Group III	0.00285±0.00151	0.00441±0.00171
Group IV	0.00148±0.00072	0.00555±0.00415

MDA

nmol mg/protein

	Left	Right
Group I	7.85639±1.91217	10.953±0.96485
Group II	7.94317±1.73354	8.64009±2.12478
Group III	3.04689±2.15978	10.71049±3.54912
Group IV	6.13172±2.10088	12.50003±0.92522***

Hypoxic preconditioning and Hypoxia-ischemia model

Hypoxic preconditioning was performed on randomized SD rat pups from four litters at P6 as previously described (14). The pups were placed in an 8% O_2 / 92% N_2 humidified atmosphere in pre-prepared closed chambers. The jar was partially submerged in a water-bath to maintain the gas mixture temperature at 37°C for 2.5 h. A maximum of 3 animals were allowed per chamber. We used the modified Levine preparation described by Rice et al. to produce the HI injury (23). The pups were anesthetized with isoflurane (4% in the cylinder followed by 2% by mask). We then made a midline cervical incision and double-ligated the right common carotid artery (CCA) with 5-0 surgical sutures, coagulating and severing the artery

between the two ligatures. Throughout this procedure the rats were kept at a temperature of 37°C. The operating microscope was used to confirm the surgical procedure. The duration of the anesthesia and surgery did not exceed 5 min per pup. Following 2.5 h of 8% oxygen, the animals were allowed to recover in open jars for 15 min before being returned to their dams until being humanely put down. The pups were allowed to recover with their dams for 2 hours. At the end of the recovery period, the brains of the rat pups were removed to be evaluated for lipid peroxidation levels and antioxidant enzyme (superoxide dismutase and glutathione peroxidase activities). A schematic diagram of experimental steps is shown in Figure 1.

Determination of SOD and GPX activity

SOD and GSH-Px activities were determined using Randox kits [Antrim, UK]. Briefly, the determination of SOD activity was based on the production of O_2- anions by the xantine/xantine oxidase system (22). GSH-Px was catalysed by the oxidation of reduced glutathione in the presence of cumene hydroperoxide. The generation of nicotinamide adenine dinucleotide phosphate was measured spectrophotometrically at 340 nm. The activity of GSH-Px and SOD were expressed as U/mg protein.

Determination of MDA activity

Brain tissue MDA levels were determined by the thiobarbituric acid method, and expressed as nmol MDA/mg protein. Brain tissue homogenates were prepared as follows; 0.25 g of tissue sample was homogenized in 2.5 ml of ice-cold 1.15 % potassium chloride buffer. Aliquots of homogenates were then used for analysis of lipid peroxides (20).

The degree of hypoxic ischemic injury was examined histopathologically to confirm damage. Hippocampal specimens of 1mm thickness were
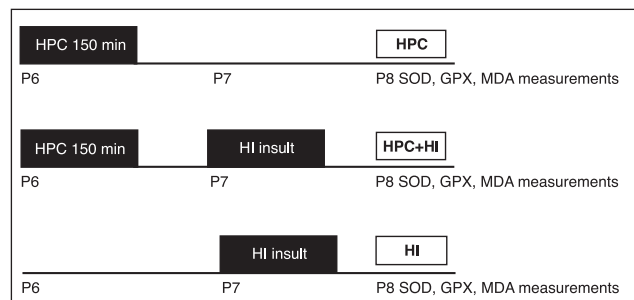
Figure 1: : Schematic diagram of experimental steps.

obtained from 2mm anterior and 2mm posterior to the bregma. From each specimen, 5Ìm-thick sections were stained with Hematoxylin and Eosin. We quantified CA1-CA3 neuronal loss by counting abnormal neurons at five hippocampal levels. Observing the slides under 400X magnification, eosinofilic neurons were counted in five different fields. Light microscopic examination was performed by an examiner blinded to the study (Figure 2 and Figure 3).
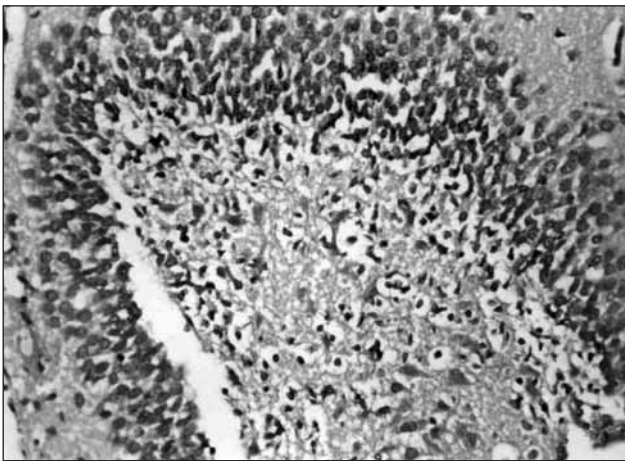

Figure 2: *Normal hippocampal cell structure in the left hemisphere (H&E) (X100).*

Statistical Analysis

Comparisons between groups were performed using the Kruskal–Wallis H-test analysis of variance by ranks. In case of a significant H-value (significance level was set at $p \leq 0.05$), the Mann–Whitney U-test was used for matched pairs post hoc comparisons.

RESULTS

The findings of the present study suggest increased lipid peroxidation and/or decreased antioxidant activity in the brain of the HI rats. Furthermore, HPC might exert protective effects, since MDA levels of the HPC+HI group were not significantly higher than those of the control and the HPC groups. Although the statistical difference did not reach the significance level, the lower MDA levels in the HPC and HPC+HI groups when compared with those of the control and the HI groups respectively, might reflect the protective effects of HPC on the HI situation. The beneficial effects of HPC might not be related to the early alterations in the antioxidative activity, since SOD
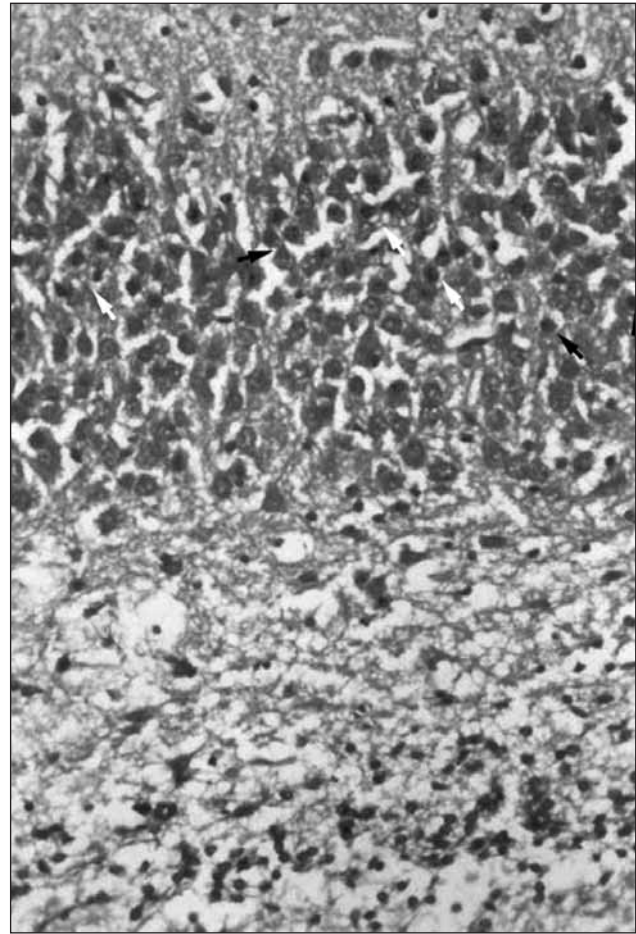

Figure 3: *Diffuse hippocampal neuronal degeneration, black arrows indicate shrunken nucleus and loss of cytoplasm, white arrows indicate increase of vacuolisation in GroupIV (H&E) (X400).*

activities were significantly lower in the HPC+HI and HI groups compared with the control and HPC groups (Figure 4,5,6).

DISCUSSION

Preconditioning is an endogenous strategy in which brief periods of hypoxia render a tissue more resistant to a subsequent ischemic/hypoxic insult and describes a powerful sublethal treatment that induces neurons to become more resistant to a subsequent ischemic/hypoxic insult. Hypoxic preconditioning is an endogenous protection against subsequent lethal hypoxia, but the mechanism involved is not understood. Preconditioning with repetitive episodes of mild hypoxia improves resistance of the organism to subsequent severe hypoxia (17) including structural and functional resistance of brain neurons (11;16;24). This treatment has been shown to protect the newborn rat brain
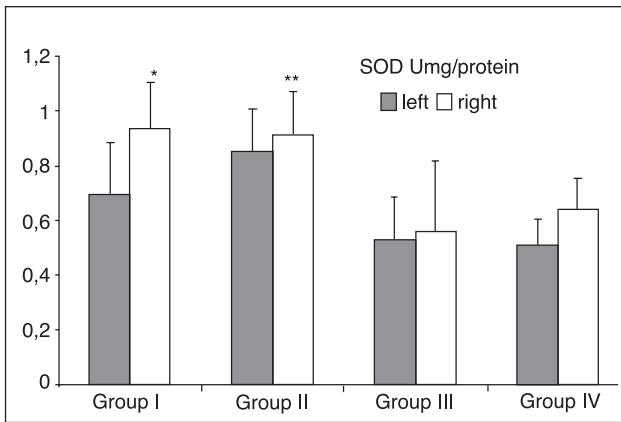
Figure 4: *Neonatal rat pups brain tissue SOD levels in groups; * Significantly different from Group III and IV Rt hemispheric measurements (p<0.05)*
** Significantly different from Group III and IV Rt hemispheric measurements (p<0.05)
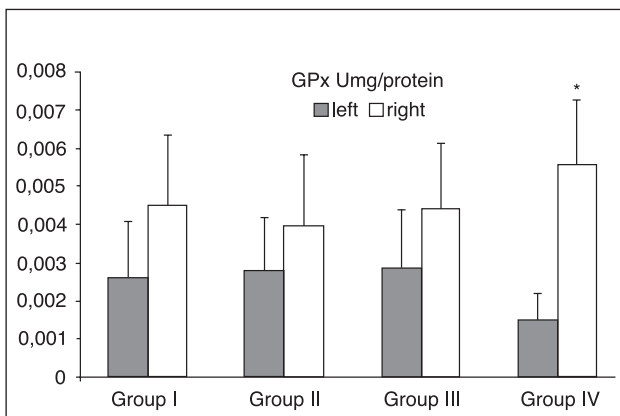
Figure 5: *Neonatal rat pups brain tissue GPx levels in groups; * Significantly different from Group IV left hemispheric measurements (p<0.05)*
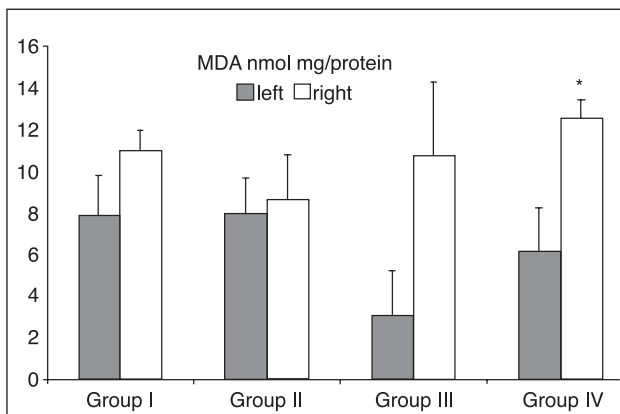
Figure 6: *Neonatal rat pups brain tissue MDA levels in groups; * Significantly different from Group I and II Rt hemispheric measurements (p<0.05)*

against subsequent hypoxic–ischemic brain injury (11;12;14;15;26;29).

Many theories have been put forward to explain the mechanism of hypoxic preconditioning; spreading depression (7;11;30) NMDA receptors (21), hypothermia (1;19) and low dose lipopolysaccharide (3). Cimarosti et al, in their research for safe pharmalogical preconditioning agents, showed that EAAT2 and ER are co-regulated and therefore maybe contributory factors in hypoxia-induced tolerance (6). Jones and Bergeron conducted a study investigating the involvement of MAPK and PI3K /Akt signaling pathways in hypoxia-induced ischemic tolerance. No clear results were shown so further studies are required (15).

One possible neuroprotective mechanism induced by hypoxic preconditioning is stated to involve up-regulating antioxidant enzymes to reduce the oxidative stress associated with ischemia and reperfusion. In support of this concept is the progressive and sustained increase in the level of Mn superoxide dismutase (MnSOD) following transient exposure to hypoxia in the gerbil brain (5;10).

We used MDA levels to show damage to the brain caused by lipid peroxidation in our study.

Various studies have looked at the SOD and GPx levels in adult animal brain tissues. Ustun et al. observed a decrease in these levels one hour after head trauma whereas Fan et al. showed an increase 24 hours after trauma (9;28).

Our study used brain tissue from 7-day old rats to resemble the newborn human brain tissue as closely as possible. These previous studies were looking at the enzyme levels following trauma but we were investigating the possible protective effects following oxidative stress. The SOD, GPx and MDA levels were measured four hours after HI insult to demonstrate the immediate short-term effects of hypoxic preconditioning.

These results suggest that the oxidative stress caused by HPC could induce tolerance to ischemia in the neonatal brain, and that the increase in the superoxide dismutase and GPx could provide a biochemical explanation of the tolerance induced under these conditions.

It is widely acknowledged that the developing brain has higher tolerance to HI (11). By studying the mechanisms of this development and subsequent time/age-dependent responses it can be established

what role SOD, GPx and MDA levels play as potential protective factors. Other studies have noted alterations as lower rates of resting glucose metabolism; increases in glucose transport, glycolytic enzymes and glycogen stores; lower densities of NMDA (N-methyl-d-aspartate) channels and channel distribution patterns that reduce overall neuronal excitability and delay depolarization; and other adaptations that slow the rate of high-energy phosphate depletion and maximize ATP homeostasis during and after ischemia (2;4;11;26;27;29).

Our study was concerned only with the effects of the changes in SOD, GPx and MDA levels together with preconditioning in cases of hypoxic ischemia.

Postnatal HI injury is known to be reduced by hypoxic preconditioning (12). However, although the mechanism of this preconditioning has not been fully clarified, it is thought to be driven by genes or proteins influencing the oxidative and metabolic occurrences (11;26;29).

ROS are a significant factor in the higher tolerance level to a potentially lethal insult that has been induced by preconditioning from a sublethal stimulus. The direct mitochondrial swelling and damage is lower so there is less inhibition of ATP synthesis which in turn prevents any increase in ROS production (3;8).

The normal adaptive response to increased SOD activity is an accompanying increase in GPx levels. This increase in GPx detoxifies the high level of hydrogen peroxide produced by the increased SOD activity. When sufficient increased GPx to scavenge these superoxide radicals is not present, there will be consequent brain damage (5;8;13).

In our study the preconditioning was performed with the aim of raising the levels of both SOD and GPx thus strengthening the antioxidant defenses. Our results show that the two groups which underwent HI (Group III and IV) had lower levels of SOD when compared to group I and II. This was an acute response as we only took one measurement at 4 hr following the HI injury.

Although there is ongoing research focusing on neuroprotection and therapy that may affect a common pathophysiological pathway of hypoxia, thus minimizing neuronal damage, further studies taking measurements over different time periods are needed to clarify any time- or age- related changes.

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