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Effect of Melatonin on Brain Oxidative Damage Induced by Traumatic Brain Injury in Immature Rats

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Summary

Progressive compromise of antioxidant defenses and free radical-mediated lipid peroxidation, which is one of the major mechanisms of secondary traumatic brain injury (TBI), has also been reported in pediatric head trauma. In the present study, we aimed to demonstrate the effect of melatonin, which is a potent free radical scavenger, on brain oxidative damage in 7-day-old rat pups subjected to contusion injury. Whereas TBI significantly increased thiobarbituric acid reactive substances (TBARS) levels, there was no compensatory increase in the antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) 24 hours after TBI in 7-day-old rats. Melatonin administered as a single dose of 5 mg/kg prevented the increase in TBARS levels in both non-traumatized and traumatized brain hemispheres. In conclusion, melatonin protects against oxidative damage induced by TBI in the immature brain.

Key words

Traumatic brain injury (TBI) • Immature rat • Lipid peroxidation • Superoxide dismutase (SOD) • Glutathione peroxidase (GPx) • Melatonin

Introduction

Traumatic brain injury (TBI) is the leading cause of morbidity in childhood. Clinical studies suggest that age is an important prognostic factor for pediatric TBI (Adelson and Kochanek 1998). Children less than 4 years old suffering from head trauma have the worst prognosis (Koskiniemi *et al.* 1995). Traumatic brain injury can be classified as primary which occurs immediately after trauma, and secondary which may appear several hours or even days later (Stein and Spettell 1995, Colicos *et al.* 1996). Several pathogenetic mechanisms, including derangements in cerebral blood flow, excitotoxicity, reactive oxygen species (ROS), inflammation, and apoptosis, have been described for secondary traumatic damage (Bayir *et al.* 2003). ROS such as superoxide, hydrogen peroxide and hydroxyl radicals are formed in the course of cellular metabolism. The hydroxyl radicals are widely accepted as being the most damaging ROS produced by cells. ROS can cause cellular injury *via* several mechanisms, including peroxidation of membrane lipids as well as oxidative damage of proteins and DNA (Feuerstein *et al.* 1997). They play an important role in secondary brain injury induced by head trauma (Shohami *et al.* 1997, Tyurin *et al.* 2000). Newborns and particularly preterm infants are very susceptible to oxidative damage induced by ROS; certainly, their antioxidant defense systems are imbalanced in favor of

oxidants (Buonocore *et al.* 2001, 2002).

Melatonin, which is secreted by the pineal gland, is a powerful scavenger of oxygen free radicals, hydroxyl radicals and peroxyl radicals (Tan *et al.* 2000, Reiter *et al.* 2000a,b, Cuzzocrea and Reiter 2001). Additionally, melatonin is able to increase the activity and expression of antioxidant enzyme activity under both physiological and conditions of elevated oxidative stress (Rodriguez *et al.* 2004). Melatonin (10 mg/kg) administration to pregnant rats has been reported to increase antioxidant enzyme activities such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and to protect against oxidative mitochondrial damage in the fetal rat brain (Okatani *et al.* 2000, Wakatsuki *et al.* 2001a,b).

The aim of the present study was to evaluate the effect of melatonin on brain antioxidant enzyme activities including SOD and GPx levels, and thiobarbituric acid reactive substance (TBARS) levels as an indicator of lipid peroxidation after TBI in immature rats.

Material and Methods

Animals

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylül 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. Twenty-one litters delivered spontaneously were reared with their dams until the time of experimentation at 7 days of postnatal age. All rats were maintained on a constant 12-h light/12-h dark cycle at constant room temperature (21 °C), and humidity (60 %). Experiments were carried out between 9:00-11:00 h in a sound-attenuated and air-regulated experimental room. Rats were divided into three groups: (1) vehicle-treated TBI group (TBI, n=7), (2) melatonin-treated TBI group (TBI+Mel, n=7), and (3) control group (n=7). Pups subjected to sham surgery served as controls.

Induction of traumatic brain injury and treatment

We used a modification of a well-described percussion trauma model in immature rats, in an attempt to model infant and early childhood head trauma (Bittigau *et al.* 1999). In this model, the severity of the trauma-triggered neurodegeneration in the brains of 3- to 30-day-old rats had been demonstrated to be age-dependent and highest in 7-day-old animals. Therefore, 7-day-old rat pups were subjected to contusion injury in our study. The contusing device consisted of a hollow tube 40 cm long, perforated at 1-cm intervals to prevent air compression. The device was kept vertical to the surface of the skull and guided a falling weight onto a circular footplate resting upon the surface of the parietal bone. The center of the footplate was stereotaxically positioned 3 mm anterior and 2 mm lateral to the lambda and was fixed in place under ether anesthesia. A force of 160 g×cm produced by a 10-g weight was selected to produce brain contusion.

Melatonin (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol and diluted with physiologic saline. The ethanol concentration in the final solution was 5 %. The melatonin solution was injected intraperitoneally at a dose of 5 mg/kg of body weight immediately after induction of TBI. Control rats were injected with an equal volume of vehicle. Animals were kept on a heating pad maintained at 37 °C until returned to their mothers 4 hours after the trauma or sham surgery.

Determination of antioxidant enzyme activities and TBARS levels

Twenty-four hours after the experiments, the rats were killed by decapitation under ether anesthesia. Right and left hemispheres were separated on an ice-cold surface. Tissue homogenates were prepared as described by Carrillo *et al.* (1991). An aliquot of the homogenate and supernatant was stored at –70 ºC until the determination of TBARS levels, and SOD and GPx enzyme activities.

Determination of SOD activity

SOD activity was determined using a RANSOD kit (Randox labs, Crumlin, UK) (Delmas-Beauvieux *et al.* 1995). To generate superoxide radicals that react with 2-4-iodophenyl-3-4-nitrophenol-5phenyltetrazolium chloride (INT) to form a red formazan dye used xanthine and xanthine oxidase. Concentration substrates were 0.075 μmol for xanthine and 0.037 μmol for INT. SOD inhibit this reaction by converting the superoxide radical to oxygen. A SOD unit inhibits the rate of reduction of INT by 50 in a complex system with xanthine and xanthine oxidase. Due to the small linearity range of the test, the sample must be diluted so that the percentage of inhibition falls between 30 % and 60 %. Using the standard provided in the kit a standard curve was prepared, and we obtained the value for the supernatant

from this curve. In the supernatant SOD activity measured at 505 nm on a Shimadzu UV-1201V spectrometer and the results were expressed as SOD U/mg protein.

Determination of GPx activity

GPx was determined using a Randox test combination (RANSEL). GPx catalyses the oxidation of glutathione (at a concentration of 5 μmol) using cumene hydroperoxide according to the method of Paglia and Valentine (1967). In the presence of glutathione reductase (at a concentration $\geq 0.75.\ 10^{-3}$ U) and 0.35 μmol of NADPH, the oxidized glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to NAD^+. The decrease in absorbance measured at 340 nm and 37 °C. The assay was performed on a supernatant. The necessary enzyme activity to convert one μmol of NADPH to NADP in one minute defined as the GPx unit and the results expressed as GPx U/mg protein.

Determination of TBARS level

TBARS level was estimated according to the method of Rehncrona *et al.* (1980). Homogenates (0.5 ml) were extracted with 0.5 ml of trichloroacetic acid (20 % wt/vol). After the centrifugation, 1 ml of acid (0.67 % wt/vol) was added to 0.9 ml of supernatant and the samples heated in boiling water for 10 min. After cooling, the recorded absorbance was measured at 532 nm. Using 1, 1, 3, 3-tetraethoxypropane prepared a standard curve and we read the value of the homogenate from this curve. The results were expressed as nmol/mg protein.

Determination of protein concentration

Protein contents of supernatant and homogenate were determined using a U/CSF Protein kit (Randox Labs, Crumlin, UK) (Watanabe *et al*. 1986).

Statistical Analysis

Results are presented as means ± SEM. All data were analyzed by one-way analysis of variance (ANOVA), Tukey-HSD (post hoc) and Mann-Whitney tests. $P<0.05$ was considered significant.

Results

Rats subjected to sham surgery or trauma recovered within 5 to 10 min. There was no mortality in all groups. TBARS levels (Fig. 1) were significantly increased by TBI in both traumatized and non-traumatized sides of the brain compared with the control group ($p<0.001$). Melatonin (5 mg/kg) prevented the increase in TBARS levels of both brain sides ($p<0.001$). There was no significant difference for TBARS levels between the traumatized and non-traumatized sides in both the non-treated and melatonin treated groups ($p>0.05$). There was no significant difference among all groups for tissue SOD and GPx levels (Figs 2 and 3).

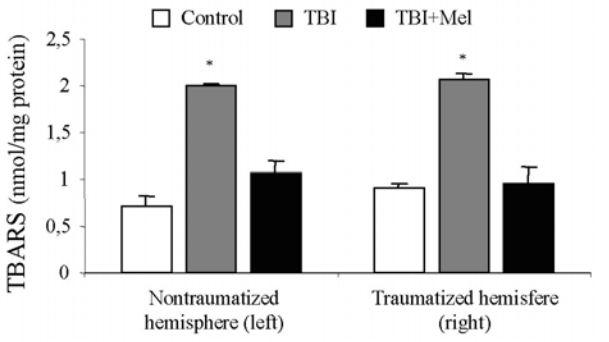

Fig. 1. Effects of traumatic brain injury (TBI) on thiobarbituric acid reactive substance (TBARS) levels. TBI increased TBARS levels in the non-traumatized and traumatized hemispheres of the brain. Melatonin prevented the increase in TBARS levels of both brain sides.

Discussion

The present study demonstrated that TBI significantly increased TBARS levels in both traumatized and non-traumatized sides without any difference between the sides of the brain, indicating that ROS produced by head trauma induced lipid peroxidation in the immature rat brain. ROS play a key role in mediating secondary brain injury induced by trauma (Shohami *et al.* 1997, Tyurin *et al.* 2000). The brain is particularly vulnerable to oxidative injury because of its high rate of oxygen consumption, intense production of reactive radicals and high levels of transition metals, such as iron, which can catalyze the production of reactive radicals. Moreover, neuronal membranes are rich in polyunsaturated fatty acids, which are a source for lipid peroxidation reactions (Evans 1993, Reiter 1998). Lipid peroxidation is supposed to cause the destruction and damage of cell membranes, leading to changes in membrane fluidity/permeability and to enhanced rates of protein degradation (Garcia *et al.* 1997). Non-radical products such as alkanes and carbonyl compounds, which

are produced during the final steps of lipid peroxidation, can be measured based on their reactivity with thiobarbituric acid. TBARS primarily reflects production of lipid peroxides, which are broken down during the assay to yield malonaldehyde (Hageman *et al.* 1992). Kasprzak *et al.* (2001) demonstrated that increased erythrocyte TBARS concentrations were correlated with the severity of injury in the patients with brain contusion.

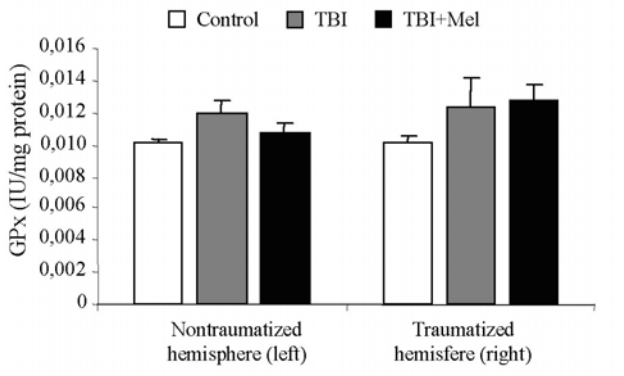

Fig. 2. Effects of traumatic brain injury (TBI) on glutathione peroxidase (GPx) activities in the non-traumatized and traumatized hemispheres of the brain. There was no significant difference among all groups.

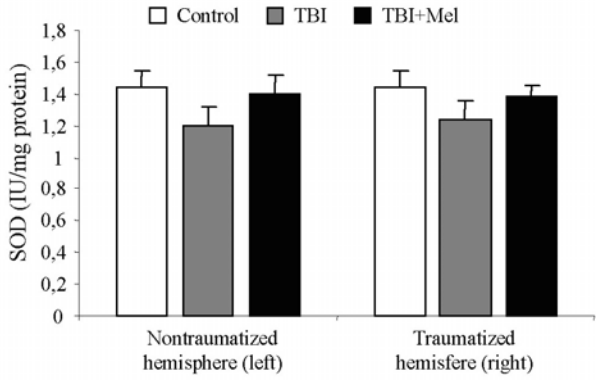

Fig. 3. Effects of traumatic brain injury (TBI) on superoxide dismutase (SOD) activities in the non-traumatized and traumatized hemispheres of the brain. There was no significant difference among all the groups.

When the tissues are exposed to oxidative stress they increase the activity and expression of antioxidant enzymes as a compensatory mechanism against free radical-mediated damage. Nevertheless, the increased activity of the antioxidant enzymes may be inadequate to counteract the potential damage in many conditions of oxidative stress (Rodriguez *et al.* 2004). Moreover, antioxidant enzyme activities have been found to be diminished under highly elevated oxidative stress conditions as a result of molecular damage. In adult animals, brain tissue SOD and GPx levels have been demonstrated to be decreased at one hour after head trauma (Ustun *et al.* 2001a,b). Recently, Fan *et al.* (2003) examined age-dependent differences in brain GPx activity on postnatal day 21 and adult murine brain after TBI. They found that GPx activity in the adult brain was increased within 24 h after trauma, whereas there was no compensatory increase in GPx activity in immature brain. In the present study we found no increase in either SOD or GPx levels 24 h after TBI in 7-day-old rats.

Melatonin is believed to be a potent free radical scavenger and a powerful antioxidant. It is rapidly absorbed regardless of the administration route since its lipophilic nature, and readily crosses the blood-brain barrier (Tan *et al* 2000, Reiter *et al* 2000a,b, Cuzzocrea and Reiter 2001). It directly scavenges the highly toxic peroxynitrite anion, which induces oxidative damage (Gilad *et al.* 1997). Melatonin treatment in the different doses (10-100 mg/kg) has been reported to reduce brain lipid peroxidation induced by various free radical generating agents and processes such as lipopolysaccharide, kainic acid, iron, potassium cyanide, L-cysteine, ischemia-reperfusion, radiation and painful stimulation (Sewerynek *et al.* 1995, Melchiorri *et al.* 1995, Kabuto *et al.* 1998, Yamamoto and Mohanan 2002, Yamamoto and Tang 1996, Wakatsuki *et al.* 1999, Undeger *et al.* 2004, Pekárková *et al.* 2001). Cirak *et al.* (1999) demonstrated that a higher dose of melatonin (200 mg/kg) helps to decrease lipid peroxidation in the very early posttraumatic period. Rogerio *et al.* (2002), however, observed that melatonin treatment in the doses of 50 and 100 mg/kg caused failure to thrive, seizures or death in neonatal rats, whereas the doses of 1, 5 and 10 mg/kg did not significantly alter postnatal growth and weight gain. The dose of melatonin used in humans is 1 or 5 mg/kg *per os* (Solar 1999). Therefore, we investigated the effect of melatonin at a dose of 5 mg/kg on brain lipid peroxidation induced by head trauma and found that melatonin prevented the increases in TBARS levels of both non-traumatized and traumatized brain sides. To our knowledge, this is the first report indicating that melatonin decreases head trauma-induced lipid peroxidation in the immature rat brain. There was no significant difference between non-treated and melatonin treated rats at 24 hours after induction of TBI for antioxidant enzyme levels. This may be due to the fact that antioxidant enzyme activities could become

normalized by inhibition of the production of ROS because melatonin is a direct free radical scavenger.

In conclusion, melatonin administered as a single dose of 5 mg/kg prevents the brain oxidative damage induced by TBI in the immature rats.

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