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RESEARCH NOTE

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Does melatonin protect or treat brain damage from traumatic oxidative stress?

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Abstract A variety of experimental studies have demonstrated the neuroprotective effects of melatonin, based on its antioxidant activity. In a prospective randomized study, the effects of melatonin were investigated in experimental head trauma-induced oxidative stress in rabbits. The experimental study was performed on 30 rabbits. The animals were divided into three groups. Group I (sham procedure): a right parietal craniotomy was performed on each animal, and the dura mater was left intact. Group II: experimental brain trauma (EBT) was performed on each animal using a 1 cm inner diameter × 10 cm long glass tube, through which a 20 g weight (0.5 cm diameter) was dropped onto the brain at the craniotomy site, causing a contusional head trauma. Group III: the same EBT model was performed, but 2.5 mg/kg melatonin was injected intraperitoneally four times (total dose 10 mg/kg); these injections were performed 20 min before the operation, during the trauma, 1 h later and 2 h later. The rabbits were sacrificed after the EBT at 24 h after the brain trauma. The activities of the three principal antioxidant enzymes—catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px)—were determined, and the levels of malondialdehyde (MDA), a product of lipid peroxidation, and glutathione (GSH) were measured in brain homogenates. MDA levels were found to be higher in the EBT group than in the EBT + melatonin group or the sham procedure group. The SOD activity was found to be higher in the EBT group than in the sham procedure group. Enzymatic parameters (except for SOD) were significantly higher in melatonin-treated animals than in EBT animals. GSH levels in melatonin-treated animals were decreased compared with EBT animals. In conclusion, the data indicate that melatonin protects against free radical-mediated oxidative changes in brain tissue by boosting antioxidant enzymes, and in particular lowering lipid peroxidation in rabbits with EBT.

Keywords Melatonin · Antioxidant enzymes · Lipid peroxidation · Experimental brain damage

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Introduction

Various experimental studies have reported that brain injury results in an increase in reactive oxygen species (ROS). Among the pathomechanisms involved in traumatic brain injury, ROS-mediated lipid peroxidation plays an important role (Hall 1993; Wada et al 1999).

Several criteria for the pathophysiologic significance of ROS have been established. These include the demonstration of increased post-traumatic levels of ROS soon after central nervous system injury, the spatial and temporal correlation between ROS formation and pathophysiologic alterations (such as loss of microvascular regulation, vasogenic edema, progressive post-traumatic ischemia development), striking similarity between post-traumatic post-ischemic central nervous system pathology and that caused by chemical peroxidative insults, and the protective efficacy of oxygen radical-scavenging agents or compounds that inhibit lipid peroxidation (Hall 1993).

Melatonin is associated with the transmission of photoperiodic information and the regulation of seasonal reproductive cycles (Reiter 1991). It has also been implicated in aging and immune responses (Reiter 1994). Recently, melatonin was discovered to be a highly efficient free radical scavenger and general antioxidant (Poeggeler et al 1993; Reiter 1993, 1998; Zang et al 1998; Franceschini 1999; Vural et al 2001). Due to its high diffusion ability and highly lipophilic nature, it passes through all membranes and barriers, including the blood–brain barrier. Melatonin acts as a non-enzymatic antioxidative agent against the devastating actions of the extremely reactive hydroxyl radical (Poeggeler et al 1993; Reiter 1993, 1998; Vural et al 2001).

Certain enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-Rd) play an important role in antioxidant defense by converting ROS to non-radical products (Vural et al 2001). GSH-Rd generates reduced glutathione (GSH) from oxidized glutathione. GSH, in turn, reduces hydrogen peroxide, lipid peroxides, disulfides, ascorbate, and free radicals. Melatonin also stimulates GSH-Px activity, which metabolizes the precursor of the hydroxyl radical, hydrogen peroxide, to water (Okatani et al 2000).

There is a need to clarify the effects of melatonin on these antioxidant enzymes and on lipid peroxidation in experimentally-induced brain injury. This study investigated the activities of different enzymes in experimentally-induced brain injury in rabbits. It was designed to examine the possible protective effects of exogenous melatonin on EBT-induced oxidative changes in the neural tissue of rabbits.

Materials and methods

Thirty male New Zealand Albino rabbits weighing 400–500 g were used in the study. The experiment was performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research at Suleyman Demirel University, Isparta. The animals were placed in a temperature (21 ± 2°C) and humidity (60 ± 5%) controlled room in which a 12:12 light:dark cycle was maintained. The animals were divided into three groups.

Group 1 ($n = 10$): under ketamin anesthesia, a right parietal craniotomy was performed on each animal in the prone position, and the dura mater was left intact (sham operation). Group II ($n = 10$): the same trauma model was performed for each animal as described by Feeney et al (1981). Through a 1 cm inner diameter, 10 cm long glass tube, a 1 cm diameter 20 g weight was allowed to fall on the craniotomy site causing contusional cerebral trauma. During the study, the body temperatures of animals were kept stable at 37 °C using a heating bag and by monitoring the body temperature via a rectal probe. Group III ($n = 10$): the same trauma was performed for these animals, but 2.5 mg/kg melatonin (Sigma Chemical Co.,USA) was injected intraperitoneally four times (total dose 10 mg/kg); 20 min before the operation, during the trauma, 1 h later and 2 h after the trauma. After induction of the brain trauma, the animals were killed with high dose phenobarbital 24 h later. Neural tissue samples of contused brain were obtained. The tissue samples obtained were stored at −70 °C until they were assayed.

Homogenization of tissue samples

Tissue samples were homogenized after thorough washing with distilled water to remove blood from the tissue. A 10% homogenate from each sample was obtained with 150 mM-cold KCl. The homogenates were then centrifuged at 6,000×*g* to separate the tissue. Protein concentrations of homogenates were determined by the method of Lowry (1951).

Biochemical analyses

SOD, GSH-Px, CAT activities, and GSH levels in the brain tissue were spectrophotometrically analyzed. Malondialdehyde (MDA) levels were determined during protein analysis. All biochemical analyses were conducted at +4 °C.

Measurement of MDA

MDA levels were measured by the double-heating method of Drapper and Hadley (1990). According to this method, thiobarbituric acid reactive substance (TBARS) concentrations were determined in tissue homogenates. Three milliliters of 1% H_3PO_4 (phosphoric acid) and 0.6% TBA solution were mixed with homogenates. The mixture was kept in a hot water bath (100 °C) for 45 min. Then it was cooled to room temperature and 4 ml *n*-butanol was added and vortexed. The absorbance was measured at 532 nm ($e = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) using a spectrophotometer (Schimadzu 1208 UV–VIS, Japan). Results are expressed as nmol/mg protein.

Measurement of SOD

SOD activity was estimated using a commercial kit (Ranso, Randox Laboratories, Crumlin, UK). Xanthine and xanthine oxidase were used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured at 505 nm on a spectrophotometer.The results were expressed as U/mg protein.

Measurement of GSH-Px

GSH-Px activity was determined using a commercial kit (Ranso, Randox Laboratories, Crumlin, UK). GSH-Px catalyses the oxidation of GSH by cumene hydroperoxide in the presence of glutathione reductase and NADPH. The oxidized glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured. Results were expressed as U/mg protein.

Measurement of CAT

CAT activity was measured using the method of Aebi (1984). Reduction of H_2O_2 by catalase occurs when exposed to 240 nm wavelenght; this change was recorded at 15-s intervals for 2.5 min. CAT activity was determined as k/ml extracts by taking appropriate absorbance readings based on regression analysis, and it was finally expressed as k/mg protein.

Measurement of glutathione

GSH levels were measured according to the enzymatic recycling method of Beutler et al (1963). The amount of total GSH was determined from a standard curve obtained with known amounts of GSH standards. GSH levels were expressed as nmol/mg protein.

Student's *t* test was performed for statistical analysis. Statistics were analyzed using SPSS 7.5 for Windows. The results are given as mean ± SD.

Results

The results are presented in Table 1. MDA levels of EBT rabbits were increased compared to both the control and the melatonin-treated animals. Melatonin significantly decreased lipid peroxidation in the brain tissue of EBT-treated rabbits.

The SOD activity was found to be higher in rabbits with EBT than in sham-operated animals. There was no statistically significant difference in GSH-Px activity and GSH level between the sham-operated and EBT groups. However, the GSH-Px and CAT activities were significantly higher while the SOD activity was lower in the EBT + melatonin-treated animals than in the EBT-alone rabbits. Melatonin treatment prevented the decrease of CAT and GSH-Px after brain injury.

Discussion

Neurotrauma is the most frequent cause of morbidity. Two kinds of injury are thought to occur in the nervous system following an impact. The first is the primary injury, which occurs at the time of impact; the second is referred to as secondary injury and is a result of the pathophysiological alterations secondary to trauma, some of which are hypotension, ischemia, and some reactants produced by the primary injury (Bullock 1993). Monoamines, ROS, neuropeptides, arachidonic acid metabolites, and changes in extracellular calcium are some of the alterations caused by the primary injury which are thought to take place in the induction of secondary injury. Some studies report that free radicals play a role in cerebral ischemia and trauma. Free oxygen radicals either disrupt the blood-brain barrier or cause brain edema by affecting the neurons (Ikeda et al 1989a; Ikeda and Long 1990; Farooqui and Horrocks 1998; Mass 1999).

The brain is particularly sensitive to lipid peroxidation due to its high concentrations of polyunsaturated fatty acids, its low antioxidant capacity, and its high rate of oxygen consumption. Furthermore, brain tissue contains high levels of iron and cupper, which promote the formation of oxygen free radicals. This suggests that the use of free radical scavengers such as melatonin might have therapeutic significance.

Antioxidant enzymes such as SOD, CAT, and GSH-Px are present in mammalian cells. They protect the cells from the toxic effects of free radicals. When free radicals are produced in the cell membrane the lipids undergo peroxidation which can ultimately cause cell death. Increased antioxidant enzyme activity is a protective response to free radical formation.

Table 1 Mean ± SD of MDA, CAT, SOD, GSH-Px, and GSH levels in sham-operated (Group I) rabbits, rabbits that underwent craniotomy and EBT (Group II), and rabbits that underwent EBT and melatonin treatment (Group III)

	Group	Mean ± SD	*P* value	Group	Mean ± SD	*P* value
MDA (nmol/mg prot.)	I	0.028 ± 0.014	$P < 0.05$	II	0.051 ± 0.027	$P < 0.001$
	II	0.051 ± 0.027		III	0.020 ± 0.013	
CAT (k/mg prot.)	I	0.067 ± 0.032	$P > 0.05$	II	0.044 ± 0.016	$P < 0.001$
	II	0.044 ± 0.016		III	0.069 ± 0.039	
SOD (U/mg prot.)	I	8.77 ± 1.66	$P > 0.001$	II	14.85 ± 2.85	$P > 0.001$
	II	14.85 ± 2.85		III	8.80 ± 2.85	
GSH-Px (U/mg prot.)	I	0.20 ± 0.04	$P > 0.05$	II	0.18 ± 0.07	$P > 0.05$
	II	0.18 ± 0.10		III	0.30 ± 0.10	
GSH (mg/dl)	I	2.08 ± 0.51	$P > 0.05$	II	2.29 ± 0.70	$P > 0.05$
	II	2.29 ± 0.70		III	1.42 ± 0.72	

There is extensive experimental support for the early occurrence and pathophysiologic importance of oxygen radical formation and cell membrane lipid peroxidation in the injured nervous system. Free oxygen radical-induced damage has been suggested in traumatic cell injury and cell death, while free radical scavengers such as SOD, CAT, and GSH-Px are associated with partial ameloriation of traumatic injury (Faden 1989).

Potential sources of oxygen radicals within the injured nervous system include the arachidonic acid cascade (prostaglandin synthetase and 5-lipoxygenase activity). Recently, some pharmacologic agents have been used to prevent secondary injury. Kontos and Wei (1986) used indomethazine and SOD to prevent secondary injury; they also reported that superoxide radical formation continued for at least 1 h after trauma.

Melatonin is a very potent and efficient endogenous free radical scavenger and affords protection of molecules, especially DNA, from oxidative damage. Melatonin is the most powerful and effective endogenous free radical scavenger detected to date. It provides on-site protection for all biomolecules due to its lipophilic nature (Tan et al 1993; Reiter 1994; Zang et al 1998; Franceschini et al 1999).

Pro-oxidant factors are subject to rapid changes and a lag time exists before biological systems can adapt to them. Permanent and irreversible injury, however, occurs only if the pro-oxidant factors are chronically higher than the maintenance and repair systems of an organism. Therefore, it is of great importance that antioxidative mechanisms, like the effects of melatonin, operate throughout life. Melatonin was shown to exert a protective effect against radical-induced lipid peroxidation, and to inhibit a pathologically-increased influx of ions (Reiter 1994; Zang et al 1998; Franceschini et al 1999; Vural et al 2001).

The hydroxyl radical-scavenging potency of melatonin is much greater than that of classical hydroxyl radical scavengers like glutathione, an important endogenous radical scavenger. Also, in humans, if in fact free radicals play a major role in the degenerative processes after brain injury (Cadet 1988), which they seem to do, then exogenously-administered melatonin may have some ameliorative effects on free radical damage.

Previous literature reports (Long et al 1972; Chan et al 1987; Ikeda et al 1989b) show that oxygen free radical species cause lipid peroxidation in brain edema. Long et al (1972) proposed that antioxidant compounds could be used in the treatment of brain edema in the posttraumatic period. For this purpose, several therapeutic methods were considered. SOD was tested as the first treatment alternative, but Chan et al (1987) and Ikeda et al (1989b) showed that SOD does not reduce oxygen free radical levels sufficiently. This is because the molecular weight of SOD is 31,000, and the enzyme cannot pass through the blood–brain barrier under normal conditions. Additionally, SOD has a very short biological half-life which limits its clinical utility.

In recent years, α-tocopherol (vitamin E) and its analogs have been considered as an alternative treatment method for ischemic neural damage in many studies (Hara et al 1990; Meydani et al 1990; Shin et al 1994). It has been demonstrated that α-tocopherol donates a hydrogen atom to the chain-propagating lipid peroxyl radicals, giving rise to the relatively stable α-tocopherol radical. This maintains the stability of the cell by protecting the lipid components of neural tissue against lipid peroxidation. Melatonin used as a free radical scavenger agent following head trauma has not been studied extensively. There have only been a few experimental studies in rats (Messenge et al 1998; Horrokova et al 2000; Sarrafzadeh et al 2000).

An increased MDA level is a marker of lipid peroxidation occurrence in traumatized tissue. In the present study, we observed an increase in antioxidant enzyme activities and TBA reactive substances or slightly increased MDA levels in injured animals. This indicates that self defense mechanisms did not sufficiently protect the neural tissue againist oxidative injury, because TBA reactive substances were used as a convenient index of the oxygen free radical-mediated lipid peroxidation in cell membranes. After melatonin administration (in Group III), as a measure of melatonin protection, we determined the drop in the MDA level. This result is in good agreement with those in the literature (Ikeda et al 1989b; Messenge et al 1998; Horokova et al 2000). This makes melatonin advantageous as an antioxidant.

In this study, it was established that melatonin potentiates and increases the activity of GSH-Px, which metabolizes H_2O_2 to H_2O, and reduced GSH, which also reduces free radicals. This is in agreement with the results of Barlow-Walden et al (1995), who showed that exogenous administration of melatonin in rats doubles GSH-Px activity in the brain. They proposed two possible explanations for the induction of GSH-Px activity: (1) the increase in GSH-Px activity is elicited by the antioxidative activity of melatonin, which protects the enzyme from inactivation by hydroxyl radicals, and (2) an enzyme induction is mediated by changes in GSH-Px gene expression. In agreement with these theories, the present study showed that GSH-Px activity was increased in melatonin-treated animals. CAT is an important scavenging enzyme that mops up reactive oxygen species, since it removes the H_2O_2 produced during metabolic processes (Ikeda et al 1989a; Ikeda and Long 1990; Wada et al 1999; Horokova et al 2000). The increase in CAT and GSH-Px activity and the decreased MDA and GSH levels observed in the melatonin-treated group must be related to the benefical effect of melatonin on free radical formation in brain tissue, which is thought by Reiter (1993, 1994, 1998) and Poeggeler et al (1993) to occur due to its lipophilic nature. These findings support the assumed protective and therapeutic effects of melatonin against experimental trauma-induced neural damage.

The activity of SOD, which catalytically converts superoxide anions to H_2O_2, was increased after head

trauma. However, we determined that melatonin administration could not prevent the decrease of SOD. Melatonin was shown to decrease SOD activity in the damaged brain. However, Nishibe (1989) showed that the SOD activity is generally much lower than the CAT activity in neural tissues. Melatonin acting as a free radical scavenger may lessen the need for extensive production of SOD. It is therefore speculated that melatonin administration would be benefical and enough to protect against lipid peroxidation.

We therefore conclude that melatonin is a potent antioxidant that prevents and decreases lipid peroxidation formation in experimental cerebral trauma. Since secondary injury and the production of free oxygen radicals are the result of primary injury, early introduction of melatonin seems to be helpful. It helps to protect against secondary injury and against therapeutic neuronal injury following secondary injury. Further investigations into the time/dose-dependency of melatonin are needed before its clinical use in cerebral trauma as a free radical scavenger is proposed.

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