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Original Article

The protective role of melatonin in experimental hypoxic brain damage

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Abstract

Background: It is known that oxygen-derived free radicals play an important role in the pathogenesis of brain injury. Melatonin is a powerful scavenger of the oxygen free radicals. In this study, the protective effect of melatonin against the damage inflicted by reactive oxygen species during brain hypoxia was investigated in newborn rats using biochemical parameters.

Methods: For biochemical analyses, the levels of lipid peroxidation product (malondialdehyde ([MDA]), levels of reduced glutathione (GSH) and the activities of superoxide dismutase (SOD) and catalase (CAT) were estimated.

Results: After the third day of brain hypoxia, the brain levels of MDA increased. Pretreatment of animals with melatonin abolished the rise in MDA induced by hypoxia. GSH concentration did not increase by pretreatment with melatonin. Additonally, the activities of two antioxidative enzymes (SOD and CAT) decreased after the experimental period with melatonin only preventing the change of CAT. The activity of SOD was not influenced by melatonin administration as expected.

Conclusion: In this experimental study, exogenously administered melatonin effectively protected against brain injury by oxidative stress. This protective effect of melatonin may be due to its direct scavenger activity and activation of CAT. Thus, melatonin may potentially be useful in the treatment of neurodegenerative conditions that may involve free radical production, such as perinatal hypoxia.

Key words antioxidant enzyme, hypoxia, lipid peroxidation, melatonin.

Perinatal encephalopathy, induced by hypoxia or ischemia, is a major cause of childhood neurological disability. Oxygen free radicals play an important role in the pathogenesis of hypoxic/ischemic brain damage during the perinatal period.[1,2] Thus, inhibition of free radical generation may be a potential mechanism for reducing the pathophysiologic effects of hypoxic-ischemia. Melatonin, the chief secretory product of the pineal gland, has been found to be effective in protecting against pathological states due to reactive oxygen species release.[3–6]

In the present study, we examined the protective effect of melatonin against oxidative stress during brain hypoxic injury using biochemical parameters as follows: the levels of malondialdehyde (MDA) as an index of lipid peroxidation, related to levels of glutathione (GSH) and antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT).

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Materials and methods

Animals

In the present study, 35 7-day-old Spraque–Dawney rat pups of either sex, weighing 15–20 g, were used. Trakya University Animal Research Committee approved all experimental procedures. The pups suckled until the start of the experiment.

Preparation of the drug

Melatonin was purchased from Sigma (St. Louis, MO) and dissolved freshly in pure ethanol and later liquidized with isotonic sodium chloride (0.9% NaCl) amounting to a final concentration of 1:10 in a freshly prepared solution form. Melatonin (10 mg/kg bodyweight [BW], 20 mg/kg BW) or vehicle were administered intraperitoneally (IP) 30 min before hypoxia.

Experimental design

Hypoxic brain injury model

The hypoxic exposure was achieved by placing littermates (n = 8–9) in a 20 L airtight plastic box submerged into a 37°C water bath and flushed for 2 h with a humidified mixture of 8% oxygen and 92% nitrogen delivered at 1.0 L/min. After 30 min recovery in the 37°C water bath, they were returned to their cages and suckled until they were sacrified. The first dose of melatonin was applied IP followed by intramuscularly on the first and second days of experiment in groups 3 and 4. We designed the procedure of the experiment and decided the melatonin dose would be taken from the previous reported studies.[7,8]

A total of 35 pups were divided randomly into four groups: Group 1 (n = 8), hypoxia process (HP) was not applied to the pups of the control group; Group 2 (n = 9; untreated group), HP was applied and physiologic saline (0.2 mL) was given P 30 min before hypoxia; Group 3 (n = 9; melatonin pretreated group), 10 mg/kg of BW melatonin in addition to HP was applied; and Group 4 (n = 9; melatonin pretreated group), 20 mg/kg of BW melatonin in addition to HP was applied.

At the end of Day 3, all pups were sacrified by cervical dislocation. The cranium was opened and the cerebrum and cerebellum were immediately removed from the skull. After being washed two times with ice cold 0.9% NaCl solution, all brain tissue was frozen at –70°C in polypropylene tubes until assay.

Determination of tissue malondialdehyde, reduced glutathione, activities of superoxide dismutase, and catalase enzymes

The frozen tissues were separately weighed and then homogenized in 10 volumes of cold phosphate buffer (pH 7.4) in a Potter-type homogenizer. Samples were centrifuged at 11 000 g for 10 min at 4°C.

Measurement of lipid peroxidation

Tissue lipid peroxidation was done by the method of Ohkawa *et al*.[9] The color formed by the reaction of thiobarbituric acid with breakdown products of lipid peroxidation was measured spectrophotometrically at 532 nm. Standard curves were constructed with 1,1,3,3-tetraethoxypropane as a standard (Sigma, St. Louis, USA). The results were expressed as nanomol/mg tissue protein in the homogenate supernatants of the brain.

Determination of reduced glutathione

For determination of GSH concentrations, a precipitating solution was added to tissue homogenate to precipitate all proteins in the sample. After centrifugation at 11 000 g, 15 min and 4°C, glutathione content of clear supernatants were assayed by the Beutler method.[10] In this system, glutathione is oxidized by 5,5′dithiobis-2-nitrobenzoic acid (DNTB 10 mm; Merck, Darmstadt, Germany), and then 2-nitro-5-thiobenzoic acid is formed, which can be detected spectrophotometrically by a change of absorption at 412 nm. Standard curves were constructed using reduced glutathione (0.25–10 μM; Sigma, St. Louis, USA). The results were expressed as nanomol/mg tissue protein in the homogenate supernatants of the brain.

Determination of enzyme activities

SOD activity was measured by the inhibition of nitroblue tetrazolium reduction due to the superoxide anion generated by the combination xanthine + xanthine oxidase.[11] One unit of SOD activity was defined as the quantity of enzyme capable of decreasing the reduction of nitroblue tetrazolium by 50%. The results were expressed as unit/mg protein in the homogenate of supernatants of the brain.

CAT activity was measured according to the method of Aebi and Luck by spectrophotometrically following up the decrease in the H_2O_2 concentration at the 240 nm.[12,13] The results were expressed in unit/mg protein in the homogenate of the supernatants of the brain. The protein content of the tissues was measured as described by Lowry *et al*.[14]

Statistical analysis

All statistical analyses were performed using SPSS for Windows, Version 8.0 (Referans 105 192) at the Trakya University Faculty of Medicine Data Processing Center. All values were expressed as mean ± standard deviation (SD). The groups comparisons were done by Kruskal–Wallis ANOVA because data did not show normal distribution. Mann–Whitney U-test was used to determine the origin of the difference in the comparisons if a significant difference was observed by Kruskal–Wallis. $P < 0.05$ was considered significant.

Results

Mean MDA level in Group 1 (the control group) was 0.39 ± 0.2 nmol/mg protein (range, 0.18–0.74 nmol/mg protein). In Group 2 (the hypoxic group) mean MDA was 0.87 ± 0.32 nmol/mg protein (0.41–1.31). In both pretreated melatonin groups (Group 3 and 4), mean MDA levels were almost equal (0.37 ± 0.07 nmol/mg protein [0.25–0.48], 0.34 ± 0.06 nmol/mg protein [0.25–0.43], respectively). The brain tissue mean MDA level was significantly higher in Group 2 compared with Groups 1, 3 and 4 (P = 0.001) as shown in Fig. 1.

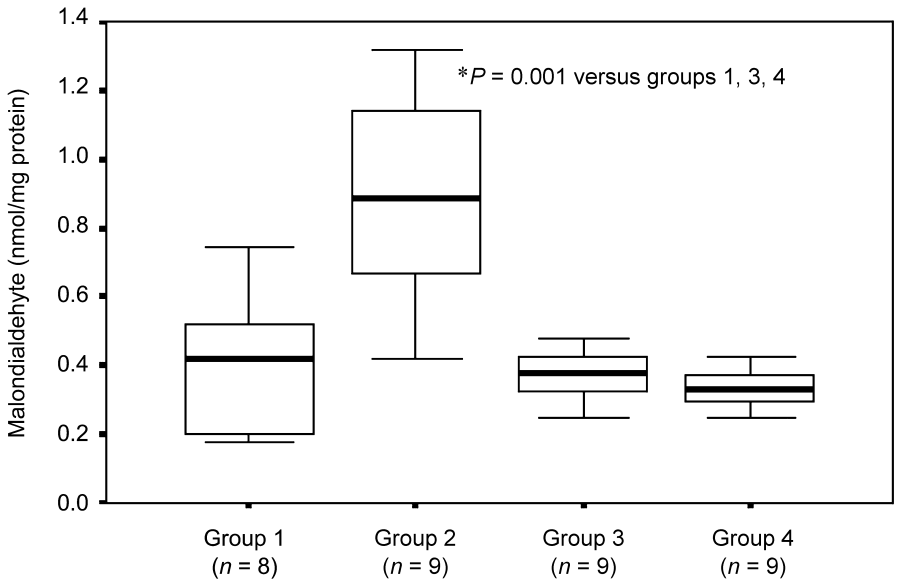

Fig. 1 The box plot graphic illustrates the effect of melatonin on the malonaldehyte (MDA) levels of brain tissue induced by hypoxia. The MDA level is expressed as nmol/mg protein. The tissue MDA level and mean ± standard deviation in the control (Group 1), hypoxic (Group 2), melatonin pretreated 10 mg/kg of bodyweight (Group 3), and melatonin pretreated 20 mg/kg of bodyweight (Group 4) groups. Other experimental details are given in the text. **P* = 0.001 versus Groups 1, 3 and 4.

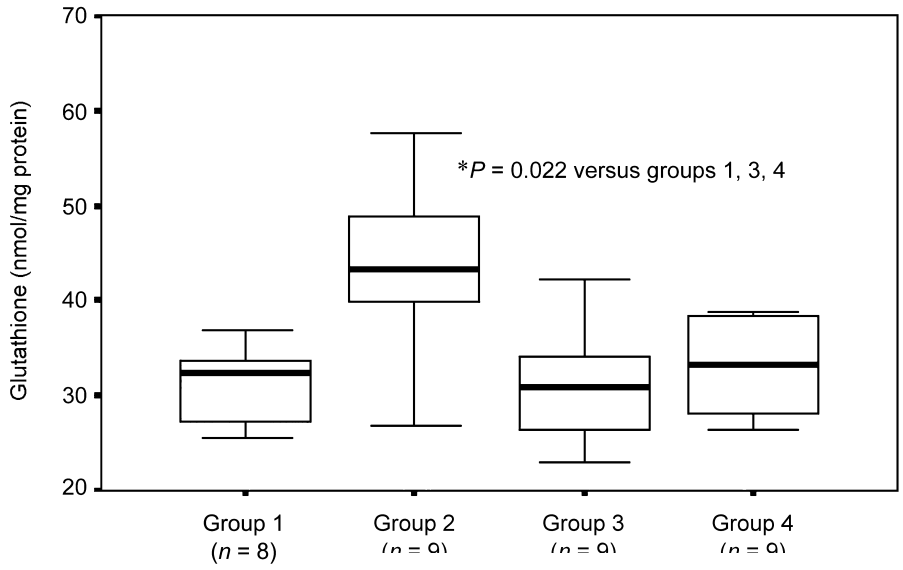

Fig. 2 The box plot graphic illustrates the effect of melatonin on the glutathione (GSH) level of brain tissue induced by hypoxia. The GSH level is expressed as nmol/mg protein. The tissue GSH level and mean ± standard deviation in the control (Group 1), hypoxic (Group 2), melatonin pretreated 10 mg/kg of bodyweight (Group 3), and melatonin pretreated 20 mg/kg of bodyweight (Group 4) groups. Other experimental details are given in the text. **P* = 0.022 versus Groups 1, 3 and 4.

The brain tissue mean GSH level in Group 1 was 31 ± 3.98 nmol/mg protein (25.66–36.95). In Group 2, the mean GSH level was 43. 08 ± 10.56 nmol/mg protein (26.8–57.6). In Group 3, the mean GSH level was 31.17 ± 6.08 nmol/mg protein (22.95–42.29) and in Group 4, the mean GSH level was 33.46 ± 5.23 nmol/mg protein (26.4–38.75). The mean GSH level was significantly higher in Group 2 compared with Groups 1, 3 and 4 ($P = 0.022$) as shown in Fig. 2.

The brain tissue mean SOD level in Group 1 was 0.82 ± 0.31 U/mg protein (0.47–1.49). In Group 2, the mean SOD level was 0.48 ± 0.53 U/mg protein (0.02–1.46). In Groups 3 and 4, the mean SOD levels were 0.32 ± 0.16 U/mg protein (0.18–0.67) and 0.23 ± 0.14 U/mg protein (0.07–0.45), respectively. The mean SOD level in Group 1 was significantly different than in Groups 2, 3 and 4 ($P = 0.008$). The SOD level significantly decreased in Group 2, 3 and 4. The mean SOD level, either in the hypoxic group (Group 2) or both melatonin pretreated groups (Groups 3 and 4), was significantly decreased as shown in Fig. 3. However, in both pretreated with melatonin groups, mean SOD levels were not found to be significantly different than the hypoxic group (Group 2).

The brain tissue mean CAT level in Group 1 was 5.4 ± 0.25 U/mg protein (5.1–5.8). In Group 2, the mean CAT level was 3.36 ± 0.45 U/mg protein (2.8–4.2). The mean CAT level in Groups 3 and 4 were 3.78 ± 0.25 U/mg

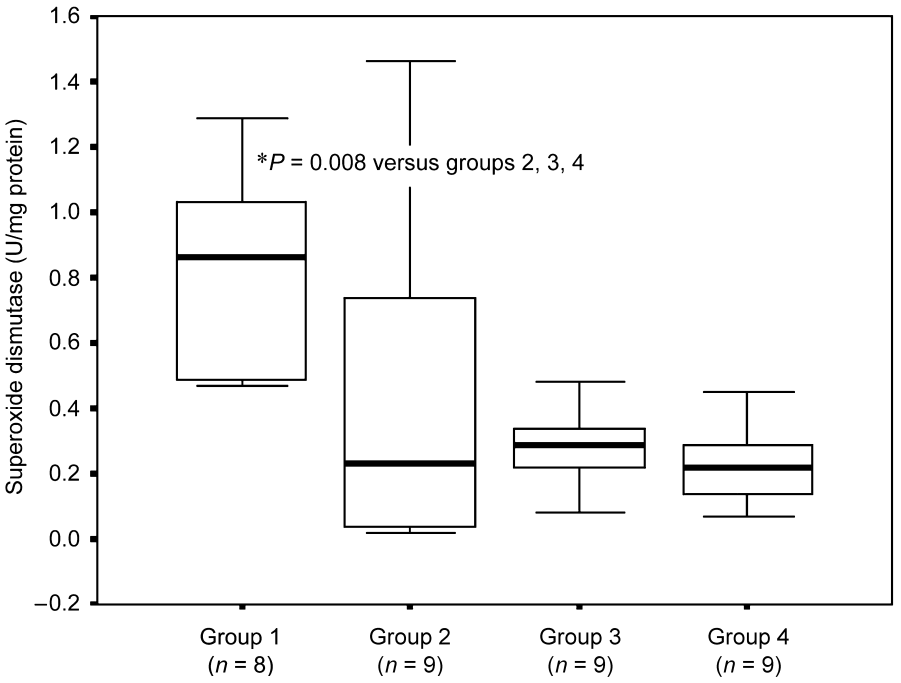

Fig. 3 The box plot graphic illutrates the effect of melatonin on the superoxide dismutase (SOD) activity of brain tissue induced by hypoxia. SOD activity is expressed as U/mg protein. The tissue SOD activity and mean ± standard deviation in the control (Group 1), hypoxic (Group 2), melatonin pretreated 10 mg/kg of bodyweight (Group 3), and melatonin pretreated 20 mg/kg of bodyweight (Group 4) groups. Other experimental details are given in the text. $*P = 0.008$ versus Groups 2, 3 and 4.

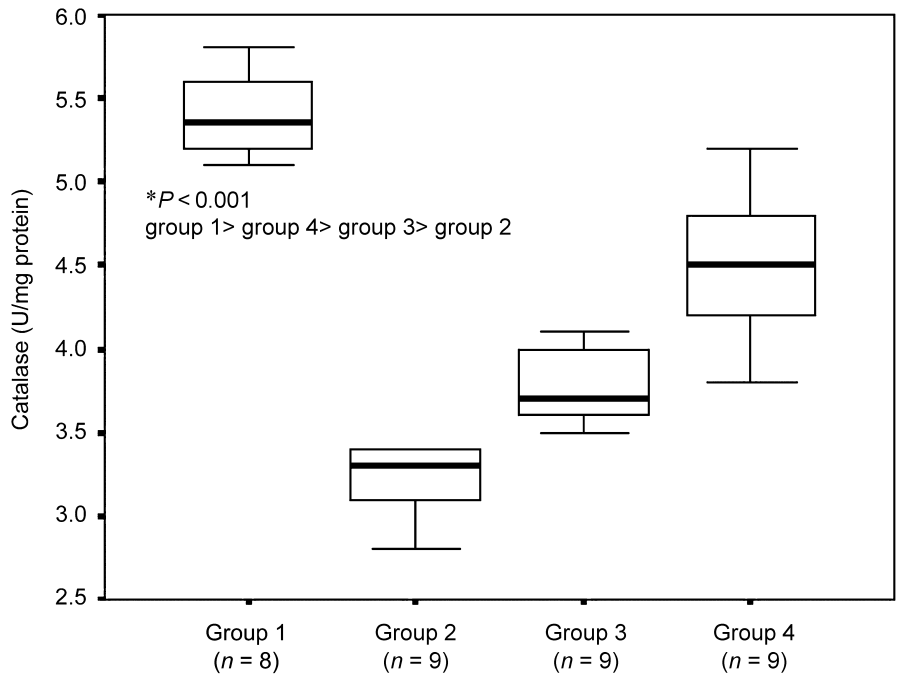

Fig. 4 The box plot graphic illustrates the effect of melatonin on the catalase (CAT) activity of brain tissue induced by hypoxia. CAT activity is expressed as U/mg protein. The tissue CAT activity and mean ± standard deviation in the control (Group 1), hypoxic (Group 2), melatonin pretreated 10 mg/kg of bodyweight (Group 3), and melatonin pretreated 20 mg/kg of bodyweight (Group 4) groups. Other experimental details are given in the text. $*P < 0.001$ versus Groups 2, 3 and 4; $**P < 0.001$ versus Group 2; $***P < 0.001$ versus Group 3.

protein (3.5–4.1) and 4.5 ± 0.51 U/mg protein (3.8–5.2), respectively. The mean CAT level was found to be significantly different in all groups when compared to each other. The mean CAT level in Group 1 was significantly higher than in Group 3, which was higher than Group 2 ($P < 0.001$) as shown in Fig. 4.

Discussion

Several pathways converge in the pathogenesis of perinatal brain damage. Lipid peroxidation caused by free radicals is one of the most important pathways. Many oxygenated compounds, particularly aldehydes such as MDA, are produced

during attacks of free radicals to membrane lipoproteins and polyunsaturated fatty acids. An MDA test is probably the single most widely used assay for the measurement of lipid peroxidation.[15] Our present study demonstrated that when hypoxia caused brain injury in pups, the lipid peroxidation levels in the brain were significantly increased as compared to that of the control group. This observation is in agreement with previous studies.[1,2] Melatonin is a potent endogenous free radical scavenger.[16] There has been increased interest in melatonin since it was shown to be a potent scavenger of toxic free radicals.[17] In the last several years, hundreds of publications have confirmed that melatonin is a broad spectrum antioxidant.[16] In previous studies, which were performed to test the antioxidant capacity of melatonin, it was given at different doses. While in some studies melatonin was used at a dose of 10 mg/kg of BW,[18–20] in other studies, which used the ischemia-reperfusion injury model, it was given at 10 mg/kg of BW and 20 mg/kg of BW.[7,8] We used a similar application of melatonin dose in our study. In the present study, we found that the mean MDA level in the untreated hypoxic group showed a statistically significant increase when compared with the control group, whereas the MDA level in both the melatonin pretreated groups did not increase (Fig. 1). Observing no increase in MDA levels in the brain tissue of both melatonin pretreated groups implies that melatonin has eliminated H_2O_2 and OH. These findings are consistent with findings in the previous studies where melatonin has attenuated the increased levels of MDA in brain tissue.[21–24] Kazez *et al*.[7] and De La Lastra *et al*.[8] reported that the highest antioxidant effect of melatonin was seen at a 20 mg/kg dose regimen. However, we did not find any significant difference between the two dose regimens of melatonin.

Reduced GSH plays an important role in the protection process against endogenous and exogenous oxidative destruction of the cell. Oxide GSH is deranged by gluthation reductase.[25,26] Glutathione is probably more of an aqueous phase indicator than thiobarbituric acid – reactive substances,[27] and it can also directly reduce hydrogen peroxide using the enzyme glutathione peroxidase.

In our study, GSH levels in the brain of hypoxic pups were significantly increased as compared to that of other groups. This may be explained by the emergence of the defence systems against the rise of free radicals due to hypoxia. There was no significant difference in the GSH levels between the two different dose melatonin groups and the control group. This result suggests that either the organism does not need to raise the GSH levels because melatonin removes the free radicals by the direct or indirect pathways, namely, the level of free radicals is not enough to promote a defence mechanism. Some authors reported that melatonin is more potent in discharging hydroxyl radicals than glutathion and mannitol, the two well-known free radical scavengers.[3,4]

All the tissues in the organism contain some antioxidant enzymes to protect themselves from the hazardous effect of oxidative attack. SOD and CAT, two natural enzymes, catalyze subsequent reactions in the antioxidative defence mechanism.[28] Besides being a scavenger of hydroxyl and peroxyl radicals, melatonin has been demostrated to increase the mRNA levels of antioxidant enzymes such as SOD and gluthathione peroxidase.[29] Okatani *et al*.[19] showed that melatonin given to the mother increases antioxidant enzyme activities in the fetal brain and may thereby provide indirect protection against free radical injury. These findings contrast with those reported here, where we found activity of SOD did not change significantly in both pretreated with melatonin groups as expected. Based on our findings, we speculated that antioxidant activity of melatonin should be due to a direct scavenger effect rather than to increased antioxidant enzyme activity such as SOD. Wakatsuki *et al*.[18] also showed that the activity of SOD did not change significantly after melatonin administration.

CAT activity within the brain is considered to have minimal influence as an antioxidative process because of its low activity.[30] Recent preliminary studies suggest that melatonin may also stimulate the H_2O_2 metabolizing enzyme, CAT, in the brain.[31] In our study, marked decrease of these antioxidant enzymes (SOD and CAT) after hypoxia, means that these protein structures were degraded in combating with antioxidant attack developed during reoxygenation. We demonstrated that pretreatment with melatonin increases the CAT activity in hypoxic rat brain tissue. We also found that this effect of melatonin is dose dependent. Based on the these findings, we might propose that melatonin has been effected to increase the activity of CAT but not the activity of SOD as expected. We also speculated that antioxidant activity of melatonin should be due to its direct scavenger effect and in increasing other antioxidant enzyme activities except SOD. A few authors indicate that although free radical generation can be seen during the hypoxic-ischemic insult before the reoxygenation in a neonatal animal model[32] and lipid peroxidation injury was seen only after an ischemic insult,[32,33] we found a significant increase in pups brain tissue levels of the lipid peroxidation products after hypoxia. Therefore, we hypothesized that lipid peroxidation injury not only occurred during the hypoxic-ischemic injury but also it could be during hypoxia without ischemic injury.

In conclusion, the present study shows that administration of melatonin before hypoxia significantly decreases lipid peroxidation. This protective effect is not dose dependent. Second, melatonin increases only activity of CAT but not activity of SOD as expected. We conclude that the antioxidant effect of melatonin could be due to its direct free radical scavenger activity and activation of CAT. The choice of melatonin used alone or in combination with other antioxidants and conventional treatments may lead to the development of a new therapeutic approach for hypoxic brain injury.

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