

Melatonin Modulates Hippocampus NMDA Receptors, Blood and Brain Oxidative Stress Levels in Ovariectomized Rats

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Abstract We investigated the effects of melatonin administration on ovariectomy-induced oxidative toxicity and *N*-methyl-D-aspartate receptor (NMDAR) subunits in the blood of rats. Thirty-two rats were studied in three groups. The first and second groups were control and ovariectomized rats. Melatonin was daily administered to the ovariectomized rats in the third group for 30 days. Blood, brain cortical and hippocampal samples were taken from the three groups after 30 days. Brain cortical, erythrocyte and plasma lipid peroxidation (LP) levels were higher in the ovariectomized group than in controls, although the LP level was decreased in the ovariectomized group with melatonin treatment. Brain cortical and plasma concentrations of vitamins A, C and E as well as the NMDAR 2B subunit were lower in the ovariectomized group than in controls, although, except for plasma vitamin C, they were increased by the treatment. Brain cortical and erythrocyte reduced glutathione (GSH) levels were lower in the ovariectomized group than in controls, although erythrocyte GSH levels were higher in the melatonin group

than in the ovariectomized group. Brain cortical and erythrocyte glutathione peroxidase activity and NMDAR 2A subunit concentrations were not found to be different in all groups statistically. Oxidative stress has been proposed to explain the biological side effect of experimental menopause. Melatonin prevents experimental menopause-induced oxidative stress to strengthen antioxidant vitamin and NMDAR 2A subunit concentrations in ovariectomized rats.

Keywords Oxidative stress · Melatonin · Hippocampal NMDA receptor · Brain · Reproduction

Introduction

Erythrocytes and the brain may be vulnerable to oxidative stress induced by menopause and become exposed to reactive oxygen species (ROS) continuously generated via the autooxidation of hemoglobin and polyunsaturated fatty acids (PUFAs) (Nazıroğlu et al. 2004; Gordon et al. 2005). Because of their high rate of oxygen consumption, high content of PUFAs and poor enzymatic antioxidant defense, the brain and erythrocytes exhibit increased vulnerability to oxidative stress (Halliwell 2006; Nazıroğlu 2007a). Erythrocytes are also extremely susceptible to oxidative damage induced by these ROS because they contain hemoglobin and PUFAs, which can readily be peroxidized (Chung and Wood 1971). Lipid peroxidation (LP) causes injury to cells and intracellular membranes and may lead to cell destruction and subsequently cell death (Halliwell 2006; Kovacic and Somanathan 2008). Brain and erythrocytes are protected by antioxidants against peroxidative damage (Halliwell 2006; Nazıroğlu 2007b). Glutathione peroxidase (GSH-Px) catalyzes the reduction of hydrogen

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peroxide to water. GSH-Px can also remove organic hydroperoxides (Kovacic and Somanathan, 2008). Reduced glutathione (GSH) is a hydroxyl radical and singlet oxygen scavenger and participates in a wide range of cellular functions (Rayman 2009). Vitamin E (α -tocopherol) is the most important antioxidant in the lipid phase of cells. Vitamin E acts to protect cells against the effects of free radicals, which are potentially damaging by-products of the body's metabolism (Halliwell 2006; Nazıroğlu 2007b). Vitamin C (ascorbic acid), as well as being a free radical scavenger, also transforms vitamin E to its active form (Frei et al. 1989). The brain ascorbic acid concentration is extremely low according to body tissues such as liver and kidney (Frei et al. 1989). Vitamin A (retinol) serves as a prohormone for retinoids and is involved with signal transduction at cytoplasmic and membrane sites (Tafti and Ghyselinc 2007).

Melatonin, the main secretory neurohormone of the pineal gland, has been considered a potent antioxidant that detoxifies a variety of ROS in many pathophysiological states (Ekmekcioglu 2006; Mollaoglu et al. 2007). For example, melatonin plays an important role in influencing circadian rhythmicity and reproduction, especially in seasonally breeding animals (Reiter 1991). Melatonin has also been studied in relation to eye disease (Kükner et al. 2004), neurodegenerative diseases as an antioxidant (Tan et al. 2007) and in relation to the toxemia of pregnancy (Nakamura et al. 2001). Melatonin may also influence cholesterol metabolism in peri- and postmenopausal women (Tamura et al. 2008). It has also been shown that melatonin is superior to vitamin E as a peroxy radical scavenger (Pieri et al. 1994). We hypothesized that, if melatonin is a potent scavenger, it could prevent or ameliorate experimental menopause-induced brain oxidative injury, counteracting the impairment of the antioxidant endogenous system.

In women with normal reproductive function, the estrogenic compounds are secreted as estrogen in great quantity, mainly by the ovaries. Estrogen exerts diverse nonreproductive actions on multiple organs, including the brain (Rohr and Herold 2002). There are changes in hypothalamic *N*-methyl-D-aspartate receptor (NMDAR) subunit mRNA levels and coexpression of NMDARs in rats (Spencer et al. 2008). It was reported that estrogen deprivation is implicated in the pathogenesis of oxidative stress-induced neurodegenerative diseases such as Alzheimer disease and cerebral ischemia (Christen 2000). The Women's Health Initiative Study reported that hormone replacement does not improve, and may actually impair, cognitive function in postmenopausal women (Shumaker et al. 2003). Melatonin instead of hormone replacement therapy may improve oxidative stress-induced cognitive brain function in postmenopausal women.

It has not been studied whether melatonin in ovariectomized rats modifies alterations in the antioxidant enzyme system and LP of plasma, erythrocyte and cortex as well as of NMDAR subunits of the hippocampus. We evaluated the effects of melatonin on oxidative stress, enzymatic antioxidants and NMDAR subunits (namely, NR2A and NR2B) in an ovariectomized rat menopause model.

Materials and Methods

Animals

Thirty-two female Wistar albino rats weighing 200 ± 20 g were used for the experimental procedures. Rats were allowed 1 week to acclimatize to the surroundings before beginning any experimentation. Animals were housed in individual plastic cages with bedding. Standard rat food and tap water were available ad libitum for the duration of the experiments. The temperature was maintained at $22 \pm 2^\circ\text{C}$. A 12/12 h light/dark cycle was maintained, with lights on at 06.00, unless otherwise noted. The experimental protocol of the study was approved by the ethical committee of the Medical Faculty of Suleyman Demirel University (protocol 2009:6–24). Animals were maintained and used in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory animals prepared by Suleyman Demirel University.

Experimental Design

Melatonin was dissolved in ethyl alcohol (0.9% w/v). Thirty-two rats were randomly divided into three groups as follows:

Group I: Control group ($n = 10$). Placebo (physiological saline) was intraperitoneally supplemented.

Group II: Ovariectomized group ($n = 11$). Animals were ovariectomized, and placebo (physiological saline) was intraperitoneally supplemented.

Group III: Ovariectomized + melatonin group. Animals were ovariectomized, and melatonin (10 mg/kg/day) was intraperitoneally given for 30 consecutive days.

After 12 h of the last melatonin dose administration, all rats were killed and blood and brain samples were taken.

Anesthesia, Blood Collection and Preparation of Blood Samples

Rats were anesthetized with a cocktail of ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) administered i.p. before death and removal of blood samples. Blood (4–6 ml) was taken from the heart, using a sterile

injector and anticoagulant-coated tubes protected against light.

Blood samples were separated into plasma and erythrocytes by centrifugation at $1,500 \times g$ for 10 min at $+4^{\circ}\text{C}$. Erythrocyte samples were washed three times in cold isotonic saline (0.9%, v/w), then hemolyzed with a ninefold volume of phosphate buffer (50 mM, pH 7.4). After addition of butylhydroxytoluol (4 $\mu\text{l}/\text{ml}$), hemolyzed erythrocytes and plasma samples were stored at -33°C for <1 month pending measurement of vitamin assays. Hemolyzed erythrocytes and plasma samples were used immediately for LP and enzymatic activity.

Preparation of Brain Samples

The brain was also taken as follows. The cortex was dissected out after the brain was split in the mid-sagittal plane. Following removal of the cortex, the cortex and hippocampus were dissected from the total brain as described in our previous studies (Sutcu et al. 2006; Naziroğlu et al. 2008).

Cortical and hippocampal tissues were washed twice with cold saline solution, placed into glass bottles, labeled and stored in a deep freeze (-33°C) until processing (maximum 10 h). After weighing, half of the cortex was placed on ice, cut into small pieces using scissors and homogenized (2 min at 5,000 rpm) in five volumes (1:5, w/v) of ice-cold Tris-HCl buffer (50 mM, pH 7.4), using a glass Teflon homogenizer (Caliskan Cam Teknik, Ankara, Turkey). All preparation procedures were performed on ice. The homogenate was used for determination of LP and antioxidant levels.

After addition of butylhydroxytoluol (4 $\mu\text{l}/\text{ml}$), brain cortical homogenate was used for immediate LP levels and enzyme activities. Antioxidant vitamin analyses were performed within 3 months.

LP Determinations

LP levels in hemolyzed erythrocytes, plasma and brain homogenate were measured with the thiobarbituric acid reaction by the method of Placer et al. (1966). The quantification of thiobarbituric acid-reactive substances was determined by comparing the absorption to the standard curve of malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. LP values in the plasma, erythrocytes and cortex were expressed as nanomoles per milliliter, micromoles per milliliter hemolyzate and micromoles per gram tissue, respectively. Although the method is not specific for LP, measurement of thiobarbituric acid reaction is an easy and reliable method (Naziroğlu et al. 2008), which is used as an indicator of LP and ROS activity in biological samples.

GSH, GSH-Px and Protein Assay

The GSH content of the plasma, erythrocytes and cortex was measured at 412 nm using the method of Sedlak and Lindsay (1968). GSH-Px activities of RBCs were measured spectrophotometrically at 37°C and 412 nm according to the Lawrence and Burk (1976) method.

Plasma Vitamin A, Vitamin C and Vitamin E analyses

Vitamins A (retinol) and E (α -tocopherol) were determined in the plasma and cortical samples by a modification of the method described by Desai (1984) and Suzuki and Katoh (1990). About 250 μl of plasma and 250 μg of brain samples were saponified by addition of 0.3 ml of 60% (w/v in water) KOH and 2 ml of 1% (w/v in ethanol) ascorbic acid, followed by heating at 70°C for 30 min. After cooling the samples on ice, 2 ml of water and 1 ml of *n*-hexane were added and mixed with the samples, which were then rested for 10 min to allow phase separation. An aliquot of 0.5 ml of *n*-hexane extract was taken, and vitamin A levels were measured at 325 nm. Then, reactants were added and the absorbance value of the hexane extract was measured in a spectrophotometer at 535 nm. Calibration was performed using standard solutions of all-*trans* retinol and α -tocopherol in hexane.

Vitamin C (ascorbic acid) in the plasma and cortical samples was quantified according to the method of Jagota and Dani (1982). The absorbance of the samples was measured spectrophotometrically at 760 nm.

Hippocampal NR2A and NR2B Analyses by Western Blot

Hippocampal samples were homogenated in ice-cold buffer (50 mM Tris-HCl [pH 7.5]), and an aliquot was taken for protein determination. Equal amounts of protein for each sample (20 μg protein/lane) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Images of immunoblots were analyzed with a computerized image analysis system (Uviphoto MW V.99; Ultra-Violet Products, Cambridge, UK) (Sutcu et al. 2006).

Statistical Analysis

All results were expressed as means \pm SD. Significance in three groups was first checked by ANOVA using the Kruskal-Wallis test. Then, significant values in three groups were assessed with the unpaired Mann-Whitney *U*-test. Data were analyzed using the SPSS statistical program (version 9.05; SPSS, Inc., Chicago, IL). $P < 0.05$ was regarded as significant.

Results

The mean plasma LP values and antioxidant vitamin concentration in the three groups are shown in Table 1, and erythrocytes and cortical LP values are shown in Tables 2 and 3, respectively. The results showed that plasma ($P < 0.05$), erythrocyte ($P < 0.01$) and brain ($P < 0.05$) LP levels in the ovariectomized group were significantly higher than those in the control (intact) group. Melatonin administration caused a decrease in the LP levels of plasma, erythrocytes and cortex ($P < 0.05$) relative to the ovariectomized group.

The mean GSH levels and GSH-Px activities in erythrocytes and cortex of the three groups are shown in Tables 2 and 3, respectively. The results showed that the erythrocyte ($P < 0.05$) and brain ($P < 0.01$) GSH levels in the ovariectomized group were significantly lower than those in the control group. Administration of melatonin caused an increase in the erythrocyte and brain GSH levels of rats ($P < 0.05$). There was no statistically significant difference in erythrocyte and cortical GSH-Px activity among the groups.

The mean vitamin A, vitamin C and vitamin E concentrations in plasma and cortex of the three groups are shown in Tables 1 and 3, respectively. Vitamin C ($P < 0.01$) and vitamin E ($P < 0.05$) concentrations in plasma and cortex were significantly lower in the ovariectomized group than in the control group. Decreased plasma and cortical vitamin E and cortical vitamin C concentrations were improved by melatonin administration ($P < 0.01$), although decreased plasma vitamin C concentration was not. Cortical vitamin A levels were significantly ($P < 0.05$) higher in the melatonin treatment group than in controls. There were no significant differences in cortical vitamin A concentration between the control and ovariectomized groups. Plasma vitamin A concentrations were significantly ($P < 0.05$) lower in the ovariectomized group than in controls and were increased by melatonin administration ($P < 0.05$).

Western blot analysis of NR2A and NR2B is shown in Figs 1 and 2. The density of the protein band in the control group was accepted as 100%, and data from other groups were calculated as percentages of the control value. The NR2B percentage was significantly lower in the

Table 1 Effects of melatonin on plasma LP and antioxidant vitamin concentrations in ovariectomized rats (mean \pm SD)

| Parameters | Control ($n = 10$) | Ovariectomized ($n = 11$) | Ovariectomized + melatonin ($n = 11$) |
|--------------------------|----------------------|--------------------------------|---|
| LP (nmol/ml) | 1.41 \pm 0.24 | 1.68 \pm 0.27 ^a | 1.36 \pm 0.08 ^c |
| Vitamin A (μ mol/l) | 1.05 \pm 0.13 | 0.71 \pm 0.07 ^a | 0.85 \pm 0.16 ^c |
| Vitamin E (μ mol/l) | 9.45 \pm 1.26 | 4.28 \pm 0.99 ^b | 7.64 \pm 1.4 ^{d,e} |
| Vitamin C (μ mol/l) | 66.58 \pm 19.84 | 32.51 \pm 10.49 ^b | 39.74 \pm 8.02 ^f |

^a $P < 0.05$ and ^b $P < 0.01$ vs. control group, ^c $P < 0.05$ and ^d $P < 0.01$ vs. ovariectomized group, ^e $P < 0.05$ and ^f $P < 0.01$ vs. control group

Table 2 Effects of melatonin on erythrocyte hemolysate GSH-Px activity, GSH and LP levels in ovariectomized rats (mean \pm SD)

| Parameters | Control ($n = 10$) | Ovariectomized ($n = 11$) | Ovariectomized + melatonin ($n = 11$) |
|----------------|----------------------|------------------------------|---|
| LP (nmol/ml) | 4.54 \pm 0.92 | 6.26 \pm 0.82 ^b | 5.47 \pm 0.53 ^c |
| GSH (nmol/ml) | 3.62 \pm 0.39 | 3.14 \pm 0.48 ^a | 3.64 \pm 0.24 ^c |
| GSH-Px (IU/ml) | 2.83 \pm 0.26 | 2.79 \pm 0.20 | 2.81 \pm 0.22 |

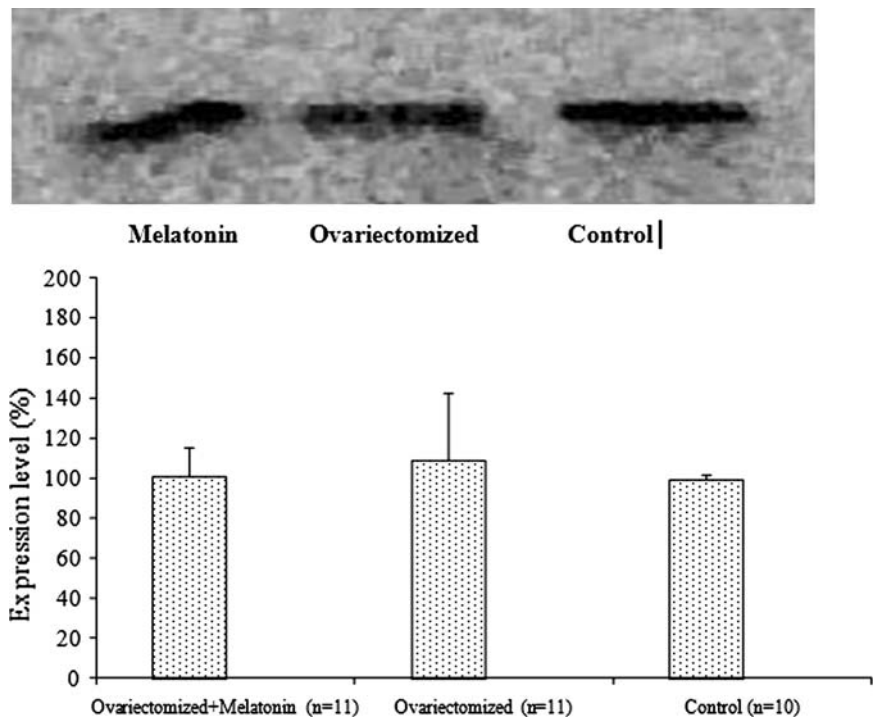
^a $P < 0.05$ and ^b $P < 0.01$ vs. control group, ^c $P < 0.05$ vs. ovariectomized group

Table 3 Effects of melatonin on cortical GSH-Px, GSH, LP and antioxidant vitamin concentrations in ovariectomized rats (mean \pm SD)

| Parameters | Control ($n = 10$) | Ovariectomized ($n = 11$) | Ovariectomized + melatonin ($n = 11$) |
|---------------------------------|----------------------|--------------------------------|---|
| LP (μ mol/g tissue) | 2.89 \pm 0.72 | 3.33 \pm 0.75 ^a | 2.86 \pm 0.34 ^c |
| GSH (μ mol/g tissue) | 1.87 \pm 0.34 | 1.34 \pm 0.20 ^b | 1.47 \pm 0.35 ^c |
| GSH-Px (IU/g) | 3.29 \pm 0.36 | 3.37 \pm 0.41 | 3.39 \pm 0.48 |
| Vitamin A (μ mol/g tissue) | 0.89 \pm 0.32 | 0.89 \pm 0.30 | 1.20 \pm 0.36 ^{e,e} |
| Vitamin C (μ mol/g tissue) | 98.22 \pm 9.66 | 83.61 \pm 12.20 ^a | 98.07 \pm 12.20 ^c |
| Vitamin E (μ mol/g tissue) | 5.31 \pm 0.76 | 4.36 \pm 0.49 ^a | 7.72 \pm 0.48 ^{d,f} |

^a $P < 0.05$ and ^b $P < 0.01$ vs. control group, ^c $P < 0.05$ and ^d $P < 0.01$ vs. ovariectomized group, ^e $P < 0.05$ and ^f $P < 0.01$ vs. control group

Fig. 1 Effects of melatonin on hippocampal NMDAR 2A subunit concentrations in ovariectomized rats (mean ± SD). The density of the protein band in the control group was accepted as 100%, and data from other groups were calculated as a percentage of the control value (mean ± SD). There was no statistical change among the groups ($P > 0.05$)



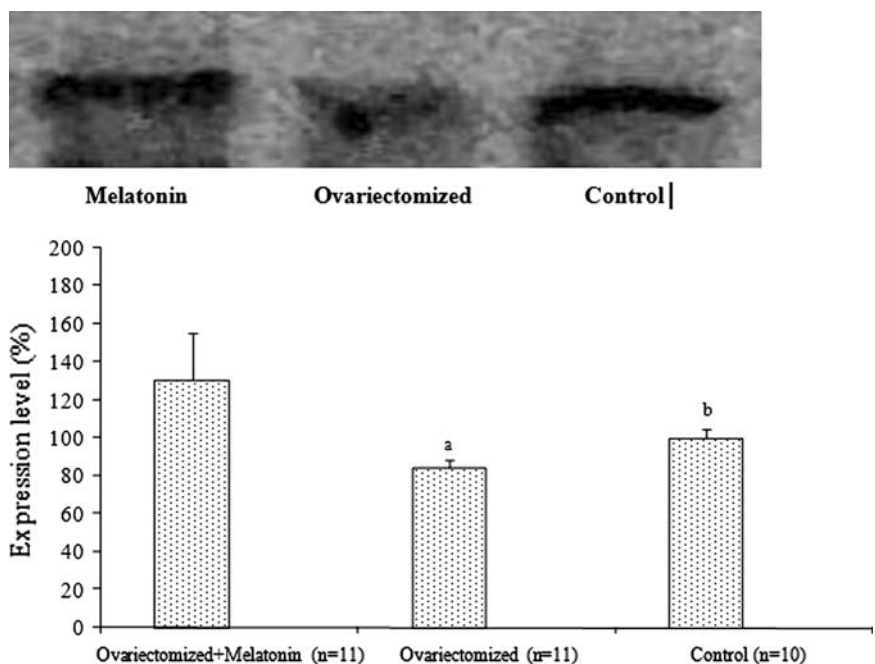
ovariectomized group than in controls, although it was increased by melatonin treatment. The NR2A percentage in the three groups did not change statistically.

Discussion

We found that cortical, erythrocyte and plasma LP values were increased by experimental menopause, whereas

cortical and plasma vitamin A, vitamin C, vitamin E and NMDAR 2B subunit concentrations decreased. Hence, the experimental animal menopause model is characterized by increased LP and decreased vitamin A, vitamin C, vitamin E and hippocampal NMDAR 2B subunit concentrations. Administration of melatonin caused an increase cortical and plasma vitamin A and vitamin E, cortical vitamin C and hippocampal NMDAR 2B subunit concentrations.

Fig. 2 Effects of melatonin on hippocampal NMDAR 2B subunit concentrations in ovariectomized rats (mean ± SD). The density of the protein band in the control group was accepted as 100%, and data from other groups were calculated as a percentage of the control value (mean ± SD). ^a $P < 0.05$ vs. control and ^b $P < 0.05$ vs. ovariectomized group



Steroid hormones, especially estriol and estradiol, are natural antioxidants (Mooradian 1993). Kume-Kick et al. (1996) reported that all female brain areas increased ascorbate loss after gonadectomy, indicating enhanced oxidative stress. Incubation of primary neuronal cultures with 17β -estradiol showed an increased survival of cells, reducing LP (Vedder et al. 1999). These reports provided evidence for the hypothesis that protection against oxidative damage is afforded by ovarian sex hormones. The current study indicated that ovariectomy in rats produced a significant increase in LP levels of plasma, erythrocyte and cortical preparations. Our results are in accordance with previous reports of LP increment in brain, erythrocytes and plasma during ovariectomy of animals or postmenopausal women (Kume-Kick et al. 1996; Nazıroğlu et al. 2004).

Moreover, the present study was also designed to explore the protective effects of a well-known free radical scavenger, melatonin, on ovariectomy-induced oxidative brain injury. We showed that administration of exogenous melatonin restored the situation from an antioxidant point of view. In the current study, melatonin treatment prevented the high level of LP in erythrocytes, cortex and plasma. All data reported here help us to discuss a possible antioxidant role played by melatonin against ovariectomy-induced oxidative brain injury. Ovariectomy of rats and surgical postmenopause produced an excess of ROS production according to data (Nazıroğlu et al. 2004; Irwin et al. 2008) via the mitochondrial respiratory chain and impaired antioxidant defense system. It has been hypothesized that melatonin, a lipophilic compound (Reiter 1991), acts as a direct or indirect mechanism for ROS production; in fact, melatonin can directly scavenge free radicals or it can induce antioxidant enzymes via specific melatonin receptors (Tan et al. 2007). Moreover, it has been shown that melatonin has an antioxidant effect in other experimental models, such as in retinal edema during experimental uveitis, and plays a protective role against microwave-induced brain injury (Kükner et al. 2004; Köylü et al. 2006) through its effect on mitochondrial chain and antioxidant enzymes (Ekmekcioglu 2006; Mollaoglu et al. 2007; Tan et al. 2007).

Vitamin C has been shown to be an important antioxidant, to regenerate vitamin E through redox cycling and to raise intracellular GSH levels (Halliwell 2006; Nazıroğlu 2007a). Thus, vitamin C plays an important role in protein thiol group protection against oxidation. The GSH and vitamin E concentrations in cortex and plasma were higher in the melatonin treatment group than in the ovariectomized group. Taking into consideration the data given here, the increased concentrations of erythrocyte GSH and cortical and plasma vitamin C and vitamin E in the melatonin treatment group indicate an essential role of melatonin

administration in normalizing GSH and antioxidant vitamin concentration in ovariectomized rats.

In the current study, concentrations of vitamins A, C and E in the cortex and plasma of ovariectomized rats were lower than in controls and LP levels in cortex and plasma were higher in ovariectomized rats than in controls. Thus, cortical and plasma concentrations of vitamins A, C and E in ovariectomized rats may be decreased as a result of their action in inhibiting free radicals. Plasma vitamin C concentrations did not change in the current study. Because of its high rate of oxygen consumption and its high content of PUFAs, the brain exhibits increased vulnerability to oxidative stress (Halliwell 2006). Compared to tissue antioxidant levels, plasma antioxidant levels are a less sensitive antioxidant indicator (Frei et al. 1989).

A number of reports have implicated NMDAR-mediated neurotransmission in estrogen's ability to regulate hippocampal spine density. Ovariectomy causes a decrease in NMDAR binding and expression of the NR1 and NR2B receptor subunits in rat hippocampus (Cyr et al. 2001a). These effects on NMDAR binding and subunit expression correlate with increased NMDAR-mediated input that may modulate dendritic spine density (Cyr et al. 2001a). Similarly, we observed in the current study that NR2B concentrations were lower in the ovariectomized group than in controls. Hence, our results were supported by the results of recent studies (Cyr et al. 2001a, b).

In the brain, melatonin binding sites have been found in regions implicated in cognition and memory (Christen 2000; Cabrera et al. 2000). One of the brain structures that may be especially vulnerable to melatonin's action is the hippocampus. Because of its proximity to the ventricles, the hippocampus is bathed by fluctuating levels of melatonin, which appears to influence hippocampal physiology (Cyr et al. 2001a). Melatonin receptors were indeed found in the hippocampus of various animals. To our knowledge, there is no report on the effects of melatonin on NR2A and NR2B concentration in ovariectomized rats. We observed in the current study that NR2B concentrations in ovariectomized rats were increased by melatonin treatment. Similarly, Sutcu et al. (2006) reported that melatonin increased NR2B subunit concentration in rat hippocampus.

Melatonin is capable of rapidly crossing the blood-brain barrier, and it has also been reported that melatonin accumulates in high concentrations in hippocampal cells after entering the brain. Cabrera et al. (2000) and Maharaj et al. (2006) found that 30 min after a subcutaneous injection of 0.5 mg/kg of melatonin, the concentrations of melatonin in the cell nuclei in rat cerebellum and hippocampus were five times higher than those of control rats. This study also provides evidence that melatonin is most able to protect the hippocampal NR2B against LP by ovariectomy-induced oxidative toxicity.

In conclusion, our blood and brain results in the ovariectomized group are consistent with a generalized antioxidant abnormality in different tissues of ovariectomized animals. However, melatonin supplementation has a protective effect on NMDAR subunits via regulation of oxidative stress and antioxidant redox systems in erythrocytes, plasma and cortex. The results in blood and brain may be of help to physicians in the treatment of oxidative stress-induced toxicity with melatonin and of oxidative stress-dependent postmenopausal toxicity.

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