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Olfactory Bulbectomy Induced Oxidative and Cell Damage in Rat: Protective Effect of Melatonin

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Summary

In this study we analyzed the effects of melatonin (Mel, 1 mg/kg ip) on behavioral changes as well as cell and oxidative damage prompted by bilaterally olfactory bulbectomy. Olfactory bulbectomy caused an increase in lipid peroxidation products and caspase-3, whereas it prompted a decrease of reduced glutathione (GSH) content and antioxidative enzymes activities. Additionally, olfactory bulbectomy induced behavioral changes characterized by the enhancement of immobility time in the forced swim test and hyperactivity in the open field test. All these changes were normalized by treatment of Mel (14 days). Our data show that Mel has a beneficial neuropsychiatric action against oxidative stress, cell damage and behavior alterations.

Key words

Antioxidant • Depression • Melatonin • Olfactory bulbectomy • Oxidative stress

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Introduction

Oxidative stress has been implicated in the pathogenesis of various diseases and may be a common pathogenic mechanism underlying many psychiatric disorders, a theory which is strongly supported by studies carried out in animals (Gladkevich *et al*. 2007, Nunomura *et al*. 2007, Wang 2007, Song and Leonard 2005).

Depressive disorders are amongst the leading causes of disability and mortality worldwide and are associated with different neuropsychiatric illnesses such as Alzheimer's disease, Huntington's disease, Parkinson's disease, diabetes and vascular disease. The World Health Organization (WHO) has indicated that depressive disorders will soon be the second leading cause of disability worldwide (Akiskal 2005, Nowak *et al*. 2003), with a prevalence of between 9 % and 18 % (Schloss and Henm 2004). Major depression has been linked to oxidative stress (Ng *et al*. 2008) and changes in melatonin levels (Carvalho *et al*. 2006).

N-acetyl-5-methoxytryptamine (melatonin) is a neurohormone secreted and released by the pineal gland. This indoleamine is derived from serotonin, has a characteristic circadian rhythm with high concentrations during the night and low levels during the day, and might be critically involved in mood regulation (Zeng *et al*. 2008). Disturbances in its level and circadian profile have been associated with neurodegenerative disorders such as Alzheimer's disease (Furio *et al*. 2007), but with regard to mood disorder, especially major depression (Carvalho *et al*. 2006, Crasson *et al*. 2004, Szymanska *et al*. 2001).

In this study, we investigated the effect of melatonin treatment on oxidative and cell damage biomarkers present in the depression induced by olfactory bulbectomy and it characterizes by behavioral changes in Wistar rat. To fulfill our aim we quantified: i) oxidative

stress biomarkers (lipid peroxidation products; reduced glutathione, GSH; glutathione peroxidase, GSH-Px; superoxide dismutase, SOD); ii) cell damage (lactate dehydrogenase, LDH; caspase-3); and iii) behavioral changes (open field test, forced swim test).

Materials and Methods

Animals

Male Wistar rats (purchased from Charles River, Barcelona, Spain) weighing 220-250 g were used throughout all experiments. The rats were housed five per cage at a constant temperature (20-23 ºC), illumination (12-h light/12-h dark cycle, light on at 08:00 h) and were provided with food (Purine, Barcelona, Spain®) and water *ad libitum*. All animals welfare and procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC) and RD 223/1988, and were approved by the University of Cordoba's Bioethics Committee, Spain.

To carry out this study, 35 rats were used. These rats were divided into seven groups as follows: i) control; ii) vehicle (6 % ethanol); iii) melatonin-treated; iv) sham-operated; v) olfactory bulbectomy; vi) olfactory bulbectomy + vehicle, and vii) olfactory bulbectomy + melatonin.

Melatonin was supplied from Sigma (St. Louis, MO, USA). The pineal indole was freshly dissolved in saline containing 6 % ethanol (total volume of 1 ml/kg) and administered intraperitoneally (i.p.) in a daily dose of 1 mg/kg for 14 days beginning two weeks after surgery. The dose of melatonin used was selected on the basis of our previous reports demonstrating *in vivo* neuronal protection and reduction of oxidative stress (Túnez *et al*. 2004).

Surgical procedure and experimental design

Surgery took place one week after arrival of the animals in the laboratory. Bilateral olfactory bulbectomy was performed with rats anesthetized under ketamine (50 mg/kg/i.p.; ketolar®, Pfizer S.A., Madrid, Spain). The top the skull was shaved and swabbed with an antiseptic, after which a midline frontal incision was made in the scalp and the skin was retracted bilaterally. Burr holes (2 mm) were drilled into the skull at the points 7 mm anterior to bregma and 2 mm lateral to the bregma suture, after which the olfactory bulbs were separated from the frontal cortex, removed and skin was closed with surgical clips. Sham-operated animals underwent the same procedure except for excision and removal of the olfactory bulbs. Two weeks after surgery, melatonin was administered daily for 14 days. All animals were sacrificed at the end of behavioral procedure by decapitation and their brain were rapidly removed, frozen on dry ice, and stored frozen (–80 ºC) until being assayed.

Biochemical parameters

Lipid peroxidation products

Levels of brain lipid peroxidation products were quantified as malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HDA). They were determined using LPO-586 kit (Oxis International, Portland, OR, USA). Data were expressed as nmol/mg protein.

GSH levels

GSH in brain tissue was determined using reagents purchased from Oxis International (Portland, OR, USA), i.e. GSH-400 kits. Results were expressed in nmol/mg protein.

GSH-Px activity

GSH-Px (EC, 1.11.1.9) activity was evaluated by the method of Flohé and Gunzler (1984). Briefly, the tissues were homogenized in ice-cold buffer (0.1 M KH_2PO_4/K_2HPO_4, pH 7.0 plus 29.2 mg EDTA in 100 ml of distilled water and 10.0 mg digitonin in 100 ml of distilled water, final volume, 2000 ml). The homogenates were then centrifuged at 10,000 x g for 10 min at 4 ºC. The GSH-Px assay is based on the oxidation of NADPH to NAD^+, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm. The activity of GSH-Px was expressed as U/mg protein.

SOD activity

SOD (E.C.: 1.15.1.1) was assayed by the Sun *et al.* (1998). Briefly, brain tissue was homogenized in ice cold isotonic saline. The homogenates were then centrifuged at 10,000 x g for 10 min at 4 ºC. SOD assay is based on the ability for SOD to inhibit the reduction of nitroblue tetrazolium (NBT) reduction by superoxide generator. Data were expressed in U/mg protein.

LDH activity

LDH in the brain homogenate was assayed using kit purchased from BioVision Inc. (Mountain View, CA,

USA), i.e. LDH-Cytotoxicity assay kit. The assay is based on measurement of activity of lactate dehydrogenase (LDH) which is a stable enzyme normally found in the cytosol of all cells. The activity of LDH was expressed in U/mg protein.

Caspase-3 activity

The caspase-3 activity in the brain homogenates were measured using reagents purchased from BioVision Inc. (Mountain View, CA, USA), i.e. Caspase-3/CPP32 colorimetric assay kit. The activity is expressed as optical density arbitrary units per milligram of protein (O.D. arbitraty units/mg protein).

Protein estimation

The protein concentration was determined according to Bradford (1976) using kit purchased from Sigma Co. (St. Louis, MO, USA), i.e., Bradford reagents B6916 assay kit, using bovine serum albumin as a standard.

Behavioral tests

Open field test

The rats were subjected to an open field test on the 14th day of treatment chronic with melatonin. Each rat was placed individually into the center of the open field apparatus. The open field apparatus was a circle made of wood, 90-cm in diameter. The test was performed between 09:00 and 12:00 h. A 60 W light bulb was positioned 90-100 cm above the center, and provided the only source of illumination in the testing room. Each animal was placed in the center of the open field apparatus, and the ambulation scores (the number of squares crossed) were measured during a 3-min period (Nowak *et al*. 2003, Xu *et al*. 2005).

Forced swim test

Forced swim test was carried out according to the method described by Porsolt *et al*. (1978). The rats were placed, after the open field test, in Plexiglas cylinders (height: 40 cm, diameter: 18 cm) containing 25 cm water, maintained at 23-25 ºC. After 15 min in the water they were removed and allowed to dry 15 min in a heated container before being returned to their home cages. They were again placed in the cylinders 24 h later and the total duration of immobility was measured during a 5-min test. A rat was judged to be immobile when it remained floating passively in the water.

Statistical analysis

Statistical analysis of the data was accomplished by means of the SPSS® statistical software package (SPSS Iberica, Madrid, Spain). All results were expressed as mean ± SEM. To evaluate variations in data, a one-way analysis of variance (one-way ANOVA) was corrected with the Bonferroni test. The level of statistical significance was set at $P<0.05$.

Results

Effects triggered by olfactory bulbectomy

The bilaterally olfactory bulbectomy caused significant enhancements in forced swim and open field test when animals were compared with intact control ($P<0.001$ and $P<0.001$, respectively) (Fig. 1). Additionally, olfactory bulbectomy induced oxidative stress which was characterized by increases in lipid peroxidation products ($P<0.001$) and reductions in GSH content ($P<0.001$) and antioxidative enzymes activities (GSH-Px: $P<0.001$; and SOD: $P<0.001$) (Table 1). LDH and caspase-3 were used as an indicator of cell damage. Removal of olfactory bulbs in rats did not affect LDH activity (Fig. 2), whereas caspase-3 activity was significantly enhanced ($P<0.001$) (Fig. 2).

Neuroprotective effects of melatonin

The administration of melatonin to intact controls caused significant changes in behavior (forced swim test: 89.8±4.20 s of immobility time in the control group vs. 67.8±0.86 s of immobility time in the melatonin group, $P<0.05$; and open field test: 15.3±0.20 number of ambulation counts in the control group vs. 13.0±0.25 number of ambulation counts in the melatonin group, $P<0.01$) (Fig. 1), whereas it did not modify the biochemical parameters evaluated in the present work (Fig. 2, Table 1).

Behavioral changes provoked by olfactory bulbectomy were neutralized by melatonin administration (open field test: 159.5±11.01 ambulation counts in olfactory bulbectomy group vs. 73.7±4.61 ambulation counts in the olfactory bulbectomy + melatonin group, $P<0.001$; and forced swim test: 29.8±1.45 s of immobility time in olfactory bulbectomy group vs. 15.0±0.45 s of immobility time in olfactory bulbectomy + melatonin group, respectively; $P<0.001$) (Fig. 1).

Furthermore, the enhancement in biomarkers of oxidative stress (lipid peroxidation products, GSH, SOD and GSH-Px) triggered by olfactory bulbectomy was

Table 1. Changes in the levels of oxidative stress biomarkers.

	Lipid peroxidation products (nmol/mg protein)	GSH (nmol/mg protein)	GSH-Px (U/mg protein)	SOD (U/mg protein)
Control	8.30 ± 0.47	7.00 ± 0.10	22.18 ± 0.95	39.45 ± 0.49
Vehicle	8.54 ± 0.13	6.57 ± 0.12	23.60 ± 0.51	41.60 ± 0.81
Melatonin	8.74 ± 0.25	6.49 ± 0.14	23.80 ± 0.86	44.00 ± 1.26
Sham operated	8.83 ± 0.17	6.37 ± 0.10	21.97 ± 1.66	49.87 ± 0.65
Olfactory bulbectomy	24.57 ± 0.36[a]	4.21 ± 0.16[a]	12.53 ± 0.43[a]	23.12 ± 0.34[a]
Olfactory bulbectomy + vehicle	22.95 ± 0.42	4.16 ± 0.18	12.80 ± 0.37	21.40 ± 0.51
Olfactory bulbectomy + melatonin	8.29 ± 0.29[b]	6.90 ± 0.29[b]	21.40 ± 0.75[b]	38.00 ± 0.70[b]

The results are presented as mean ± SEM; n=5 animals per group; [a]P<0.001 vs. control group; [b]P<0.001 vs. olfactory bulbectomy group.

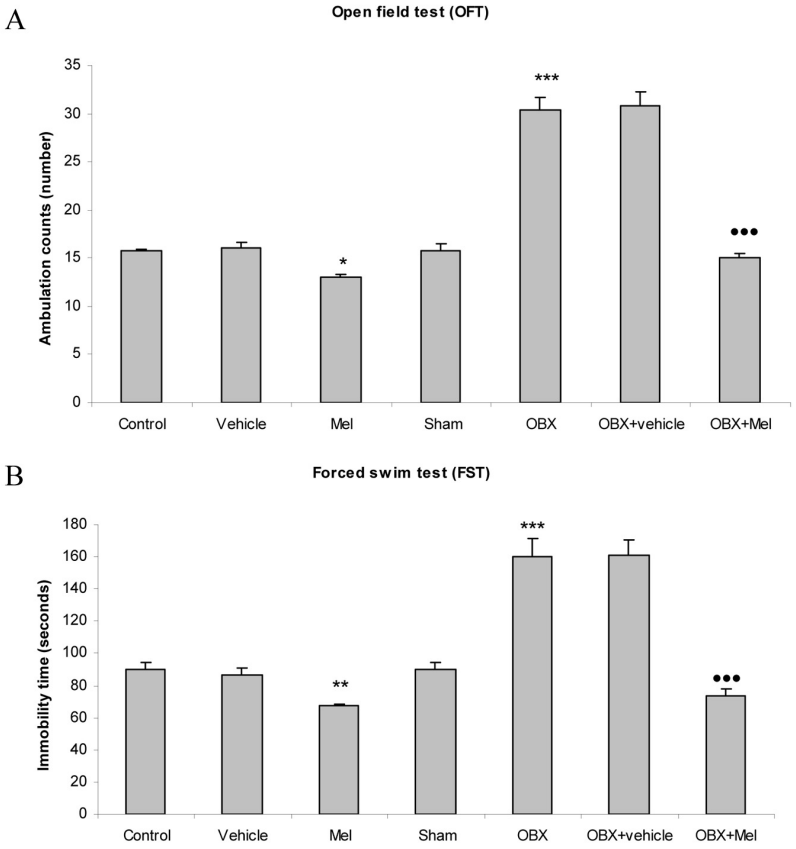

Fig. 1. The effects of chronic melatonin (Mel) administration on the ambulation in open field test (it was evaluated as the number of squares crossed) **(A)** and immobility time (determined as seconds) in forced swim test in the olfactory bulbectomy (OBX) model **(B)** of depression in rats. The ambulation scores was evaluated as the number of squares crossed during a 3-min period the open field sessions. The immobility time was recorded during a 5-min period the swim sessions. Each value represents mean ± SEM; n=5 animals per group. [*]P<0.05 versus control; [**] P<0.01 versus control; [***]P<0.001 versus control; [•••]P<0.001 versus OBX.

reversed toward normality by chronic administration of melatonin (P<0.001) (Table 1). Finally, the administration of melatonin caused a reduction in caspase-3 activation induced by olfactory bulbectomy (0.38±0.003 OD arbitrary units/mg protein in olfactory bulbectomy group vs 0.29±0.005 OD arbitrary units/mg protein in olfactory bulbectomy + melatonin group, P<0.001) (Fig. 2).

Discussion

The present study shows that melatonin reduces immobility time in the forced swim test and movement in the open field test, as well as cellular and oxidative damage. Our data suggest the antidepressive, antioxidant and neuroprotective effects of melatonin, indicating that this pineal indole could be a useful tool in the treatment

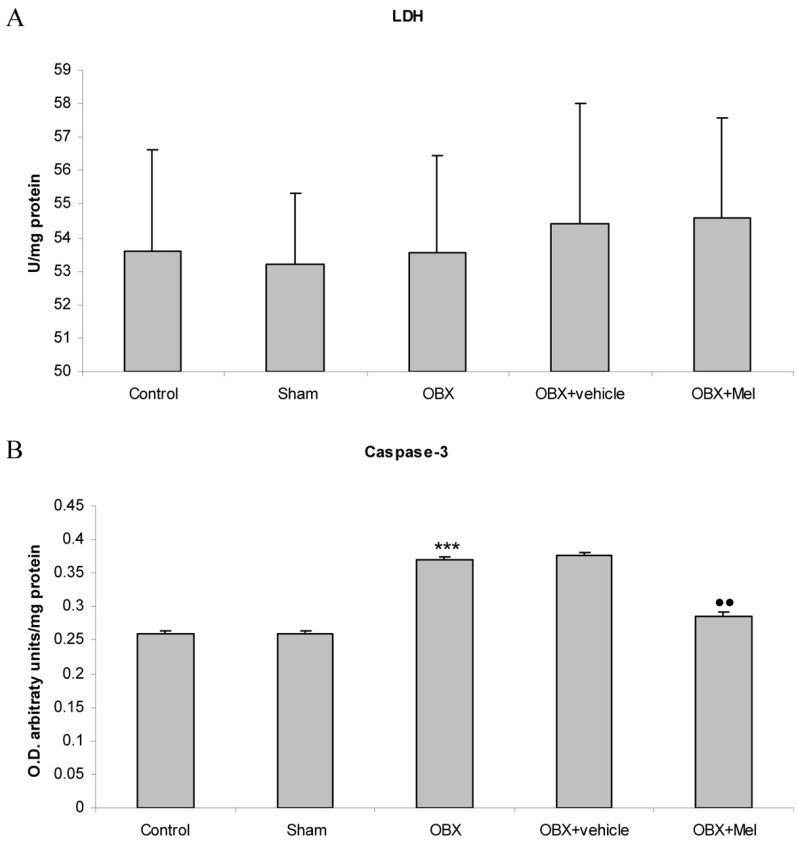

Fig. 2. Effects of olfactory bulbectomy (OBX) and melatonin (Mel) on LDH **(panel A)** and caspase-3 activity **(panel B)**. Values are mean ± SEM, n=5 animals per group. [a]P<0.001 versus control group; [b]P<0.001 versus OBX group.

and control of depression.

In addition, our results agree with those reported by Song and Leonard (1995, 2005) that olfactory bulbectomy generates immunological, neurochemical, hormonal and behavioral changes.

It is well known that oxygen free radicals are involved in the pathogenesis of numerous illnesses, and are currently linked to different neuropsychiatric disorders, such as depression, in both humans and experimental models (Atmaca *et al*. 2004, Bilici *et al*. 2001, Eren *et al*. 2007, Khanzode *et al*. 2003, McIntyre *et al*. 2007, Túnez *et al*. 2007, Zafir and Banu 2007). Recently, Sarandol *et al.* (2007) showed the presence of oxidative stress in patients diagnosed with severe depression using the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV), and demonstrated that they are characterized by increased plasma MDA levels and SOD activity. These data support the existence of oxidative stress in the course and evolution of depression, and suggest the presence of oxidative stress in olfactory bulbectomy rats. Nevertheless, our study shows a significant decrease in SOD and GSH-Px activity, whereas Sarandol *et al*. (2007) and Szuster-Ciesielska *et al*. (2008) found increased SOD activity. This difference could be either due to a different subject of the study (the rat vs. human beings) or due to the different cause of the depressive process (olfactory bulbectomy in our study, whereas in the others it was endogenous depression). Thus olfactory bulbectomy might be associated with a more intense oxidative stress, characterized by a higher production of oxygen reactive species, which could cause the saturation of antioxidant enzymatic systems and the decrease of their activity. These phenomena are reflected by changes in the studied oxidative stress biomarkers which occur simultaneously with increases in caspase-3 activity, the enzyme present in the route which leads to programmed cell death – apoptosis. These data are also found in our previous studies carried out in the depression model caused by olfactory bulbectomy (Tasset *et al*. 2008, Túnez *et al*. 2007), where we have seen that, along with the depressive and anxious state, there was also an intense oxidative stress, as the other authors have observed in major depression (Szuster-Ciesielska *et al*. 2008) and other character disorders (Forlenza and Miller 2006, McIntyre *et al*. 2007, Vawter *et al*. 2006). Furthermore, olfactory bulbectomy has been associated with an intense cell loss due to increased apoptosis through caspase-3 activation, along with a decrease in neurogenesis (Borders *et al*. 2007).

Our study also evaluated possible antidepressive effects of melatonin in two behavioral situations (forced

swim test and open field test). The chronic administration of melatonin caused a reduction of immobility time in the forced swim test and of the activity in the open field test in both intact control and those with OBX. Our findings agree not only with the results of Micale *et al*. (2006), who reported that Wistar rats treated with melatonin decrease their immobility time in the forced swim test, but also with the results obtained by Zeng *et al*. (2008) in another model of depression occurring in Wistar-Kyoto rats (WKY) (a substrain of Wistar rats which develops a spontaneously depressive state). These authors observed that the peripheral levels of melatonin and the expression of receptors in the anterior cingular cortex were lower in WKY than in normal Wistar rats. Additionally, this study put forward the idea that the administration of melatonin in the anterior cingular cortex prevents the behavioral changes shown by the open field test, forced swim test and other tests. These results are also indirectly endorsed by the studies which show that mice, whose MT1 melatonin receptors are blocked, develop symptoms similar to those of depression (Weil *et al*. 2006). Similarly, the treatment with agonists of melatonin receptors improves depressive symptomatology (Olié and Kasper 2007).

Melatonin is a powerful antioxidant with neuroprotective capacity in different models of neuropsychiatric disorders. Its antioxidant effect, which is responsible for at least a part of its beneficial effect, is achieved in different ways: i) by scavenging of oxygen-reactive species; ii) by stimulating the activity and expression of antioxidant enzymes; and iii) by inhibiting the activity of enzymes which produce reactive species like nitric oxide synthase (Reiter *et al*., 2007). It seems that the antioxidant effects can be exerted by the metabolites of melatonin, e.g. AMK, AFMK, etc, rather than by melatonin itself (Tan *et al*. 2007, Peyrot and Ducrocq 2008). These data support our present results that the chronic treatment with this indoleamine, besides reverting the animal's behavioral test back to normal, also causes a significant reduction in the oxidative stress associated with olfactory bulbectomy as can be documented by reduced lipid peroxidation, and increases in GSH and the activity of the studied antioxidant enzymes.

On the other hand, melatonin blocks the activation of caspase-3. Bearing in mind that this is the unifying point between the extrinsic and intrinsic routes which lead to cell death by apoptosis, it is possible to think that the decrease in activity of this protease is accompanied by a decrease in apoptosis and neuronal loss. This is further supported by several studies, including that by Das *et al*. (2008) who reported that melatonin prevents proteolysis and apoptosis in C6 cells of astroglia incubated with hydrogen peroxide. Kilic *et al*. (2008) demonstrated that melatonin improves cell survival and neurogenesis in animal models with cerebral ischemia, a phenomenon that is associated with an improvement in motor deficiencies, hyperactivity, coordination and behavioral changes. Finally, Jou *et al.* (2007) found that melatonin blocks cytochrome c release, caspase-3 activation, the condensation and karyorrhexis of the nucleus and apoptotic fragmentation of nuclear DNA.

Although it is not clearly established how melatonin acts in a similar way as the antidepressants, we believe that a large part of these therapeutic effects is due to its antioxidant effect, prevention of cell damage and cell death (facilitating not only cell survival, but also neurogenesis) and due to the reduction of hyperactivity symptoms indicative to the depression in animals.

To summarize, our results suggest that melatonin possesses an antidepressant effect whose molecular mechanism is in part due to its antioxidant and anti-apoptotic effects, at least in the model of animal depression induced by olfactory bulbectomy. However, more studies on this effect are required to establish and clarify the mechanisms underlying the beneficial effect of melatonin.

Conflict of Interest

There is no conflict of interest.

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