

Olfactory bulbectomy induced oxidative and cell damage in rat: Protective effect of melatonin.

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Running Title: Oxidative stress, depression and melatonin

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SUMMARY

In this study we analyzed the effects of melatonin (Mel, 1 mg/kg ip) on behavioral changes and 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 reduction in reduced glutathione (GSH) content and antioxidative enzymes activities. Additionally, olfactory bulbectomy induced behavior changes characterized by 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 neuropsychiatry action against oxidative stress, cell damage and behavior alteration.

Keywords: Antioxidant, depression; melatonin; olfactory bulbectomy; oxidative stress

1. 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 tests carried out on 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-metoxytryptamine (melatonin) is a neurohormone secreted and released by the pineal gland. This indoleamine is derived from serotonin, presenting a characteristic circadian rhythm with high concentrations during the night and low levels during the day, as well as potentially being 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, the results are reversed (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 behavioural changes in Wistar rat. To fulfill our aim we quantified: i) oxidative stress biomarkers (lipid peroxidation products; reduced glutathione, GSH;

glutathione peroxidase, GSH-Px; and superoxide dismutase, SOD); ii) cell damage (lactate dehydrogenase, LDH; and caspase-3); behavioral changes (open field test and forced swim test)

2. Materials and methods

2.1. Animals

Male Wistar rats (purchased from Charles River, Barcelona, Spain) weighing 220 – 250 g were used throughout all experiments. The rats were housed five to a cage at a constant temperature (20 – 23° C), 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) treated with melatonin; 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 dose of 1 mg/kg for 14 days beginning two weeks after surgery; the doses of melatonin used was selected on the bases of previous our reports demonstrating *in vivo* neuronal protection and reduction of oxidative stress (Túnez *et al.*, 2004).

2.2. Surgical procedure and experimental design

Surgery took place 1 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 severed from the frontal cortex, removed and skin was closed with surgical clips. Sham operated animals underwent the same procedure except for excision and removed of the olfactory bulbs. Two weeks after surgery, Mel were 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° C) until being assayed.

2.3. Biochemical parameters

2.3.1. Lipid peroxidation products

Brain lipid peroxidation products levels were quantified as malondialdehyde (MDA) + 4-hydroxyalkenals (4-HDA). They were determined using reagents purchase from Oxis International (LPO-586 kit; Oxis International, Portland, OR, USA). The levels are expressed as nmol/mg protein.

2.3.2. 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.

2.3.3. GSH-Px activity

GSH-Px (EC, 1.11.1.9) activity was evaluated by the Flohé and Gunzler method (1984). Briefly, the tissues were homogenated in ice-cold buffer (0.1M KH₂PO₄/K₂HPO₄, 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) to produce a homogenate. The homogenates were then centrifuged at 10,000xg for 10 min at 4° C. 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 is expressed as U/mg protein.

2.3.4. SOD activity

SOD (E.C.: 1.15.1.1) was assayed by the Sun et al. (1998). In sum, brain tissue was homogenized in ice cold isotonic saline. The homogenates were then centrifuged at 10,000xg for 10 min at 4° C. 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.

2.3.5. LDH activity

LDH in the brain homogenate were 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 is expressed in U/mg protein.

2.3.6. 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. arbitrary units/mg protein).

2.3.7. Protein estimation

The protein concentration was determined by the Bradford method (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.

2.4. Behavior tests

2.4.1. 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).

2.4.2. Forced swim test

Forced swim test was carried out according to the methods describe 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° C. 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 replaced 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.

2.5. Statistical analysis

Statistical analysis of the data was accomplished by means of the SPSS[®] statistical software package (SPSS Iberica, Madrid, Spain). 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 $P < 0.05$. All results are expressed as mean \pm SEM.

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 prompted an intensity oxidative stress 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 control prompted significant changes in the behavioral (forced swim test: 89.8 ± 4.20 seconds of immobility time in control group versus 67.8 ± 0.86 seconds 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 versus 13.0 ± 0.25 number of ambulation counts in the melatonin group, $P < 0.01$) (Fig. 1), whereas it did not caused modification in the biochemical parameters evaluated in the present work (Fig. 2; and 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 versus 73.7 ± 4.61 ambulation counts in the olfactory bulbectomy+melatonin group, $P < 0.001$; and forced swim test: 29.8 ± 1.45 seconds of immobility time in olfactory bulbectomy group versus 15.0 ± 0.45 immobility time in olfactory bulbectomy+melatonin group, respectively; $P < 0.001$) (Fig. 1).

Furthermore, the enhancement in biomarker of oxidative stress (lipid peroxidation products, GSH, SOD and GSH-Px) triggered by olfactory bulbectomy was 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 an olfactory bulbectomy+melatonin group, $P < 0.001$) (Fig. 2).

DISCUSSION

The present study shows that melatonin, when administered systematically in a model of depression for olfactory bulbectomy in rats, reduces immobility time in the forced swim test and movement in the open field test, as well as cellular and oxidative damage. However, the data suggests an depressive, antioxidant and neuroprotecting effect by melatonin, indicating that this pineal indole could be a useful tool in the treatment and control of depression.

Rats with bilateral olfactory bulbectomy show behaviour similar to that charted in cases of behavioral tests. These results agree with those referred to by Song and Leonard (1995; 2005), who appreciate that olfactory bulbectomy generates immunological, neurochemical, hormonal and behavioral changes.

It is well known that free radicals of oxygen are found in the pathogenesis of numerous illnesses, and are currently being linked to different neuropsychiatric disorders, such as depression, both in humans and in 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). A recent study by 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 showed that it is characterized by the increase in the plasmatic levels of MDA and SOD activity. This data supports the existence of oxidative stress in the course and evolution of depression, and indirectly proves the presence of oxidative stress in olfactory

bulbectomy rats. Our study shows a significant decrease in SOD and GSH-Px activity whereas, as we see above, Sarandol finds increases in SOD activity in a similar way to the Szuster-Ciesielska *et al.* (2008) group. This difference could be due to: i) the subject of the study, in our case the rat and in the case of Sarandol *et al.* (2007) and Szuster-Ciesielska *et al.* (2008), human beings; or ii) the cause of the depressive process which in our study is olfactory bulbectomy, whereas in the others it is endogenous. Certain situations could cause the olfactory bulbectomy to set off a more intense oxidative state, caused by a higher production of oxygen-reactive types, which could provoke the saturation of antioxidising enzymatic systems and the decrease in its activity. These phenomena are processed using changes in the oxidative stress biomarkers studied which occur simultaneously with increases in caspase-3 activity, the enzyme present in the tracts which lead to programmed cell death, or apoptosis. This data is found in previous studies carried out in the depression model caused by olfactory bulbectomy (Tasset *et al.*, 2008; Túnez *et al.*, 2007), where we see that, along with the depressive and anxious state, there is also an intense oxidative state, as 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 increase in apoptosis through caspase-3 activation, along with a decrease in neurogenesis (Borders *et al.*, 2007).

Our study also evaluates the possible antidepressive effects of melatonin in two behavioral models (forced swim and open field test), using them to evaluate the effect of behavioral changes. The chronic administration of melatonin caused a reduction in immobility time in the forced swim test and of activity in the open field test in both intact control and those with OBX. These findings agree with the studies carried out by

Micale *et al.* (2006), which show how Wistar rats treated with melatonin decrease their immobility time in the forced swim test, as in the results obtained by the Zeng *et al.* (2008) group in the model of depression provoked in Wistar Kyoto rats (WKY) (a variation of the Wistar rat which develops a spontaneous depressive state). These authors observe that the peripheral levels of melatonin, like the expression of receptors in the anterior cingulate cortex, were lower in the WKY than in normal Wistar rats. Additionally, this study put forward the idea that the administration of melatonin in the anterior cingulate 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), just like those which show that treatment with analogues of melatonin receptors improves depressive symptomatology (Olié and Kasper, 2007).

Melatonin is a powerful antioxidant with neuroprotective capacities in different models of neuropsychiatric disorders. Its antioxidant effect, upon which lies part of its beneficial effect, is developed in different ways: i) with a round-up of oxygen-reactive types; ii) by stimulating the activity and expression of antioxidising enzymes; iii) by inhibiting the activity of enzymes which produce reactive types like synthase nitric oxide (Reiter *et al.*, 2007); and iv) as a consequence of the metabolites of melatonin, eg, AMK, AFMK, etc, rather than to melatonin itself (Tan *et al.* , 2007; Peyrot and Ducrocq 2008). This data supports the results reported in the present study, where the chronic treatment with this indoleamine, as well as reverting the animal's test behavior back to normal, also causes a significant reduction in the oxidative stress associated with olfactory bulbectomy and characterized by reductions in the levels of 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, and 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. A situation which is supported by various studies, including the one carried out by Das *et al.* (2008) which argues that melatonin prevents proteolysis and apoptosis in C6 cells of astroglia incubated with hydrogen peroxide, just like in the study by Kilic *et al.* (2008), which showed that melatonin causes 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. Additionally, Jou et al found (2007) that melatonin blocks cytochrome c release, caspase-3 activation, the condensation and karyorrhexis of the nucleus and apoptotic fragmentation of nuclear DNA.

Although how melatonin acts in a similar way to antidepressants is not clearly established or defined, and basing ourselves on the data shown here, we believe that a large part of these therapeutic effects are due to its antioxidant effect, in the same way that it prevents cell damage and cell death, facilitating not only survival, but also neurogenesis, and with it the reduction of symptoms of hyperactivity indicative to depression in animals.

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

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FIGURES AND LEGENDS

Legends

Figure 1.-

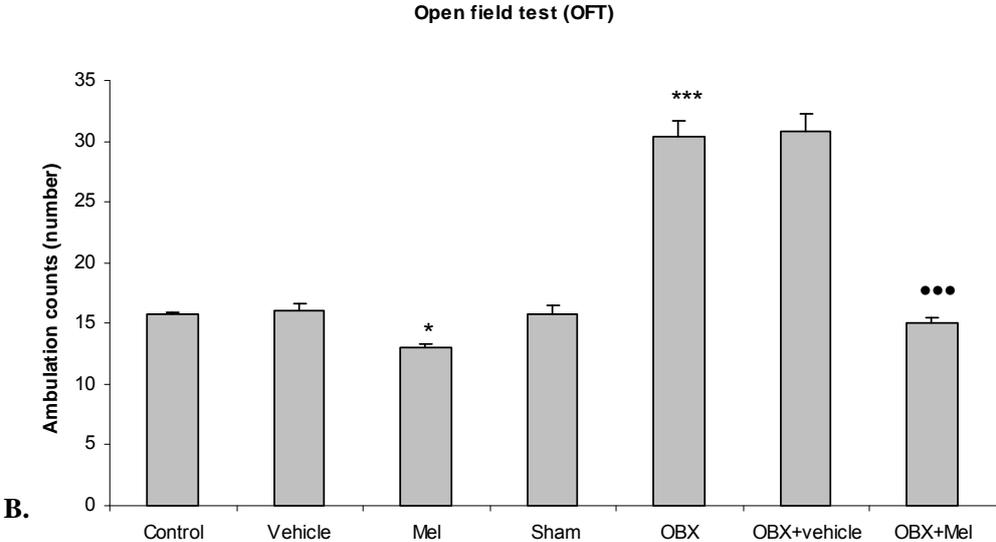
The effects of chronic melatonin (Mel) administration on the ambulation in open field test (it was evaluated as the number of squares crossed) (panel A) and immobility time (it determined as seconds) in forced swim test in the olfactory bulbectomy (OBX) model (panel B) of depression in rats. The ambulation 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 \pm 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.

Figure 2.-

Effects of OBX and melatonin (Mel) on LDH (panel A) and caspase-3 activity (panel B). Values are mean \pm SEM, n= 5 animals per group. ^a P < 0.001 versus control group; ^b P < 0.001 versus OBX group.

Figure 1.-

A.-



B.

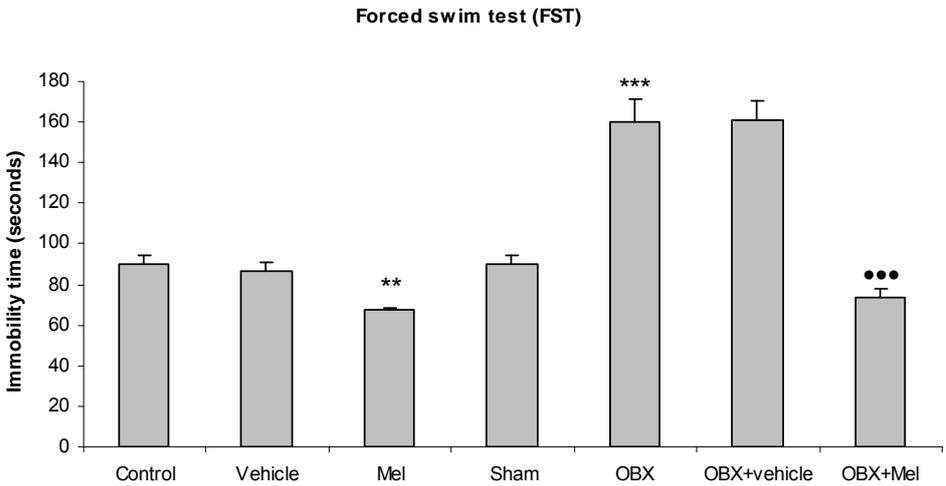
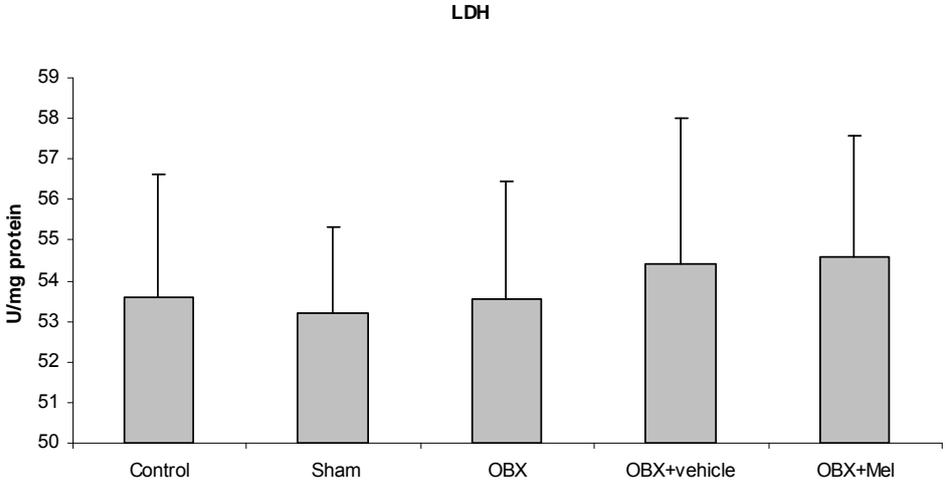
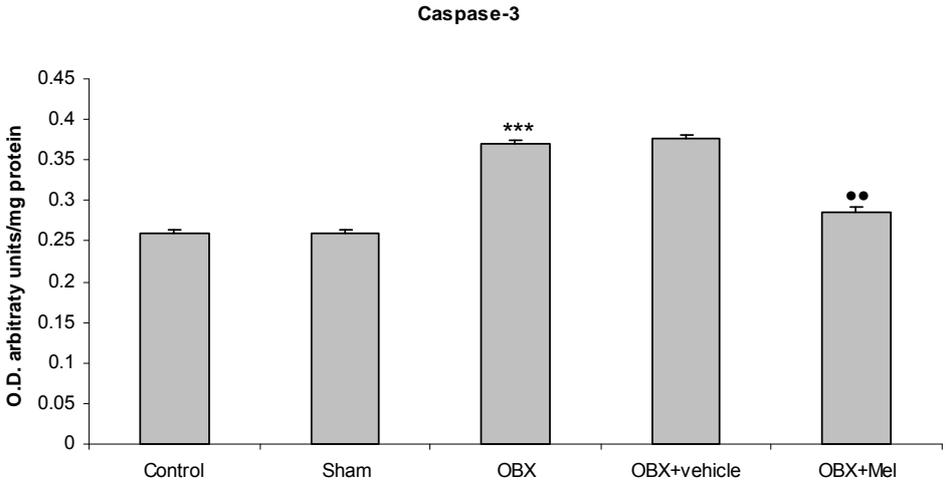


Figure 2.-

A.-



B.-



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

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

The results are presented as mean ± SEM; n=5 animals per group.

^a*P*< 0.001 versus control group.

^b*P*< 0.001 versus olfactory bulbectomy group