# Effect of 17β-estradiol on olfactory bulbectomy-induced oxidative stress and behavioral changes in rats

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**Abstract:** The present study evaluated 17β-estradiol ($17\beta E_2$) (2.5 mg/kg sc) effects on bilateral OBX-induced behavioral changes and oxidative stress. OBX in male Wistar rats produced an increase in lipid peroxidation products and a decline in reduced glutathione (GSH) content and glutathione peroxidase (GSH-Px) activity, together with an increase in caspase-3 activity. Additionally, OBX triggered changes of behavior such as an enhancement of immobility time in the forced swim test and hyperactivity in the open field test. These changes were reversed by treatment with $17\beta E_2$ (14 days). Our results reveled that $17\beta E_2$ has a protective effect against oxidative stress, cell damage and behavioral changes induced by OBX, and present antidepressant and antianxiety properties.

**Keywords:** behavioral, depression, 17β-estradiol, oxidative stress

## Introduction

Among models of depression have been described, the olfactory bulbectomy (OBX) rat is among well validated animal models that exhibit a number of behavioral, neuroendocrine and neurochemical changes with great relevance to clinical depression (Song and Leonard 2005). OBX results in degeneration of many areas such as the cortex, hippocampus, amygdale, locus coeruleus, and dorsal raphe nuclei, and produces enlargement of the lateral and third ventricules. The degenerative changes in the amygadala, hippocampus, and cortex induce in turn alterations in memory and behavior as well as cognitive deficits. Additionally OBX triggers a reduction in the number of synapses and the levels of brain noradrenaline and serotonin while the levels of glutamate are increased. OBX also triggers hyperactivity of the central cholinergic system and enhanced GABA turnover (Song and Leonard 2005). These changes may be reversed by chronic, but not acute, antidepressant treatments (Harkin et al 2003; van der Stelt et al 2005). It is therefore an attractive proposition to study pathophysiology of depression as well as to assay the effects of different antidepressive drugs.

The World Health Organization predicts that depression will be the second cause of loss in human disability-adjusted life years worldwide (Nowak et al 2003; Akiskal 2005). Affective disorders, especially major depression, are the most common psychological disorders affecting 10% of all patients seeking treatment at primary healthcare facilities worldwide (Murray and Lopez 1997). Major depression is a heterogeneous disorder. The classification is based on a cluster of symptoms described by the Diagnostic and Statistic Manual of Mental Disorders (DSM-IV) (APA 1994).

Women have higher rates of depression than men (Weismann and Klerman 1977) with this disorder affecting approximately twice as many women as men. Additionally, epidemiological and clinical studies have reported that the perimenopause in women is associated with an increased vulnerability to depression. Thus, estrogen replacement therapy

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has been proposed as a potentially effective treatment for mood disorders occurring in this period (Rasgon et al 2002).

Estrogens have been proved potent neuroprotective and antioxidant agents. The female sex hormone 17β-estradiol ($17\beta E_2$) has shown cytoprotective activities in animal models of neurodegeneration. A recent study of our group found that sex ovarian hormone has a neuroprotective effect against the changes induced by ovariectomy and ovariectomy plus 3-nitropropionic acid (Túnez et al 2006).

On the other hand, the studies of Fernández-Guasti and colleagues have shown that sex hormones estradiol and testosterone show antidepressant- and anxiolytic-like action in rats (Martínez-Mota et al 2000; Fernandez-Guasti and Martínez-Mota 2005; Estrada-Camarena et al 2006). In addition, a recent work by this group has found that estrogens participate in the antidepressant-like effect of desipramine and fluoxetine in the forced swimming test (Martinez-Mota et al 2008). Walf and Frye (2005, 2007a) have observed a similar effect of estrogen treatment. They have also recently shown that estradiol enhanced the spatial learning ability of rats (Frye et al 2007).

Based on this evidence, we designed the current study to test our working hypothesis that $17\beta E_2$ administration may result in an improvement in the oxidative stress biomarkers, caspase-3 activity, and OBX-induced behavioral changes.

# Materials and methods

## Chemical reagents and administered products

$17\beta E_2$ and all other chemicals were purchased from Sigma Aldrich Co., Ltd. (St. Louis, MO, USA).

## Animals

Male rats of the Wistar strain (purchased from Charles River, Barcelona, Spain) weighing 220–250 g were used throughout all experiments. For at least 1 week prior to 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, 25 rats were used. These rats were divided into five groups as follows: i) control; ii) treated with $17\beta E_2$; iii) sham operated; iv) olfactory bulbectomy (OBX); and v) OBX+$17\beta E_2$.

## Surgical procedure and drug treatment

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. The rats, housed five per cage, were given 2 weeks to recover from the surgical procedure and develop the OBX syndrome prior to drug administration. They were handled daily throughout the recovery period to eliminate any aggressiveness that would otherwise arise, because the degree of irritability depends on the degree of handling following surgery (Leonard and Tuite 1981). Drug treatment began two weeks after surgery. $17\beta E_2$ was administered chronically once daily for 14 days at a dose of 2.5 mg/kg body weight s.c.; the doses of estradiol used was selected on the bases of previous our reports demonstrating in vivo neuronal protection and reduction of oxidative stress (Muñoz-Castañeda et al 2005; Túnez et al 2006). 24 h after last treatment and under anesthetic with ether, the animals were sacrificed by decapitation and their brain were rapidly removed, frozen on dry ice, and stored frozen (–40 °C) until assayed.

## Biochemical parameters evaluated

### Lipid peroxidation (LPO) products

The LPO levels (malondialdehyde + 4-hydroxyalkenals; MDA+4-HDA) were evaluated in brain homogenates. They have been used an indicator of lipid peroxidation. Lipid peroxidation is a mechanism of cellular injury. The levels of lipid peroxidation products were determined using reagents purchase from Oxis International (LPO-586 kit; Oxis International, Portland, OR, USA). Brain tissue were homogenized in ice-cold buffer (20 mM Tris-HCl) and they was then centrifuged at 10,000 × g for 10 min at 4 °C. the supernatant was collected and immediately tested for LPO. The kit uses a chromatogenic reagent which reacts with the LPO (MDA+4-HDA). The content of LPO is expressed as LPO nanomol per milligram of protein (nmol/mg protein), and absorbance was evaluated in a specgtrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at wavelength 586 nm.

### Reduced glutathione (GSH) levels

GSH plays an important role as a co-enzyme in different enzymes. This tripeptide present a relevant function in antioxidant system. The levels of GSH were estimated using reagents purchased from Oxis International (Portland, OR, USA), ie, GSH-400 kits. The content of GSH is expressed as GSH nanomol per milligram of protein (nmol/mg protein), and absorbance was evaluated in a specgtrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at wavelength 420 nm.

### Glutathione peroxidase (GSH-Px) activity

GSH-Px (EC, 1.11.1.9) is an enzyme whose function is to detoxify peroxides in the cell. Its activity was evaluated by the Flohé and Gunzler method (1984). The tissues were homogened in ice-cold buffer (0.1M $KH_2PO_4/K_2HPO_4$, 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) to produce a homogenate. The homogenates were then centrifuged at 10,000 × g 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 abasorbance at 340 nm. The activity of GSH-Px is expressed as units per milligram of protein (U/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan).

### Total superoxide dismutase (SOD) activity

SOD (E.C.: 1.15.1.1) is an enzyme which catalyzes the dismutation superoxide ion into oxygen and hydrogen peroxide. Its activity was assayed by Sun and colleagues (1998). Brain tissue was homogenized in ice cold isotonic saline. The homogenates were then centrifuged at 10,000 × g for 10 min at 4 °C. SOD assay is based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) reduction by superoxide generator. The activity of GSH-Px is expressed as units per milligram of protein (U/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) with maximum at 560 nm.

### LDH activity

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. LDH in the striatal homogenate were assayd using kit purchased from BioVision Inc. (Mountain View, CA, USA), ie, LDH-Cytotoxicity assay kit. The activity of LDH is expressed as units per milligram of protein (U/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) with maximum absorbance at 500 nm.

### Caspase-3 activity

The caspase-3 activity in the brain homogenates were measured using reagents purchased from BioVision Inc. (Mountain View, CA, USA), ie, 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), and absorbance was evaluated in a spectrophotometer (UV-1603 shimadzu, Kyoto, Japan) at wavelength 405 nm.

### Protein estimation

The protein concentration was determined by the Bradford method (1976) using kit purchased from Sigma Aldrich Co., Ltd. (St. Louis, MO, USA), ie, Bradford reagents B6916 assay kit. The absorbance was evaluated in a spectrophotometer (V-1603 Shimadzu, Kyoto, Japan) at wavelength 595 nm, using bovine serum albumin as a standard.

## Open field test (OFT; anxiety)

All rats (control and OBX treated or not with $17\beta E_2$) were subjected to an open field test on the 14th day of chronic $17\beta E_2$ administration. 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 place 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 (FST; depressive)

This study was carried out in rats according to the methods describe by Porsolt and colleagues (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.

## Statistical analysis

Statistical analysis of the data was accomplished by means of the SPSS® statistical software package (SPSS Iberica, Madrid, Spain). The Shapiro-Wilk test did not show a

significant departure from normality in the distribution of variance values. 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 ± SD.

# Results

## Effects of OBX and 17βE$_2$ on behavior

The administration of estrogen to healthy animals did not prompt changes in the behavioral parameters evaluated in the present work. OBX produced a significant increase of immobility time and ambulation counts (immobility: 90.20 ± 4.32 seconds in the control group vs 160.40 ± 10.84 seconds in the OBX group, $p < 0.001$; ambulation:15.60 ± 0.25 counts in the control group vs 30.40 ± 1.33 counts in the OBX group $p < 0.001$) (Figure 1). Bulbectomised rats that were given chronic 17βE$_2$ administration for 14 days (OVX+17βE$_2$) exhibited a significant reduction of immobility time in the FST (73.80 ± 0.80 seconds, ie, 53.99 % reduction) and hyperactivity in OFT (23.00 ± 1.14 counts, ie, 24.34 % reduction), compared with the OBX-group (Figure 1).

## Effects of OBX and 17βE$_2$ on oxidative stress and cell damage biomarkers

OBX significantly increased lipid peroxidation products in brain tissue (Table 1). Additionally, OBX-rat showed a reduction in GSH content, GSH-Px and SOD activities (Table 1). OBX-induced changes on oxidative stress biomarkers were reversed toward normality by chronic administration of 17βE$_2$ whereas none of these parameters were affected in control animals. LDH and caspase-3 were used as an indicator of cell damage. Removal of olfactory bulbs in rats did not affect LDH activity (Figure 2), whereas caspase-3 activity was significantly enhanced (0.26 ± 0.004 OD arbitrary units/mg protein in control group vs 0.37 ± 0.004 OD arbitrary units/mg protein in OBX group, $p < 0.001$) (Figure 2). Administration of 17βE$_2$ reversed caspase-3 activation induced by OBX (0.37 ± 0.004 OD arbitrary units/mg protein in OBX group vs 0.25 ± 0.004 OD arbitrary units/mg protein in an OBX+17βE$_2$ group, $p < 0.001$) (Figure 2).

# Discussion

Several studies investigating the role that gonadal hormones play in relation to animal models of depression show contradictory results (Kennett et al 1986; Bernardi et al 1989, 1990). OBX triggers immunological, behavioral, neurochemical, and hormonal alterations that resemble the changes observed during major depressive disorder (Song and Leonard 1995, 2005). In the present study, we have demonstrated that OBX prompts changes in behavior, oxidative stress, and caspase-3 activation, similar to those observed during major depression. Importantly, we have shown that 17βE$_2$ administration is able to reverse these OBX-induced changes, acting as an antidepressant and antioxidant agent.

It is well known that free radicals and other reactive species are involved in various physiological and pathological conditions, including aging. They have also been implicated in the pathophysiology of neuropsychiatric disorders such as mood alterations, both in human and experimental models (Khanzode et al 2003; Eren et al 2007; McIntyre et al 2007; Zafir and Banu 2007). Additionally, numerous studies have shown that reactive oxygen species (ROS) play a relevant role in the pathophysiology of depression (Bilici et al 2001; Atmaca et al 2004).

Our results show that bilateral bulbectomy triggers an oxidative stress state characterized by decreased antioxidant capacity and increased levels of lipid peroxidation products. ROS generated by OBX significantly compromise the in vivo antioxidant defenses of the animals. These data are in accordance with a previous study of our group which established that OBX causes a high increase in lipid peroxidation products, with a concomitant decline in the antioxidant defenses (Túnez et al 2007a). Furthermore, our results indirectly agree with data of clinical studies which observed a correlation between mood disorders and oxidative stress (Forlenza and Miller 2006; Vawter et al 2006; McIntyre et al 2007).Moreover, we have found that chronic 17βE$_2$ administration to bulbectomised animals resulted in enhancement of the rat antioxidant status, protecting them against oxidative damage. This effect is in agreement with previous studies which show cytoprotective and antioxidative action of 17βE$_2$ in cell and animal models of neurodegenerative diseases (Mattson et al 1997; Feng et al 2005; Simpkins et al 2005; Túnez et al 2006; Tripanichkul et al 2007) and, indirectly, with reports which indicate that estrogen deficiency as a result of ovariectomy induced severe brain oxidative damage (Kume-Kick et al 1996; Túnez et al 2006, 2007b). Additionally, our results are partly supported by studies which have found that antidepressant drugs provide protection against oxidative stress (Eren et al 2007; Túnez et al 2007a; Zafir and Banu 2007). Thus, our data show that 17βE$_2$ treatment can reduce oxidative stress generated by OBX. This antioxidative response may account, at least partially, for the beneficial effect of 17βE$_2$ in this model.

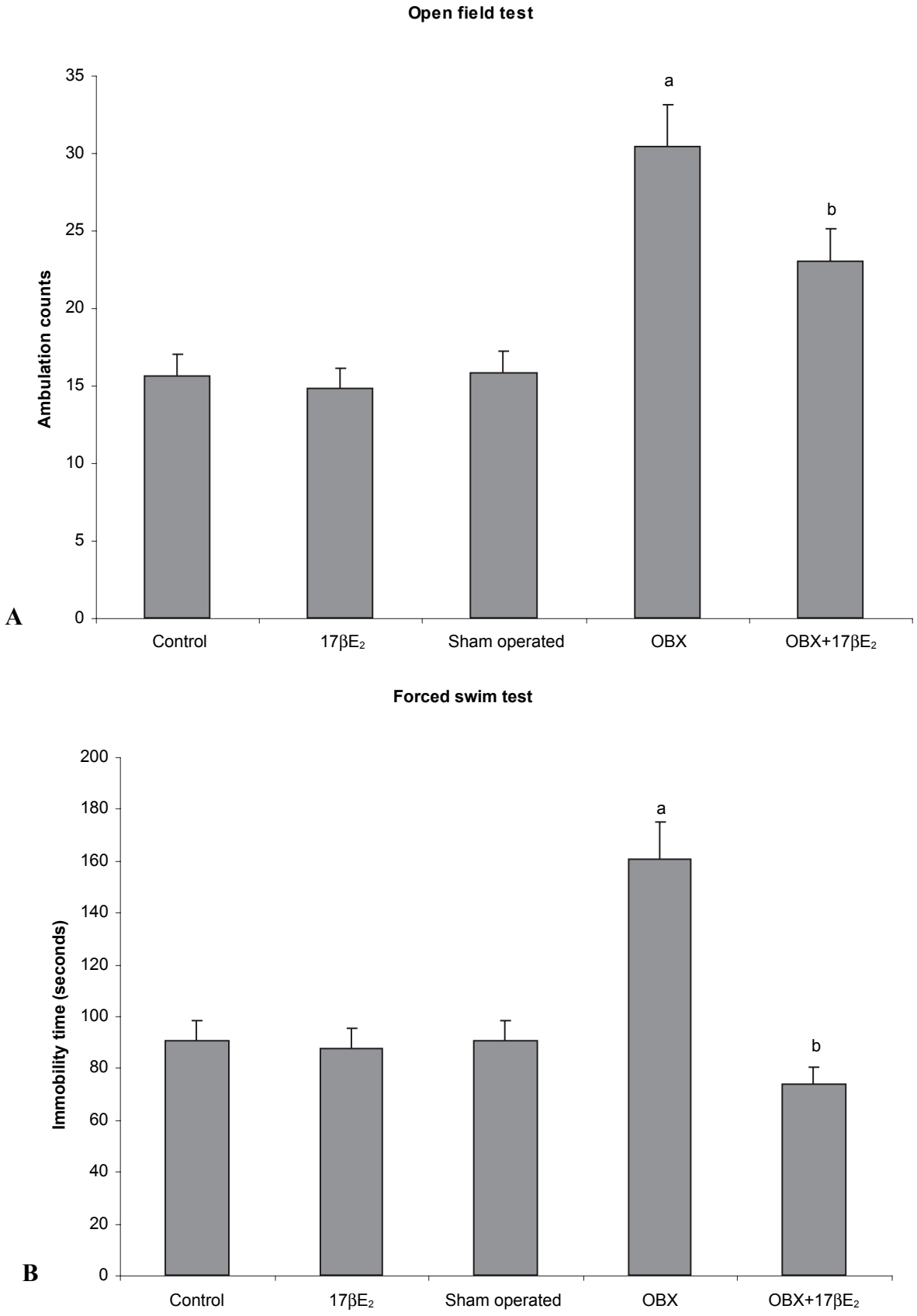


**Figure 1** The effects of chronic 17β$E_2$ administration on the ambulation in open field test (panel **A**) and immobility time in forced swim test in the olfactory bulbectomy (OBX) model (panel **B**) of depression in rats. The ambulation scores was evaluated as the number of squares crossed during 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 ± SD; n = 5 animals per group. $^{a}p < 0.001$ versus control; $^{b}p < 0.001$ versus OBX.

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

| | LPO nmol/mg protein | GSH nmol/mg protein | GSH-Px U/mg protein | SOD U/mg protein |
|---|---|---|---|---|
| **Control** | 8.12 ± 0.40 | 6.39 ± 0.09 | 23.20 ± 1.02 | 40.40 ± 0.51 |
| **17βE$_2$** | 8.47 ± 0.22 | 6.60 ± 0.12 | 22.60 ± 0.51 | 40.80 ± 0.97 |
| **Sham operated** | 8.80 ± 0.29 | 6.37 ± 0.10 | 22.80 ± 0.66 | 40.40 ± 0.60 |
| **OBX** | 22.75 ± 0.32[a] | 4.16 ± 0.12[a] | 12.80 ± 0.49[a] | 22.20 ± 0.37[a] |
| **OBX+17βE$_2$** | 8.29 ± 0.29[b] | 6.76 ± 0.24[b] | 24.75 ± 0.86[b] | 46.00 ± 1.87[b] |

**Notes:** The results are presented as mean ± SD; n = 5 animals per group.[a]$p < 0.001$ versus control group; [b]$p < 0.001$ versus OBX group.

It is possible that enhanced free radicals generation can overwhelm cell antioxidant defense systems and triggered a pathogenic cascade which leads to cell death. Interestingly, OBX did not enhance LDH levels, whereas it prompted changes in caspase-3 activity, data which indicate that OBX may be induced cell damage. These data are in concordance with other studies which suggest that olfactory bulbs removal prompts the cell damage (Carrr and Farbmen al 1992; Michel et al 1994; Túnez et al 2007a). Moreover, in the present study we found that 17βE$_2$ prevents cell damage induced by OBX. According to our data, other studies have shown that estrogen treatment may prevent apoptosis, a cellular pathway that involves to caspase-3 activity (Monroe et al 2002; Rau et al 2003; Túnez et al 2006; Gerstner et al 2007; Satoh et al 2007).

Additionally, our study evaluated the antidepressant effect of 17βE$_2$ on rats showing behavioral changes as a result of OBX. These changes included an increase of immobile time and ambulation counts in the FST and OFT tests, respectively. These results agree with a previous report from our group (Túnez et al 2007a) and with several other studies (Pieper and Newman 1999; Mucignat-Caretta et al 2006; Breuer et al 2007). 17βE$_2$ administration reversed the changes induced by OBX in male Wistar rats. Indirectly, these data are in agreement with reports which show that estrogens have a clinically significant antidepressant effect in perimenopausal women (Nagata et al 2005; Gryllstrom et al 2007; Soares et al 2007) and experimental studies showing that steroid hormones synergize the anxiolytic effect prompted by desipramine in rats, suggesting an interaction between hormones and antidepressants (Martínez-Mota et al 2000, 2005). They are also in agreement with data which reveal that gonadectomy intensify the effects triggered by OBX (Stock et al 2000, 2001).

In addition, we have observed that both behavioral disorders prompted by OBX and their restoration to normality after 17βE$_2$ administration occur concomitantly with changes in oxidative stress biomarkers. In this line, Haeser Ada and colleagues (2007) found that the drug clonazepam reduces the immobility time and improves the oxidative status in diabetic rats. Moreover, Khanzode and colleagues (2003) found evidence that indicates depression in patients with major depression is associated with oxidative stress and that this event can be reversed by treatment with fluoxetine ad citalopram. Likewise, a previous study of our group found that OBX triggers depression and oxidative stress, whereas treatment with fluoxetine reverses the increases in the immobility time and hyperactivity towards normality (Túnez et al 2007a). Taken together, these results indicate that oxidative stress play an important role in etiopathogeny of depression and the neuroprotective and antidepressant action of 17βE$_2$ may be due, at least partly, to its antioxidative properties.

On basis of our data and the studies by other researchers, the modulator effects of 17βE$_2$ on behavioral response following OBX may involve different mechanisms and pathways: i) membrane receptor, such as intracellular estrogen receptors (ERs).Indeed, the studies performed by Frye's group concluded that estradiol antianxiety and antidepressive effects may involve estrogen receptor beta (ERβ) in the hippocampus (Walf and Frye 2005, 2007b); ii) modulation hypothalamic-pituitary-adrenal axis (HPA) activity. The hippocampus is part of the limbic region which plays an important role in the regulation of emotional processing, providing inhibitory feedback to the HPA (Stock et al 2000; Figueiredo et al 2002, 2003; Walf and Frye 2005); and iii) antioxidative effects.

In this work, we have focused in the study of the antioxidative, antidepressant, and antianxiety effect of estrogen in OBX-model. In addition, we have tried to establish the possible role plays by oxidative stress in the pathogenesis of both OBX and indirectly major depression in order to evaluate the possible relationship between oxidative stress and behavioral changes. This study presents some limits which would be interesting to investigate in future work; as they are other relevant neurochemistry parameters such as serotonin, corticosterone, and estradiol levels in both brain tissue and plasma in this experimental model.

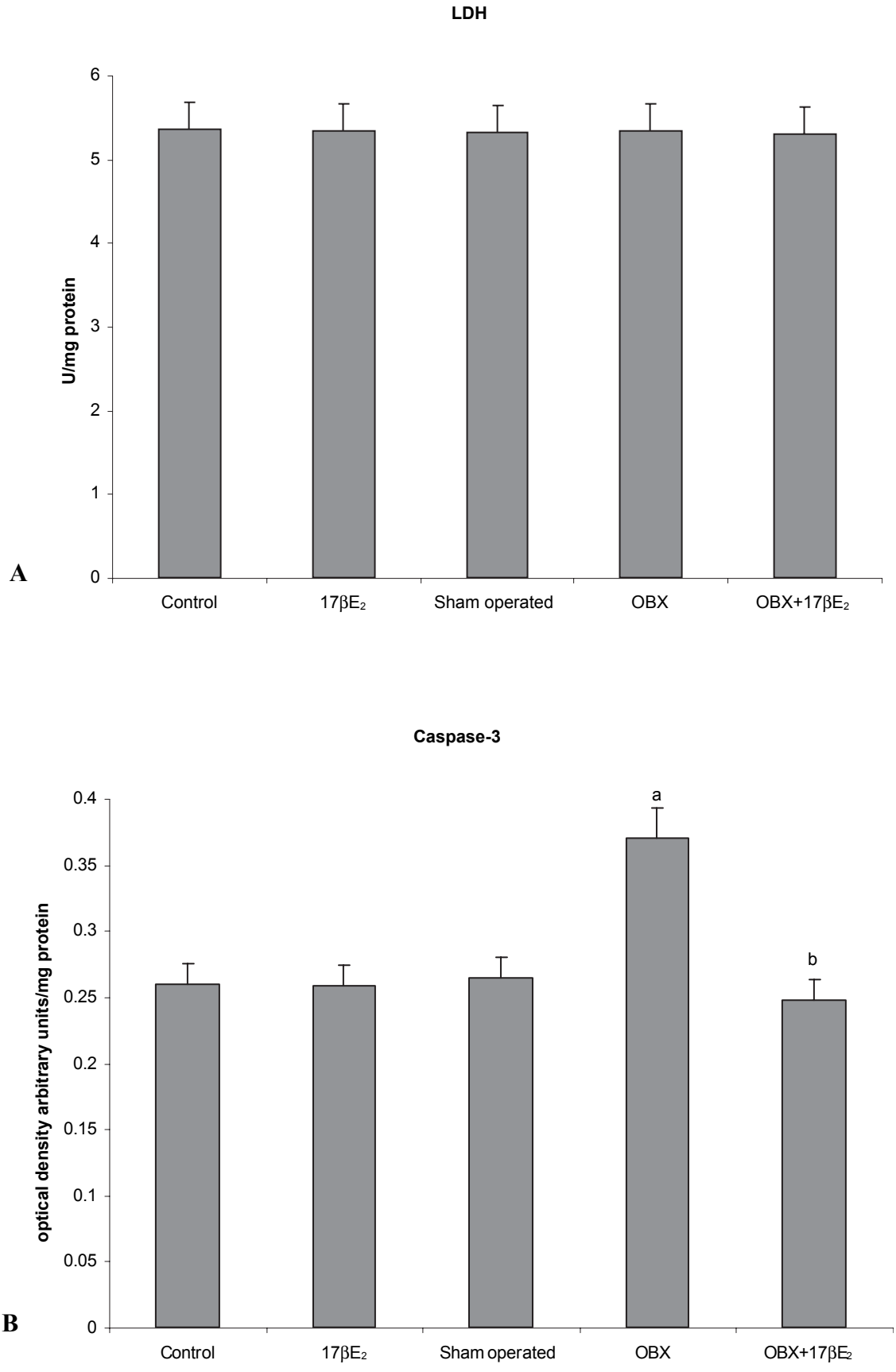


**Figure 2** Effects of OBX and 17βE$_2$ on LDH (panel **A**) and caspase-3 activity (panel **B**). Values are mean ± SD, n = 5 animals per group. [a]$p < 0.001$ versus control group; [b]$P < 0.001$ versus OBX group.

In summary, our results suggest that 17β$E_2$ exerts an antidepressant and antioxidative effect in the OBX model of depression. However, further research is required in order to better evaluate the mechanisms involved and relationship between estrogens, oxidative, and neurochemical changes after OBX context that may contribute to the behavioral changes observed in this study.

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