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ORIGINAL INVESTIGATION

Etazolate, a phosphodiesterase-4 enzyme inhibitor produces antidepressant-like effects by blocking the behavioral, biochemical, neurobiological deficits and histological abnormalities in hippocampus region caused by olfactory bulbectomy

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Abstract
Rationale Olfactory bulbectomy (OBX) is a widely used model for antidepressant screening and known to induce neurodegeneration in several brain areas. Our earlier studies demonstrated that etazolate produced antidepressant-like effects in behavioral despair models of depression; however, the potential role of etazolate on behavior and morphological changes in the hippocampus region along with its underlying mechanism(s) following OBX has not been adequately addressed.
Objectives We evaluated if etazolate could protect against OBX-induced depression-like behavioral deficits and neurodegeneration. The possible underlying mechanism of etazolate in OBX model was also investigated.
Methods The effects of etazolate were measured in a battery of behavioral paradigms, including the forced swim test (FST), sucrose consumption, open arm activity in elevated plus maze (EPM), and hyperemotionality tests. The underlying mechanisms were investigated by measuring serum corticosterone (CORT), cyclic adenosine monophosphate (cAMP), cAMP response element binding protein (CREB), brain-derived neurotrophic factor (BDNF), and oxidative/nitrosative stress (lipid peroxidation and nitrite) levels and antioxidant enzymes, like reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) levels in the hippocampus.
Result OBX rats showed depression-like behavior anomalies in behavioral paradigms. OBX rats also showed high CORT and decreased cAMP, phosphorylated CREB (pCREB), and BDNF levels. Additionally, we found increased oxidative/nitrosative stress and reduced antioxidant enzyme levels in the hippocampus. Histopathological analysis showed morphological changes and neuronal loss in the hippocampus. Etazolate (0.5 and 1 mg/kg) attenuated the OBX-induced behavioral, biochemical, neurobiological, and histopathological alterations.
Conclusion The aforesaid results suggest that etazolate produces an antidepressant-like effect and neuroprotection in OBX, which is possibly mediated by modulating biochemical and neurobiological markers in the hippocampus.

Keywords Etazolate · Neuronal survival · Olfactory bulbectomy · HPA axis · cAMP signaling · Behavioral paradigms · Oxidant/antioxidant markers · Antidepressant

Introduction

Depression is a multicausal and serious mental disorder with a lifetime prevalence of approximately 21 % (Nemeroff 2007; Patten 2008). From the last several decades, according to the monoamine hypothesis, alteration in monoamine neurotransmitter levels has been thought to be the leading cause of depression. A number of antidepressant agents were produced based on this hypothesis; however, to date, no single agent has emerged as the gold standard for first-line treatment in clinical trials and the ability of these agents to improve daily functioning and productivity is questionable. Thus, the aforementioned limitations drew attention that several other etiological and pathological mechanisms may be involved in the induction of depression.

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The neurogenesis hypothesis of depression suggested that depression is associated with neuronal degeneration (Duman and Monteggia 2006) and adult neurogenesis may have a role in the pathophysiology and pharmacotherapy of depression (Malberg and Schechter 2005; Ehninger and Kempermann 2008). Moreover, recently, neuroimaging and postmortem studies of the brain of depressed patients have demonstrated structural changes and decreased neuron densities in several brain regions, supporting the hypothesis that depression may be related to impairments of structural plasticity (Fuchs et al. 2004). There are lines of evidence, which indicate that depression may lead to reductions of the volume not only of the prefrontal cortex and hippocampal regions, but also of the amygdala region (Sheline et al. 1998; Hastings et al. 2004; Koliatsos et al. 2004). Moreover, several reports demonstrated that synaptic plasticity deficit in the prefrontal cortex, hippocampus, and amygdala, brain regions involved in mood regulation, may be due to alteration in intracellular cascades (Pittenger and Duman 2008; Hercher et al. 2009). Further, preclinical studies have demonstrated that animal models of depression also mimic the neuronal rearrangements in several brain regions.

Olfactory bulbectomy (OBX) has been proposed as a well-known animal model with high predictive validity to investigate the possible biochemical or neurobiological mechanism(s) of depression, as well as the antidepressant-like property of test molecules (Kelly et al. 1997). Removal of olfactory bulbs results in behavioral, physiological, neurochemical, and morphological changes, similar to those observed in patients with depression (Van Reizen and Leonard 1990; Song and Leonard 1994). Moreover, ablation of olfactory bulbs is associated with an increase in pro-inflammatory cytokine levels, like interleukin-1β, tumor necrosis factor-α, and others in the brain region (Myint et al. 2007) and promotes a pathological condition by accompanying various inflammatory pathways (Song et al. 2009). Further, the reports of several studies using the OBX model in rats have indicated the neuronal death and altered synaptic plasticity in the hippocampus region of OBX rats (Jaako-Movits et al. 2006). Studies addressing those behavioral and morphological changes in the OBX model may be related to alterations in several biochemical and neurobiological mechanisms, which are implicated in the regulation of neuronal protection (Jarosik et al. 2007; Koo et al. 2010).

It has been suggested that cyclic adenosine monophosphate (cAMP) level and its mediated signaling cascade play an important role in the regulation of neuronal survival and synaptic plasticity (Manji and Duman 2001). Previous research has indicated that impairment in the cAMP level or signaling mechanism is associated with morphological alteration and may contribute to neurodegeneration in depression (Shelton et al. 1996). Impaired cAMP signaling and the resulting reduced levels of cAMP response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) are proposed to be among the most common factors eliciting depressive episodes. Reports indicated that reduced levels of phosphorylated CREB (pCREB) and BDNF are associated with neuronal degeneration in the hippocampus, resulting in the progression of depression (Mattson et al. 2004).

Furthermore, the role of BDNF in depression was supported by other studies, showing reduced serum BDNF level in depressive patients (Karege et al. 2005; Aydemir et al. 2007). A growing body of data has also suggested the role of altered hypothalamic-pituitary-adrenal (HPA) axis system and imbalanced oxidant/antioxidant systems in the pathophysiology of depression (Nikisch et al. 2005; Behr et al. 2012). Elevated blood corticosterone (CORT) level and oxidative stress may lead to neuronal degeneration by lowering hippocampal BDNF levels, resulting in depressive effects (Hansson et al. 2006). Besides these, studies have demonstrated that neuroinflammatory pathways also play a major role in the pathophysiology of brain disorders related to neuroinflammation (Maes et al. 2008). The functioning of the HPA axis, intracellular cAMP signaling cascade, oxidant/antioxidant systems, and pro-inflammatory pathways, therefore, plays an important role in neuronal survival in various animal models.

Etazolate is a selective inhibitor of phosphodiesterase-4 (PDE4) enzyme and proven to be of particular importance in clinical neurosciences (Drott et al. 2010; Jindal et al. 2012). Although several other studies have reported that apart from its effect on the PDE4 enzyme, etazolate is also an inhibitor of adenosine receptors and modulator of GABA-A receptor (Chasin et al. 1972; Daly et al. 1988; Marcade et al. 2008). Etazolate has been shown to have neuroprotection and neurogenesis effects in animal models by regulating the endogenous neuroprotector levels (Siopi et al. 2013). Moreover, other studies mentioned that molecules with PDE4 inhibitory activity, including etazolate, may also represent a way to regulate neuronal protection and neuronal plasticity by increasing cAMP availability and influencing cAMP/CREB/BDNF signaling (Manji and Duman 2001). Recently, for the first time, our group has demonstrated the antidepressant-like effect of etazolate in rodent models of behavioral despair (Jindal et al. 2012, 2013a, b). However, to date, no other study has adequately explored the potential effect of etazolate on the depressive-like behavioral and morphological changes in the OBX model of depression. Thus, in this study, we explored the effect of etazolate on depressive-like behaviors and morphological changes in the OBX model of depression. Moreover, we investigated, whether this compound was capable of normalizing HPA axis hyperactivity, oxidant/antioxidant system imbalance, and the levels of cAMP, CREB, and BDNF in the hippocampus region of OBX rats.

Material and methods

Animals

Adult male Wistar Albino rats (approximately 250–275 g) were procured from Hissar Agricultural University, Haryana, India. Rats were housed under standard (temperature 24±3 °C and humidity 60±10 %) laboratory conditions, maintained on 12:12 h day/night cycle, with free access to standard diet and filtered water. All the experimental procedures were approved by the Institutional Animal Ethics Committee of Birla Institute of Technology and Science, Pilani, India (Protocol No. IAEC/RES/14/03/REV/16/08).

Schedule for drug administration and behavioral tests

Etazolate was procured from Tocris Bioscience, UK. Fluoxetine was procured from Glenmark Pharmaceuticals Ltd, India as a gift sample. Both etazolate and fluoxetine were always freshly prepared in distilled water before administration. After a postsurgical rehabilitation period of 14 days, the administration of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) was started once a day for the next 14 days (15th–28th day). The time schedule for surgery, test drug treatment, and behavioral and biochemical assay of OBX/sham rats were carried out based on the customized schedule reported earlier (Pandey et al. 2008).

Olfactory bulbectomy

Surgery

Bilateral ablation of the olfactory bulb was performed, as per the previously reported method (Kelly et al. 1997). Briefly, rats were anesthetized using a cocktail of xylazine (5 mg/kg, i.p.) and ketamine (75 mg/kg, i.p.). After anesthesia, rats were fixed in a stereotactic frame (Inco, Ambala, India) and the skull was exposed by a 1 cm midline incision. Two burr holes (2 mm in diameter) were drilled 8 mm anterior to bregma and 2 mm on either side of the midline at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs were removed by suction and the holes were filled with hemostatic sponge (AbGel, Absorbable gelatin sponge USP, Sri Krishna Laboratories, India) to control excessive bleeding and the incision was sutured. Sham-operated rats were given a similar procedure, including piercing of the dura mater, but their bulbs were left intact. After surgery, the rats were treated with sulprim injection (0.2 ml/300 g, intramuscularly), once a day for 3 days. The details of the surgical process, treatment schedule, and behavioral tests are mentioned in Table 1.

Experimental design

After 14 postoperative days (recovery period), 48 rats (six rats/group) were randomly divided into eight different groups. Group I is the sham control group and no treatment was given; groups II, III, and IV consisted of sham control rats receiving etazolate 0.5 and 1 and fluoxetine 10 mg/kg, respectively; group V comprised of OBX control rats; groups VI and VII consisted of OBX rats, receiving etazolate 0.5 and 1 mg/kg, respectively; and group VIII consisted of OBX rats receiving fluoxetine 10 mg/kg.

Behavioral assessments

Forced swim test

The forced swim test (FST) was carried out according to the method described by Porsolt et al. (1978). The rats were placed in Plexiglas cylinders (height 40 cm, diameter 30 cm) containing 25 cm water, maintained at 25±2 °C. The rats were forced to swim for 15 min on the pretest day. Rats were then allowed to return to their home cage. After 24 h from the pretest day, each rat was again placed in the cylinders and the total duration of immobility was measured during a 5 min test (Porsolt et al. 1978). A rat was judged to be immobile, when it remained floating passively in the water.

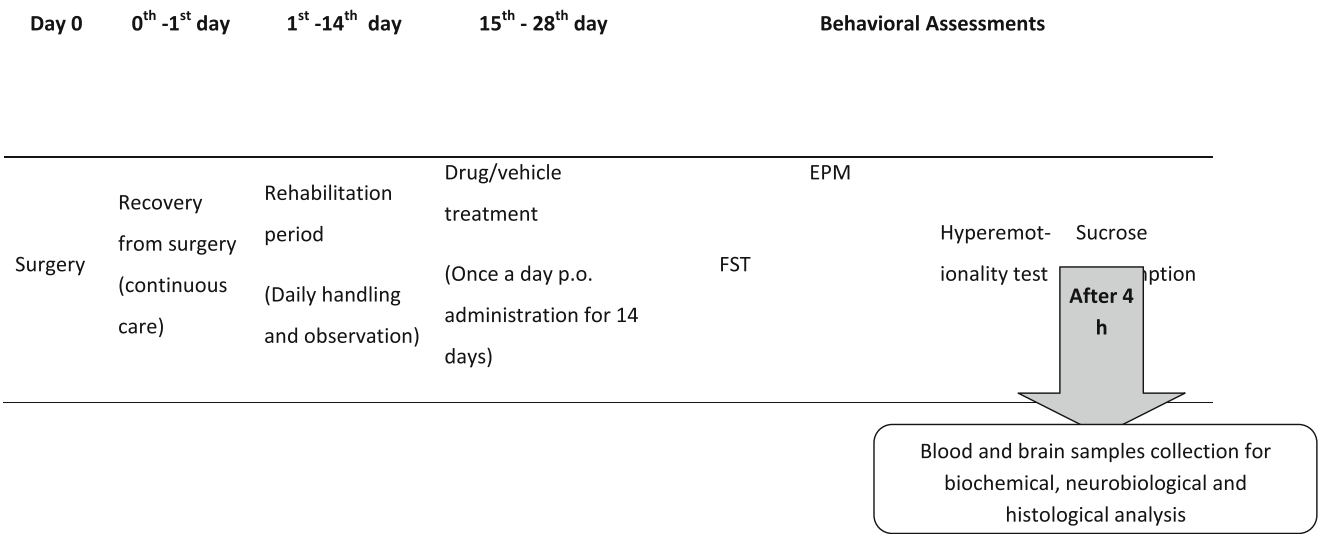
Sucrose preference test

The procedure was performed with modification as described by Willner (1997). Rats had free access to both tap water and sucrose solution (1 %) for 5 days from the commencement of drug treatment (15^{th}–19^{th} day). The position of the 250 ml bottles containing sucrose solution or distilled water was alternated each day to prevent location preference. The sucrose consumption test was performed on the 32^{nd} day of the study by presenting both sucrose solution and water in the morning (1000 hours). The bottles were weighed after 24 h (the next morning). Sucrose preference was calculated by using the following formula: percentage preference=(sucrose intake/total intake)×100.

Elevated plus maze test

The test was performed essentially as described previously (Pellow et al. 1985). The plus maze consisted of two opposite open arms (60 cm×10 cm) and two opposite enclosed arms ($60\times10\times60$ cm^3). The four arms were joined by a central platform (10×10 cm^2) which is open to all the arms. The entire apparatus was elevated to a height of 60 cm above the floor. The apparatus was indirectly illuminated with a ceiling-suspending lamp (60 W) placed at the height of 100 cm above the apparatus. At the beginning of the test, the animal was placed in the

Table 1 Schedule of surgery, drug administration, and behavioral tests



central platform facing an open arm. The behavioral parameters recorded during a 5 min test period were the percentage open arm entries and percentage time spent in the open arm. After each test, the apparatus was sprayed with dilute alcohol and wiped thoroughly to eliminate the residual odor.

Hyperemotionality test

Hyperemotionality was measured with slight modification of the previously reported procedure (Shibata et al. 1984; Pandey et al. 2008). Hyperemotionality of rats was measured by scoring responses to the following stimuli: (1) startle response: induced by a stream of air (using a 10 ml syringe) directed at the dorsum; (2) struggle response: induced by handling with a gloved hand; (3) fight response: induced by pinching the tail with forceps; and (4) bite response: induced by presenting a rod 4–5 cm in front of the snout. A trained researcher performed these operations. These responses were graded as follows: 0, none; 1, slight; 2, moderate; 3, marked; or 4, extreme. All animals in each group were observed for 1 day. The score for each animal was determined within 5 min of the observed response.

Molecular and biochemical estimation

All the molecular and biochemical estimations were carried out after completion of all behavioral assessments.

Blood sampling, dissection, and tissue extraction

The blood samples were collected by decapitation of rats 4 h after the last behavioral assessments as depicted in Table 1. Blood samples were collected and allowed to coagulate at room temperature for 30 min and were subsequently centrifuged at 4,000 rpm for 20 min. Serum was separated and stored at −80 °C until the CORT estimations were carried out.

Brains were quickly removed and washed with ice-cold sterile saline (0.9 %). The hippocampus region was isolated and then the sample homogenates were prepared as per the ELISA kit instructions and literature for the estimation of cAMP, CREB, BDNF, and oxidative and antioxidant marker levels. The homogenate was centrifuged and aliquots of the supernatant were separated and stored at −80 °C until the assays were carried out.

Molecular estimations

Estimation of CORT level

Measurement of serum CORT was performed using a commercially available ELISA kit (IBL, USA) according to the manufacturer's instructions. All samples were measured in duplicate.

Estimation of cAMP, CREB, and BDNF levels

Measurements of brain cAMP (Enzo Life Sciences Ltd, USA), pCREB (Wuhan EiAab Sciences Co., Ltd., China), and BDNF (Boster Biological Technology Co., Ltd, CA, USA) levels were performed using commercially available ELISA kits according to the manufacturer's instructions. All samples were measured in duplicate.

Biochemical estimations

Estimation of lipid peroxidation level

Malondialdehyde (MDA) content, a quantitative measurement of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substances (TBARS) by the method of Wills (1966). In this, 0.1 ml of the supernatant was incubated with 0.5 ml tris hydrochloric acid (0.1 M, pH 7.4) for 2 h. To this, 1 ml of trichloroacetic acid (10 % *w*/*v*) was added and centrifuged at 1,000×*g* for 10 min. To 1 ml of the supernatant, 1 ml (0.67 % *w*/*v*) of thiobarbituric acid (TBA) was added and kept in the boiling water bath for 10 min and cooled, and then 1 ml distilled water was added. The amount of lipid peroxidation products was measured by reaction with TBA at 532 nm using a spectrophotometer (UV-1800 Shimadzu, Japan). The values were expressed as nanomoles of MDA/milligram of protein.

Estimation of nitrite level

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide, was determined by a colorimetric assay using Greiss reagent (0.1 % *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1 % sulfanilamide, and 2.5 % phosphoric acid) as described by Green et al. (1982). Equal volumes of the supernatant and Greiss reagent were mixed, and the mixture was incubated for 10 min at room temperature in the dark and the absorbance was determined at 540 nm (UV-1800 Shimadzu, Japan). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve and expressed as micromoles/milligram of protein.

Estimation of reduced glutathione

Reduced glutathione (GSH) was estimated according to the method described by Ellman (1959); 1 ml of the supernatant was precipitated with 1 ml of 4 % sulfosalicylic acid and cold digested at 4 °C for 1 h. The samples were centrifuged at 1,200×*g* for 15 min at 4 °C. To 1 ml of this supernatant, 2.7 ml of phosphate buffer (0.1 mol/l, pH 8) and 0.2 ml of 5,5′-dithiobis(2-nitrobenzoic acid) were added. The color developed was measured immediately at 412 nm (UV-1800 Spectrophotometer, Shimadzu, Japan). Results were expressed as micromoles/milligram of protein.

Estimation of superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured by the method of Misra and Fridovich (1972). Autooxidation of epinephrine at pH 10.4 was measured spectrophotometrically at 480 nm (UV-1800 Spectrophotometer, Shimadzu, Japan). Briefly, the supernatant of the tissue was mixed with 0.8 ml of 50 mM glycine buffer, pH 10.4, and the reaction was started by the addition of 0.02 ml (−)-epinephrine. After 5 min, the absorbance was measured and the activity of SOD was expressed as percentage of activity of the sham control group.

Estimation of catalase activity

Brain catalase (CAT) activity was assayed by the method described earlier (Sinha 1972). The reaction mixture (1.5 ml) contained 1.0 ml of 0.01 mol/l phosphate buffer (pH 7), 0.1 ml of brain homogenate supernatant, and 0.4 ml of 2 mol/l hydrogen peroxide. The reaction was stopped by the addition of 2 ml of dichromate-acetic acid reagent (5 % potassium dichromate and glacial acetic acid were mixed in a 1:3 ratio). The absorbance was measured at 620 nm and expressed as micromoles of hydrogen peroxide consumed/minute/milligram protein.

Protein estimation

The protein content was measured in all brain samples for oxidant and antioxidant activity by the biuret method using bovine serum albumin as standard.

Histopathological evaluation

After the behavioral test, the rats for the histological study were deeply anesthetized and transcardially perfused with phosphate-buffered saline, followed by fixation with 4 % formaldehyde (fixative solution) in ice-cold phosphate-buffered saline (pH 7.4). After the fixation, the rats were sacrificed and brain samples were isolated and fixed overnight in 4 % of formalin. Each sample was then washed with 0.1 M PBS (pH 7.4) for 1 h, dehydrated in alcohol, and then embedded in paraffin. The paraffin sections, 5 μm thick, were cut, dewaxed, and rehydrated with alcohol for hematoxylin-eosin (H & E) staining. The samples were stained with H & E stain, and neurons in hippocampus CA1 and dentate gyrus (DG) regions were examined under bright field illumination using a microscope (Optika research microscope).

Statistical analysis

All data were expressed as mean±SEM. The data obtained in studies from various groups were statistically analyzed using two-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test for multiple comparisons in GraphPad Prism 3 software. The value of $p<0.05$ was considered statistically significant.

Results

Behavioral analysis

Effect of etazolate on the behavior of OBX rats in FST

Figure 1 shows the effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on the duration of immobility in the FST. In the FST test, two-way ANOVA revealed a significant effect of the groups for the duration of immobility [$F(1, 40)=14.01$, $p<0.05$]. The post hoc test indicated that in the FST paradigm, OBX rats exhibited a significant ($p<0.05$) increase in duration of immobility as compared to sham-operated rats. Chronic treatment with etazolate (at doses of 0.5 and 1 mg/kg, i.p.) and fluoxetine (10 mg/kg, p.o.) significantly decreased the duration of immobility as compared to OBX control rats. In addition, etazolate (at doses of 0.5 and 1 mg/kg, i.p.) and fluoxetine (10 mg/kg, p.o.) also significantly decreased the duration of immobility in drug-treated sham groups as compared to vehicle-treated sham control group (Fig. 1).

Effect of etazolate on sucrose consumption in OBX rats

In Fig. 2, two-way ANOVA revealed a significant effect of the groups for the percentage of sucrose consumption [$F(1, 40)=19.28$, $p<0.05$]. The post hoc test indicated that OBX rats show a significant reduction in the percentage of sucrose consumption as compared to sham control group (Fig. 2). Treatment with etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) significantly ($p<0.05$) increased the percentage of sucrose consumption in OBX rats as compared to the OBX control group. On the other hand, administration of etazolate as well as fluoxetine did not show any significant effect on sucrose consumption in drug-treated sham groups (Fig. 2).

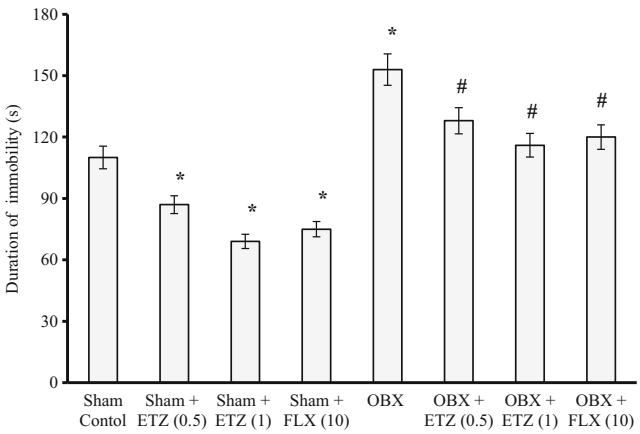


Fig. 1 Effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on behavior of OBX/sham rats in FST. *Each column* represents mean duration of immobility in FST. The *error bar* indicates SEM; *$p<0.05$ when compared with sham control; #$p<0.05$ when compared with OBX control

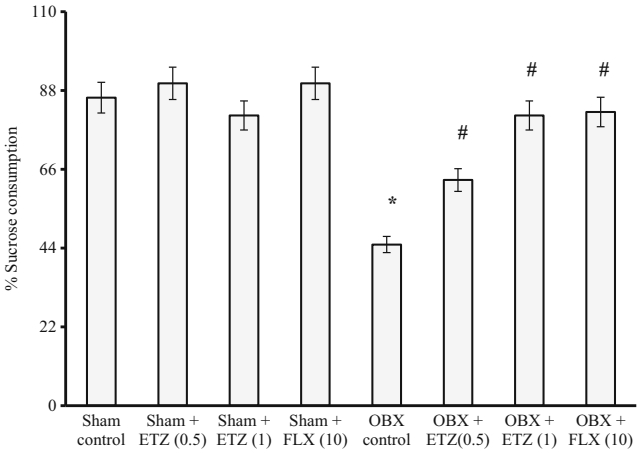


Fig. 2 Effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on sucrose preference test. *Each column* represents mean percentage sucrose consumption. The *error bar* indicates SEM; *$p<0.05$ when compared with sham control; #$p<0.05$ when compared with OBX control

Effect of etazolate on the behavior of OBX rats in elevated plus maze

In the elevated plus maze (EPM) test, two-way ANOVA revealed a significant effect of the groups for the percentage of both open arm entries [$F(1, 40)=17.12$, $p<0.05$] and time spent in the open arm [$F(7, 35)=36.19$, $p<0.05$]. OBX rats exhibited a significant ($p<0.05$) increase in the percentage of both open arm entries and time spent in the open arms as compared to the sham control group (Fig. 3a, b). Chronic administration of etazolate (0.5 and 1 mg/kg, i.p.) and fluoxetine (10 mg/kg, p.o.) significantly ($p<0.05$) decreased the percentage of both open arm entries and time spent in the open arms as compared to the OBX control group. Etazolate and fluoxetine also markedly increased the percentage of both open arm entries and time spent in the open arms in drug-treated sham groups as compared to the sham control group (Fig. 3a, b).

Effect of etazolate on the behavior of OBX rats in the hyperemotionality test

Figure 4 shows the effect of etazolate and fluoxetine treatment on the behavior of OBX/sham rats in the hyperemotionality test. Two-way ANOVA showed a significant difference among groups for the hyperemotionality scores [$F(1, 40)=14.12$, $p<0.05$] (Fig. 4). In the hyperemotionality test, OBX rats exhibited a significantly increased emotional behavior (included evaluation of the bite, startle, struggle, and fight

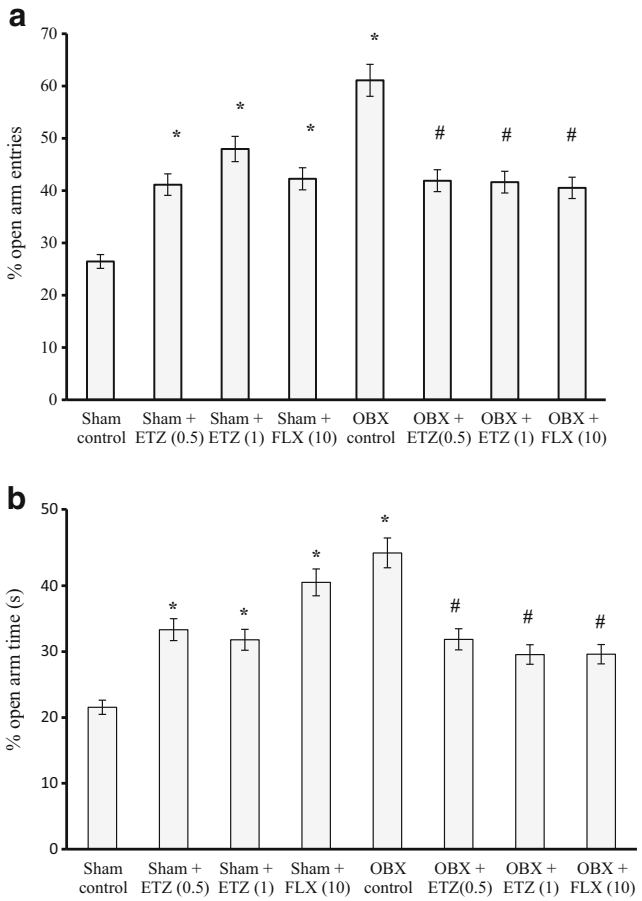


Fig. 3 Effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on behavior of OBX/sham rats in EPM. *Each column* represents mean, **a** percentage open arm entries and **b** percentage open arm time (s). The *error bar* indicates SEM; *$p<0.05$ when compared with sham control; #$p<0.05$ when compared with OBX control

response) as compared to the sham control group. Chronic etazolate and fluoxetine treatment reduced hyperemotional behavior as compared to the OBX control group. Etazolate as well as fluoxetine did not show any effect on hyperemotionality scores in drug-treated sham groups as compared to the sham control group (Fig. 4).

Molecular estimation

Effect of etazolate on serum CORT level in OBX rats

OBX rats showed a significantly [$F(1, 40)=17.22$, $p<0.05$] increased serum CORT level as compared to the sham control group. Chronic etazolate and fluoxetine treatment significantly ($p<0.05$) decreased the serum CORT level as compared to the OBX control group. However, administration of etazolate as well as fluoxetine did not show any significant effect on CORT level in the drug-treated sham groups (Fig. 5).

Effect of etazolate on the cAMP, pCREB, and BDNF levels in OBX rats

The effects of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on the cAMP, pCREB, and BDNF levels in OBX/sham rats are shown in Fig. 6a–c, respectively. Two-way ANOVA showed a significant difference among groups for cAMP [$F(1, 40)=6.29$, $p<0.05$], pCREB [$F(1, 40)=18.21$, $p<0.05$], and BDNF [$F(1, 40)=10.33$, $p<0.05$] levels. OBX rats showed significantly decreased hippocampal cAMP, pCREB, and BDNF levels as compared to the sham control rats. Chronic treatment with etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine significantly reversed the OBX-induced decrease in cAMP, pCREB, and BDNF levels

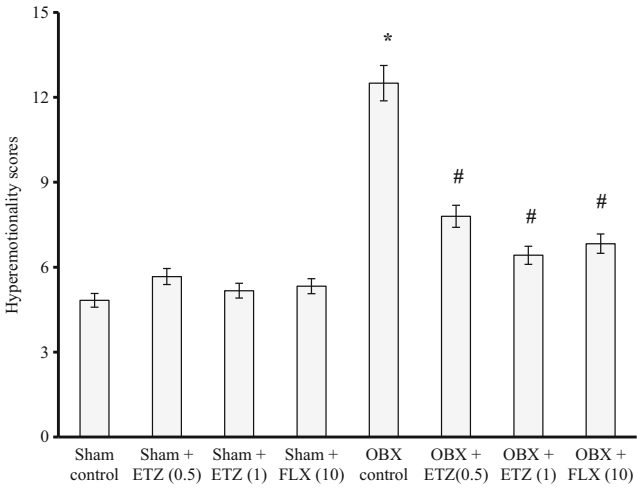


Fig. 4 Effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on behavior of OBX/sham rats in hyperemotionality test. *Each column* represents mean hyperemotionality scores. The *error bar* indicates SEM; *$p<0.05$ when compared with sham control; #$p<0.05$ when compared with OBX control

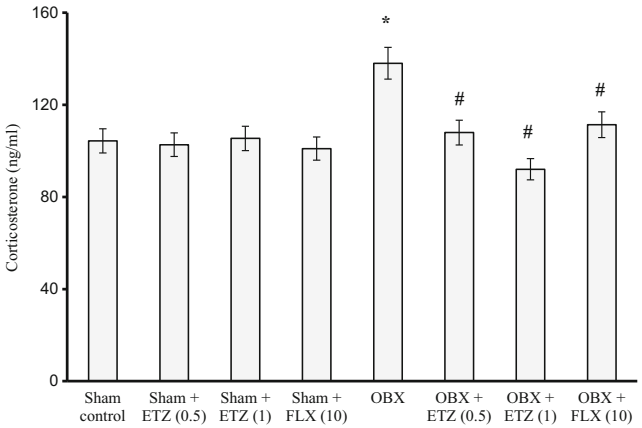


Fig. 5 Effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on serum CORT level in OBX/sham rats. *Each column* represents mean serum CORT level. The *error bar* indicates SEM; *$p<0.05$ when compared with sham control; #$p<0.05$ when compared with OBX control

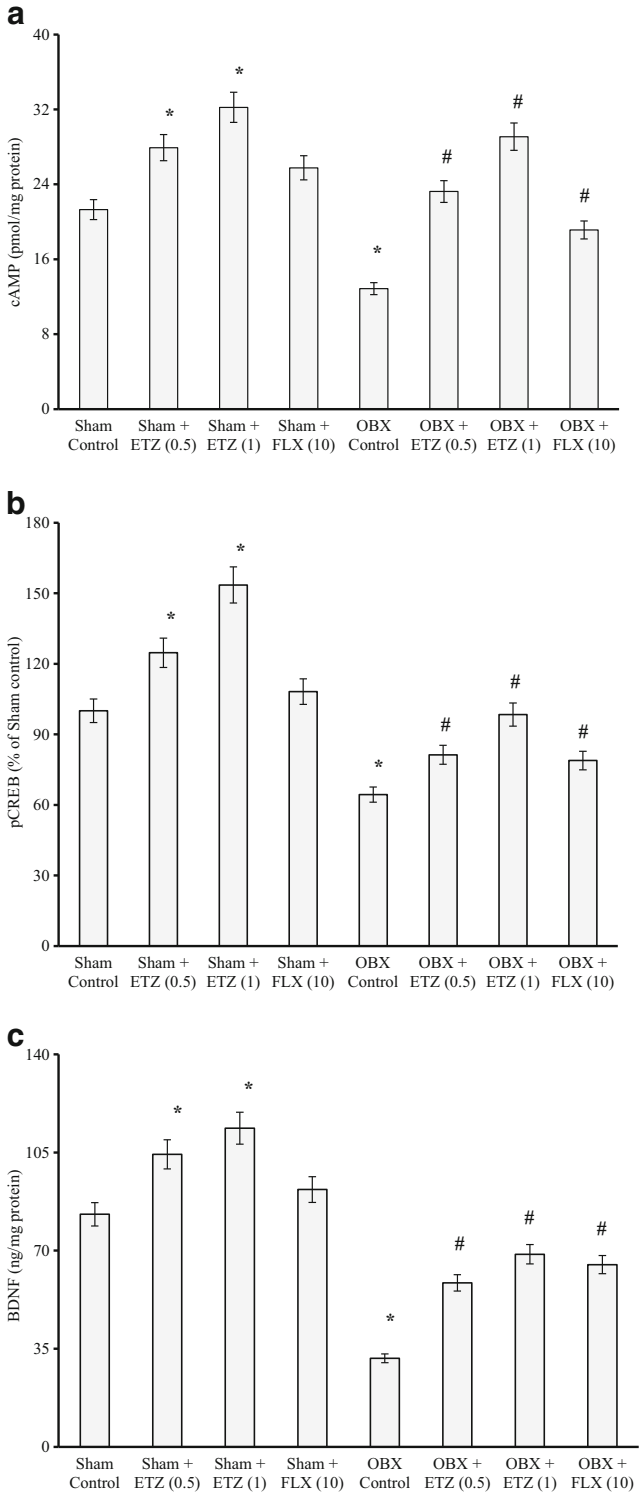


Fig. 6 Effect of etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) treatment on neurobiological aspects in OBX/sham rats; *each column* represents mean: **a** cAMP level, **b** pCREB level, and **c** BDNF level. The *error bar* indicates SEM; *$p<0.05$ when compared with sham control; #$p<0.05$ when compared with OBX control

as compared to the OBX control group. Administration of etazolate also showed a significant effect on cAMP, pCREB, and BDNF levels in drug-treated sham groups as compared to the control group (Fig. 6a–c).

Biochemical estimation

Effect of etazolate on brain lipid peroxidation level in OBX rats

OBX rats showed a significantly [$F(1, 40)=9.45$, $p<0.05$] increased hippocampal TBARS level as compared to the sham control group (Table 2). Repeated treatment with etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, i.p.) produced a significant ($p<0.05$) decrease in brain TBARS levels in OBX rats as compared to the OBX control group. No effect of etazolate was observed on TBARS level in drug-treated sham groups (Table 2).

Effect of etazolate on brain nitrite level in OBX rats

In the present study, OBX rats showed a significant [$F(1, 40)=23.17$, $p<0.05$] increase in hippocampal nitrite level as compared to the sham control group (Table 2). Etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) produced a significant decrease in brain nitrite level in OBX rats as compared to the OBX control group. Administration of etazolate and fluoxetine did not show any significant effect on nitrite level in drug-treated sham groups (Table 2).

Effect of etazolate on brain GSH level in OBX rats

Two-way ANOVA showed a significant difference among groups for GSH [$F(1, 40)=8.45$, $p<0.05$]. OBX rats showed a significantly ($p<0.05$) decreased hippocampal GSH level as compared to the sham control group. Treatment with etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) restored brain GSH level as compared to the OBX control group (Table 2). However, there was no effect of etazolate and fluoxetine observed on GSH level in drug-treated sham groups.

Effect of etazolate treatment on brain SOD level in OBX rats

OBX rats showed significantly [$F(1, 40)=15.34$, $p<0.05$] decreased hippocampal SOD levels as compared to the sham control group. Chronic treatment with etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) significantly attenuated the reduction in hippocampal SOD level as compared to the OBX control group. Repeated treatment with etazolate and fluoxetine did not show any effect on SOD level in drug-treated sham groups (Table 2).

Table 2 Effect of etazolate (0.5 and 1 mg/kg) and fluoxetine (10 mg/kg) treatment on the oxidant/antioxidant parameters of OBX/sham rats

Treatment (mg/kg)	TBARS (nmol/mg protein)	Nitrite/nitrate (μmol/mg protein)	GSH (μmol/mg protein)	Catalase (μmol/mg protein)	% SOD activity
Sham	3.63±1.01	3.71±0.57	0.238±0.08	3.03±0.56	100±4.32
FLX per se (10)	3.94±1.1	4.93±0.37	0.243±0.06	3.13±0.68	94±5.5
ETZ per se (0.5)	3.34±0.90	3.92±1.12	0.274±0.10	3.60±0.99	84±5.35
ETZ per se (1)	3.53±0.84	4.53±0.73	0.250±0.08	3.40±1.04	90±7.32
OBX	7.41±0.78*	5.94±0.37*	0.099±0.09*	0.75±0.82*	26.09±6.49*
OBX+FLX (10)	4.20±0.92**	3.93±1.04**	0.195±0.05**	2.10±0.94**	57.14±6.43**
OBX+ETZ (0.5)	4.78±0.89**	4.77±0.91**	0.168±0.09**	1.46±0.23**	52.38±7.43**
OBX+ETZ (1)	4.10±0.66**	3.83±0.86**	0.220±0.06**	2.55±0.46**	63.64±4.96**

The values are expressed as mean±SEM, n=6/group. The drug/vehicle treatments were carried out once a day for 14 days

*p<0.05 compared with sham control; **p<0.05 compared with the vehicle-treated OBX group

Effect of etazolate on brain CAT level in OBX rats

Two-way ANOVA showed a significant difference among groups for CAT [$F(1, 40)=14.26$, $p<0.05$]. Post hoc test indicated that OBX rats showed significantly decreased hippocampal CAT level as compared to the sham control group. Etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (10 mg/kg, p.o.) significantly attenuated the reduction in hippocampal CAT level as compared to the OBX control group. Etazolate and fluoxetine did not show any significant effect on brain CAT level in drug-treated sham groups as compared to the sham control group (Table 2).

Histological analysis

Effect of etazolate on morphological changes in hippocampal CA1 and DG regions of OBX rats

Figure 7 shows the effect of OBX and etazolate treatment on the morphological changes in hippocampal CA1 and DG regions of OBX rats. The H & E-stained hippocampal CA1 and DG sections showed healthy neurons in sham control (Fig. 7, A1 and B1). Healthy neurons were robust in shape and had a pale and spherical or slightly oval nucleus and a single large nucleolus with a clearly visible cytoplasm. However, the photomicrographs from the hippocampal CA1 and DG regions of the OBX group showed damaged and shrunk neurons with a pyknotic nucleus (Fig. 7, A2 and B2) as compared with the sham control group. Chronic treatment with etazolate (0.5 and 1 mg/kg, p.o.) and fluoxetine (FLX; 10 mg/kg, p.o.) in OBX rats provided histological appearances very similar to that observed in the sham control group with respect to the morphologic characteristics of the neurons (Fig. 7, A3–A5 and B3–B5, respectively). The higher dose of etazolate had displayed rounded and open nuclei with marked protection from OBX-induced neurodegenerative morphologies.

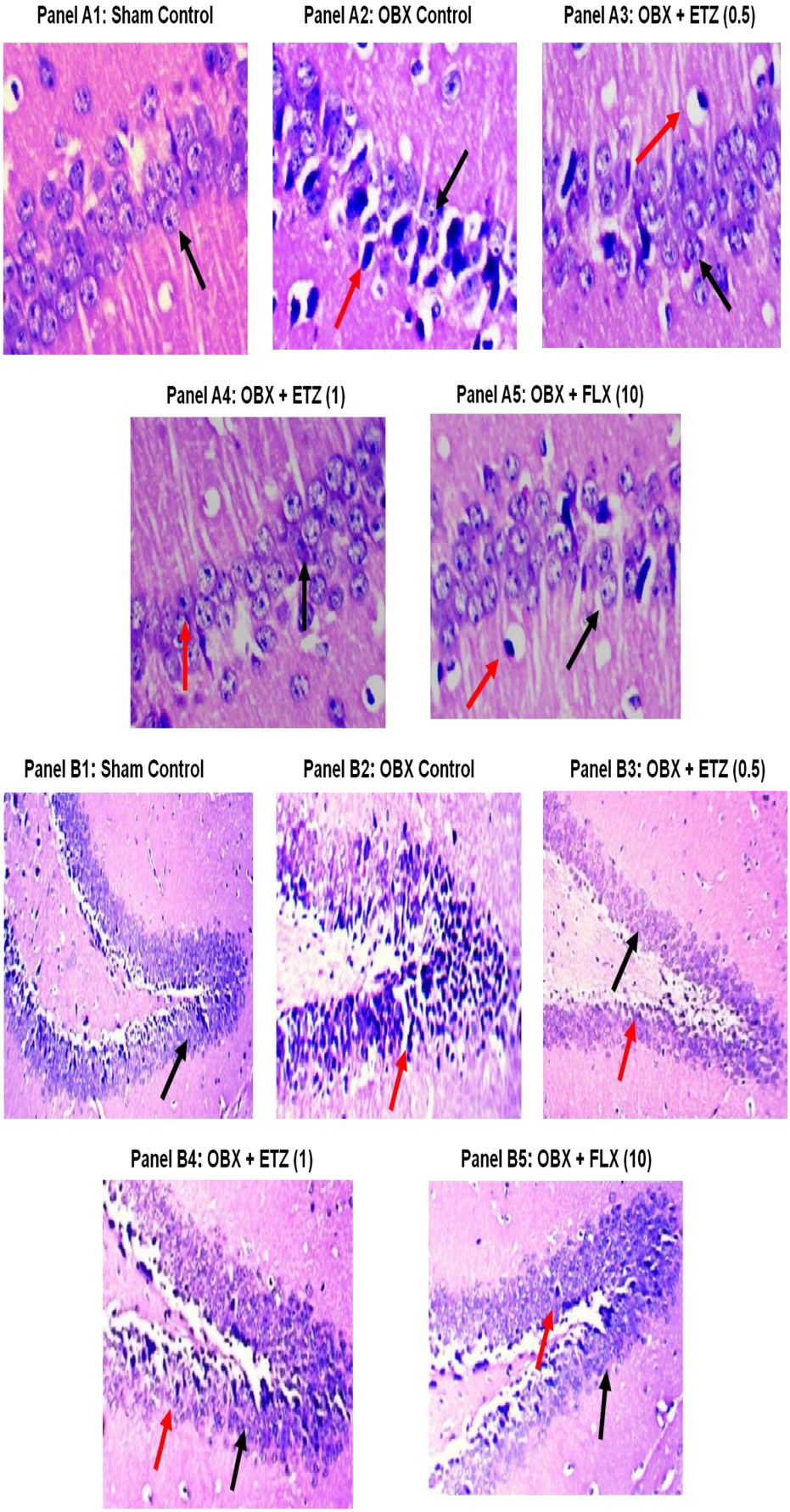
Discussion

The results of this study are consistent with the notion of PDE4 inhibitor-mediated regulation of the biochemical and neurobiological markers, which are involved in the neuronal protection and induced antidepressant-like effects in the OBX model. The results demonstrated that (1) chronic treatment with etazolate and fluoxetine reversed the behavioral anomalies in OBX rats; (2) etazolate prevented the morphological changes in the hippocampal CA1 and DG regions of OBX rats; (3) etazolate decreased the serum CORT level in OBX rats; (4) etazolate increased hippocampal cAMP, pCREB, and BDNF levels; and (5) etazolate decreased the levels of oxidative/nitrosative stress markers and increased the antioxidant enzyme levels in the hippocampal region of OBX rats.

Etazolate, which belongs to the pyrazolopyridine class, is a selective inhibitor of type 4 PDE enzyme. Several other studies demonstrated that etazolate-mediated pharmacological actions are related to its activity on GABA-A receptor or adenosine receptor (Daly et al. 1988; Marcade et al. 2008). Further, it is also observed that cAMP modulates the GABA-A receptor function (Moss et al. 1992). Hence, we cannot exclude a convergence of these pathways and the possibility that GABA-A receptor and adenosine receptor may be also involved in some of the effects shown here by etazolate.

OBX is considered as one of most validated models to evaluate antidepressant-like effects because in an experimental setup, OBX-induced hyperactivity is normalized following chronic but not acute administration of antidepressant drugs (Song and Leonard 2005). The bilateral removal of the olfactory bulbs produces an impaired state of the brain with substantial alterations in behavioral, morphological, and neurobiological correlates, many of which resemble clinical depression (Song and Leonard 2005).

In this study, our results are in accordance with earlier published reports, since it was previously indicated that OBX increases the immobility duration in Wistar rats, which


Panel A1: Sham Control
Panel A2: OBX Control
Panel A3: OBX + ETZ (0.5)
Panel A4: OBX + ETZ (1)
Panel A5: OBX + FLX (10)
Panel B1: Sham Control
Panel B2: OBX Control
Panel B3: OBX + ETZ (0.5)
Panel B4: OBX + ETZ (1)
Panel B5: OBX + FLX (10)

◀ **Fig. 7** *A1–A5*, *B1–B5* The effect of etazolate on the neuron structure in hippocampal CA1 and DG regions of rat brain section. *Red arrows* indicate degenerated neurons and *black arrows* indicate healthy neurons; *A1*, *B1* vehicle-treated sham control group showing healthy neurons; *A2*, *B2*: OBX group showing a large number of dark-stained degenerated neurons; *A3*, *B3*: etazolate (*ETZ*; 0.5 mg/kg) showing neuroprotection in OBX-treated rats; *A4*, *B4*: ETZ (1 mg/kg) showing more neuroprotection in OBX-treated rats; and *A5*, *B5*: FLX (10 mg/kg) showing neuroprotection in OBX-treated rats (*calibration bar*=50 μm)

is an indication of depressive-like behavior (Porsolt et al. 1978; Tasset et al. 2008). For the first time in this investigation, chronic administration of etazolate decreased the immobility duration in OBX rats. Porsolt et al. (1978) showed that decreased immobility duration in FST is an indication of antidepressant-like effect. In addition, etazolate also significantly decreased immobility duration in sham control rats, which is in accordance with a previous study that PDE4 inhibitor displayed a reduction in immobility time in rat FST (Xiao et al. 2011). Furthermore, several laboratories using genetically modified mice have indicated the antidepressant potential of PDE4 inhibitor in FST (Zhang et al. 2002). In addition, fluoxetine, used as reference drug, also showed potential antidepressant-like effects in both sham control and OBX rats.

In the current investigation, the effect of etazolate on anhedonia behavior, an important indicator which mimics the clinical features of human depression, in OBX rats was investigated (Willner 1997). Anhedonia behavior in OBX rats was investigated by measuring the percentage of sucrose consumption (Willner 1997; Li et al. 2011). In this study, OBX rats showed a reduced preference and consumed less amount of sucrose solution as compared to sham control rats, which is in accordance with previous reports of our laboratory (Pandey et al. 2010). Etazolate markedly attenuated the OBX-induced reduction in sucrose consumption, representing the potential antidepressant-like effect in the OBX model. Further, in our laboratory, the potential beneficial effect of etazolate on anhedonia behavior has been reported in a chronic unpredictable mild stress model of depression (Jindal et al. 2013a). In addition, fluoxetine also reversed OBX-induced decrease in sucrose consumption, which is also supported by earlier reports that antidepressants can reverse the reduction in sucrose consumption in OBX rats (Pandey et al. 2010).

The EPM test was originally described as a predictive test to detect anxiolytic/anxiety-like behavior, in which rodents normally prefer more closed arm activity than open arms, which is significantly reversed by anxiolytic treatment (Pellow et al. 1985). In the EPM test, sham rats treated with etazolate showed increase in open arm activity, such as increased percentage of both the number of entries and time spent in open arms, which is in agreement with the earlier published reports indicating anxiolytic effects of etazolate (Horovitz et al. 1972; Jindal et al. 2013c). Besides this, in this investigation, the EPM test was used to evaluate the open arm activity of OBX rats, as a marker of psychomotor agitation instead of anxiolytic activity. Interestingly, OBX rats displayed an increased open arm activity, which indicates a loss in defensive behavior in a new environment (Ramamoorthy et al. 2008), as supported by earlier reports of our laboratory (Pandey et al. 2008; Rajkumar et al. 2009). The chronic administration of etazolate significantly decreased the open arm activity in OBX rats. Etazolate-mediated decrease in open arm activity of OBX rats further corroborates the depression-like behavioral anomalies in OBX rats, which is reversed by chronic antidepressant treatment (Pandey et al. 2008; Rajkumar et al. 2009).

Hyperemotionality behavior is evident by aggressive behavior, when rodents were subjected to innocuous stimuli. Thus, there is a possibility that the hyperemotional behavior shown by OBX rats in response to innocuous stimuli may resemble the behavior of psychomotor agitation, which is a diagnostic criterion for depression (Fukuchi and Kanemoto 2000). In the current investigation, OBX rats showed a marked increase in hyperemotional score. Repeated etazolate and fluoxetine treatment significantly reduced the hyperemotional behavior displayed by OBX rats, which reflects the antidepressant-like behavior. Hence, these results are proofs that attenuation of hyperemotional behavior of OBX rats may be characterized, as a predictive test to evaluate the molecules for antidepressant-like effects.

Numerous studies reported that bilateral ablation of the olfactory bulb in rodents results in neuronal degeneration remodeling from olfactory bulb to brain regions, which are involved in the regulation of mood and emotional behaviors. A large number of studies established that in depression, the hippocampus region of the brain shows marked morphological changes. Hence, in the present study, we mainly focused on neurons' morphology in DG and hippocampal CA1 regions of OBX rats and studied the role of etazolate on morphological changes. The results of the present study provide a direct histopathological evidence of neuronal damage in hippocampal CA1 and DG regions of OBX rat brain. OBX rats showed marked morphological changes and neuronal loss in the hippocampal CA1 and DG regions as compared to sham controls which showed healthy neurons. This is supported by earlier studies that OBX leads to neuronal loss and decreases neuronal survival in the hippocampus region, which is one of the most accepted mechanisms, implicated in the induction of depression (Manji and Duman 2001; Jaako-Movits et al. 2006). Chronic 14 day treatment with etazolate remarkably attenuated OBX-induced morphological changes and neuronal loss in hippocampal CA1 and DG regions. These results supported that PDE4 inhibitors show neuronal protection by regulating neurobiological signaling.

In addition to the behavioral deficits and morphological changes, the effect of etazolate on the hyperactivity of the

HPA axis in the OBX model was also investigated. Studies reported that altered HPA axis activity is implicated in various pathological conditions, including depression (de Kloet et al. 2005; Vreeburg et al. 2010). In this relationship, modulation of HPA axis activity may be involved in the therapeutic potential of antidepressant drugs (Heim et al. 2008). Hence, the restoration of normal HPA axis function may be of critical concern in the treatment of depression. In this study, OBX rats displayed an increased serum CORT level, which is supported by earlier findings that OBX rats have high CORT level than sham controls (Cairncross et al. 1979; Kelly et al. 1997). The increase in serum CORT level in OBX rats was markedly attenuated by chronic etazolate and fluoxetine treatment. Several studies demonstrated that a high CORT level has been reported to impair the proliferation or survival of neurons and dendritic spines in the hippocampus region, via altering the expression of the neurotrophic factor (Hansson et al. 2006). In the present study, etazolate restored the normal level of the neurotrophic factor and exerts its beneficial effects in lowering HPA axis activity and CORT level.

cAMP and its mediated regulation of neurotrophic signaling cascade play a key role in neuronal survival and are implicated in the pathogenic mechanism of depression (Wachtel 1989). In this investigation, chronic treatment with etazolate significantly increased cAMP in OBX and sham control rats. Previous studies reported that chronic treatment with PDE4 inhibitors significantly increased the cAMP level and regulated mood behavior (Xiao et al. 2011).

The neurotrophic theory of depression (Nestler et al. 2002) demonstrated that BDNF and CREB, expression and function might be expected to reduce after OBX and increase after chronic antidepressant treatment (Qi et al. 2008). Similarly, in this study, OBX results in the depletion of the pCREB expression in the hippocampus region. Chronic etazolate treatment significantly increased the pCREB in OBX rats. This provides support for the possibility of the etazolate-mediated antidepressant-like action via changes in pCREB-controlled gene expression.

The role of BDNF in the pathophysiology of depression and in the mechanism of action of antidepressant drugs is well appreciated (Middeldorp et al. 2010). Several clinical investigations showed a reduced BDNF level in individuals suffering from depression and which returned to normal after chronic antidepressant treatment (Castrén et al. 2007). Similarly, in our study, OBX rats showed a decreased hippocampal BDNF level. Studies reported that reduced expression of BDNF is associated with neuronal degeneration and reduced neurogenesis and synaptic plasticity in the hippocampus region results in maladaptive changes in neural circuitry which is under the pathophysiology of depression (Kuipers et al. 2003). Repeated treatment with etazolate significantly elevated the BDNF level in both naive and OBX rats. This data is supported by earlier reports, which show high BDNF level, following chronic treatment with PDE4 inhibitors (Xiao et al. 2011). Further studies by Impey et al. (1996) have indicated that PDE4 inhibitors mediate cellular signaling progression by increasing cAMP level, which eventually regulate neuronal survival in depression by activating CREB and BDNF signaling mechanism. Etazolate-mediated neuronal survival in OBX rats, thus, may be also due to regulation of cAMP signaling.

Besides the involvement of the HPA axis and cAMP signaling, the oxidative stress hypothesis of depression demonstrated an imbalance between oxidative and antioxidant defense systems in depression, as evidenced by both human and experimental studies (Eren et al. 2007; Zafir et al. 2009). Antioxidant enzymes are implicated in the scavenging of ROS and help in neuronal survival by inhibiting neurotoxicity. Induction of high oxidative stress could decrease the activity of antioxidant enzymes like GSH, SOD, and CAT (Tasset et al. 2010). A number of data have suggested an increase in MDA (byproduct of lipid peroxidation) level in plasma and serum samples of depressive subjects (Tasset et al. 2010). Further, rats subjected to the OBX paradigm showed a marked reduction in the activity of antioxidant enzymes, such as GSH, SOD, and CAT (Túnez et al. 2010; Tasset et al. 2008). In line with these studies, our study shows that OBX impaired the oxidative/antioxidative balance, characterized by an increase in lipid peroxidation products and reduction in GSH, CAT, and SOD levels in the hippocampal region. In addition, we also found a high level of nitrosative stress in terms of nitrite (peroxynitrite), which leads to oxidative modification of proteins and ultimately causing neuronal death (Tasset et al. 2010). Repeated treatment with etazolate restored the increased oxidative/nitrosative stress marker levels in OBX rats. Moreover, previously, we have shown that etazolate attenuated the increase in oxidative stress markers in chronic unpredictable stress model of depression (Jindal et al. 2013a). In this study, etazolate also markedly restored the decreased antioxidant enzyme levels in OBX rats. Increased antioxidant enzyme activity is an important neuronal defense mechanism against neuronal degeneration; thus, high antioxidant enzyme activity may be an important mechanism in the pharmacotherapy of depression (Maes et al. 2011).

In fact, along with the oxidative/nitrosative stress, pro-inflammatory marker-mediated neuroinflammation has also been reported to influence neuronal functions in the OBX model of depression (Maes et al. 2008). It is well addressed that increased expression of pro-inflammatory mediators leads to neuroinflammation and neuronal death, impairs neurogenesis, and causes neuronal degeneration (Siopi et al. 2013). Considerable research indicated that pro-inflammatory markers mainly affect the neurochemical pathways or modulators involved in neurogenesis or neuronal survival. Mostly, pro-inflammatory marker-mediated neurotoxicity is dependent upon neurotrophic factor signaling and was found to regulate the vitality of neurons and implicated in the

pathological conditions followed by brain surgery (Shelton 2007; Siopi et al. 2013). Moreover, studies have mentioned that increased expression of the pro-inflammatory cytokines may lead to monoaminergic disturbances in depression (Maes 1993). Further, there is abundant evidence that shows that existing antidepressants, including fluoxetine and others, produce anti-inflammatory effects by decreasing the production of cytokines (Kubera et al. 2001; Diamond et al. 2006). In addition, reports demonstrated that antidepressant drugs stimulate synaptic plasticity, neuronal survival, and neurogenesis by upregulating the expression of neurotrophic factors (Kubera et al. 2011). Recently, Siopi et al. (2013) have reported the neuroprotective effects of etazolate by increasing the endogenous neuroprotector (soluble form α of the amyloid precursor protein, sAPPα) or neurotrophic factor and decreasing the pro-inflammatory markers in animal models (Siopi et al. 2013). Moreover, in this study, etazolate also showed a neuroprotective effect, possibly by regulating neurotrophic factor signaling and oxidative/nitrosative marker levels. Thus, in our study, there may be a chance of a relation between the oxidant/antioxidant system, pro-inflammatory markers, and neurobiological modulators involved in neuronal survival.

In conclusion, chronic treatment with etazolate was found to alleviate OBX-induced depression and morphological changes in the hippocampal regions. In fact, the increased level of CORT and altered cAMP signaling cascade and oxidant/antioxidant system may have contributed to neuronal degeneration, which is accountable for the depressive-like behaviors in the rat model of OBX. On the other hand, etazolate decreased high CORT level and normalized cAMP signaling cascade markers and oxidative/nitrosative/antioxidative marker levels. Thus, the antidepressant-like effect of etazolate seems to depend on the regulation of neuronal survival by modulating HPA axis activity, cAMP signaling, and oxidant/antioxidant system, which is often assumed to be a common property of clinically effective antidepressants.

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