QUERCETIN SUPPRESS MICROGLIAL NEUROINFLAMMATORY RESPONSE AND INDUCE ANTIDEPRESSENT-LIKE EFFECT IN OLFACTORY BULBECTOMIZED RATS

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Abstract-In rodents, olfactory bulbectomy (OBX) results in several behavioral and biochemical changes, useful as a screening model for antidepressants. Recent evidences suggest that guercetin; a bioflavonoid exhibits a variety of behavioral effects including anxiolytic, antidepressant, etc. Since microglia are commonly implicated in the neuroinflammation cascade of depression, we hypothesized that quercetin might involve microglial inhibition pathway in its antidepressant-like effects. To support such a possibility, we investigated the interaction of guercetin with a known microglial inhibitor (minocycline) against OBX-induced depression in male Wistar rats. In our study, ablation of olfactory bulbs caused hyperactivity in open field arena and increased immobility time in forced swim test (FST) which was coupled with enhanced serum corticosterone levels. Additionally, there were increased oxidative-nitrosative stress markers, inflammatory mediators (tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6)) along with enhanced apoptotic factor (caspase-3) in both cerebral cortex and hippocampal brain regions of OBX animals. These results were further supported by reports from histopathological analysis. After a surgical recovery period of 2 weeks. treatment with quercetin (40, 80 mg/kg; per oral (p.o.), 14 days) significantly prevented OBX-induced behavioral, biochemical, molecular and histopathological alterations. Further, combination of sub effective doses of guercetin (20, 40 mg/kg; p.o.) with minocycline (25 mg/kg; p.o.) significantly potentiated their protective effects as compared to their effects alone. Based on our results, we propose that microglial inhibitory pathway might be involved in the neuroprotective effects of quercetin and suppression of oxidative-nitrosative stress mediated neuroinflammationapoptotic cascade associated with OBX rat model of depression. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

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Key words: bioflavonoid, depression, immobility time, microglial inhibitor, neuroinflammation, olfactory bulbectomy.

INTRODUCTION

Depression is identified as a major neuropsychiatric problem which comprises a wide group of disabilities due to medical conditions or substance abuse and results in a multifactorial condition (D'Sa and Duman, 2002). World Health Organization has predicted depression as the second major cause of disability by the year 2020 (Ustun et al., 2004). Despite the availability of several animal models of depression, none of them fully imitates the manifestations of depression observed in clinical situation. Among all, olfactory bulbectomy (OBX) experimental model has been recognized widely for screening antidepressants (Song and Leonard, 2005).

Ablation of olfactory bulb induces behavioral alterations which are not only limited to the sensory deficit but also includes hyperactivity, alterations in exploration and social behavior (Mucignat-Caretta et al., 2004). Different studies on bulbectomized experimental model suggest that there is good face validity with human depressive disorder, especially the agitated depression (Kelly et al., 1997; Song and Leonard, 2005). The olfactory bulb extends to different brain regions such as cortex, amygdala and the hippocampus; consequently removal of them causes neurological deficits in these projections and perhaps results in numerous neurobiological. biochemical and behavioral alterations (Song and Leonard, 2005). Moreover, OBX has also been suggested to increase pro-inflammatory cytokines level such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) in several brain regions (Myint et al., 2007) and promotes pathological damage by accompanying inflammatory reactions (Song et al., 2009). Further, OBX alters hippocampal neurogenesis, an important pathogenic mechanisms involved in the pathogenesis of depression (Jaako-Movits et al., 2006). Since OBXinduced depression-like behavior responds to chronic and not acute antidepressant treatment, thus OBX is considered as one of most reliable models to evaluate antidepressants (Song and Leonard, 2005).

The pharmacotherapy for OBX-induced depression comprises several therapeutic agents that can block the progression of neuroinflammatory response with their

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Abbreviations: ANOVA, analysis of variance; CORT, corticosterone; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; FST, forced swim test; GSH, glutathione; H_2O_2 , hydrogen peroxide; HPA, hypothalamic-pituitaryadrenocortical; IL-6, interleukin-6; LPO, lipid peroxidation; MDA, malondialdehyde; OBX, olfactory bulbectomy; PBS, phosphatebuffered solution; pNA, p-nitroaniline; p.o., per oral; SOD, superoxide dismutase; TNF- α , tumor necrosis factor- α .

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neuroprotectant properties. However, the current management of OBX-induced depression is mainly supportive and symptomatic. Since oxidative stress and neuroinflammatory pathways are one of the major contributors in the pathogenesis of OBX, therefore search for new antioxidant and anti-inflammatory agent is urgently being investigated. Quercetin (3,5,7,3',4'pentahydroxyflavone), a naturally occurring dietary flavonol is frequently found in a variety of fruits and vegetables such as onions, berries and apples (Harwood et al., 2007). Quercetin shows strong anti-inflammatory, antiallergic and cardiovascular protective properties due to its potent antioxidant effects (Molina et al., 2003: Kumar et al., 2008). Quercetin has also been known to attenuate stress-induced depression-like behavior through reduction in hypothalamic-pituitaryadrenocortical (HPA) axis activation (Kawabata et al., 2010). Further, studies have acknowledged a potent neuroprotective effect of quercetin against ischemia and reperfusion-induced cerebral injury (Cho et al., 2006). Earlier, guercetin has also been reported to attenuate high glucose-induced expression of pro-inflammatory cytokines (Wu et al., 2009). On the other hand, minocycline, a tetracycline derivative is known to suppress activation of human and animal microglia both in vitro and in vivo (Familian et al., 2006; Seabrook et al., 2006). Minocycline is known to cause neuroprotection mainly due to its anti-inflammatory and anti-apoptotic properties (Homsi et al., 2009). However, long-term preventive effect of both guercetin and minocycline combination with their exact molecular pathways in OBXinduced depression symptoms is not yet evaluated.

Based on these evidences, we investigated the possible involvement of quercetin and its interaction with a microglial inhibitor (minocycline) against behavioral, biochemical, molecular and histopathological alterations associated with rat model of OBX.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (250-300 g) were procured from Animal House of Panacea Biotec Ltd, Lalru (Panjab, India). Animals were housed under standard (25 ± 2 °C, 60-70% humidity) laboratory conditions, maintained on a 12-h natural day-night cycle, with free access to food and water. Animals were acclimatized to laboratory conditions before the experimental tests. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the Panjab University (IAEC/282/UIPS/39 dated 30/8/12) and conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of Government of India on the use and care of experimental animals.

Bilateral OBX surgery

After the accommodation period, animals underwent either OBX or sham surgery. Animals were anaesthetized with ketamine (75 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) combination prior to surgery. Bilateral olfactory bulb ablation was performed as described by different investigators (Van Reizen and Leonard, 1990). The animals were fixed in a stereotactic frame (Stoelting Co., Wood Dale, IL, USA) and 1-cm rostral-caudal midline incision was made in the skin of the head, and two small burr holes (2 mm in diameter) were drilled into the skull 6 mm rostral of breama and 1 mm lateral of the midline. Both olfactory bulbs were removed by suction and hemostatic sponge (AbGel, Absorbable gelatin sponae USP. Srikrishna Laboratories, Andhra Pradesh, Hyderabad, India) was inserted into the cavity to control bleeding. The incision was then closed with absorbable sutures (Ethicon 4-0. Absorbable surgical sutures USP (Catgut), Johnson and Johnson, Mumbai, Maharashtra, India) and animals were injected with sulprim injection^R (each ml containing 200 and 40 mg of sulphadiazine and trimethoprim respectively), intramuscularly (0.2 ml/300 g) once a day for 3 days to prevent post surgical infection. Sham animals were given similar treatment as OBX animals except the removal of the olfactory bulbs. The success and validation of the OBX surgery was verified by using two methods: (a) dissection and direct observation of remaining olfactory bulb tissue and by (b) measurements of key behavioral variables altered by OBX, namely hyperactivity behavior during open field test. The OBX/Sham animals were housed singly in cages for 2 weeks (14 days) of surgical rehabilitation period and drug treatments were started after that. Pictogram of the entire protocol is represented in Fig. 1.

Drugs and reagents

Quercetin was purchased from Sigma (St. Louis, MO, USA) and minocycline hydrochloride from Wyeth Ltd., Mumbai, India. TNF- α , interleukin-6 (IL-6) and caspase-3 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, USA. All other chemicals used for biochemical and molecular estimations were of analytical grade.

Drug treatment schedule

The animals were randomly divided into ten experimental groups with 12 animals in each. Group I, II were designated as naïve and sham, received vehicle; Group III was considered as control (OBX); Group IV, V and VI received quercetin (20, 40 and 80 mg/kg; p.o.) respectively; Group VII and VIII received minocycline (25 and 50 mg/kg; p.o.). Further, Group IX and X received combination of quercetin (20 and 40 mg/kg; p.o.) with minocycline (25 mg/kg; p.o.) respectively. Quercetin and minocycline were prepared in peanut oil and administered orally on the basis of body weight (5 ml/100 g). Drug solutions were made freshly at the beginning of each day of the study protocol. Drugs were administered once daily for a period of 2 weeks starting from day 15 (after 2 weeks of surgical recovery period).

Behavioral tests

Open field exploration. Open field test was employed to measure the symptoms of agitated depression and hyperactivity associated with OBX. Open field behavior (started 20 h after the last drug treatment) was recorded in a circular arena of diameter 80 cm, surrounded by a 30-cm high wooden wall (Raghavendra et al., 1999). The arena painted white, was divided into 25 small sections. Each rat was carefully placed in the center of circular arena and allowed to explore the open field for 5 min. During this period, the ambulatory activity, in terms of the number of sections crossed, and the frequency of rearing was recorded along with defecation and licking episodes and values expressed as counts per 5 min.

Immobility period. Forced swim test was utilized to estimate the amount of immobility period associated with depression behavior. Forced swim test was performed as described (Porsolt et al., 1977). One day prior to the test, rats were placed for conditioning in a clear plastic tank ($45 \text{ cm} \times 35 \text{ cm} \times 60 \text{ cm}$) containing 30 cm of water ($24 \pm 0.5 \,^{\circ}$ C) for 15 min (pre-test session). Twenty-four hours later (test session); the total immobility period within a 5-min session was recorded as immobility scores (in s). A rat was judged to be immobile when its hind legs were no longer moving and the rat was hunched forward (a floating position). The immobility time was recorded manually by an observer who was blind to the drug treatment.

Dissection and homogenization

Immediately after the last behavioral test, animals were sacrificed by cervical dislocation and brain samples were rapidly removed and placed on dry ice for cerebral cortex and hippocampus dissection. 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000*g* for 15 min. Aliquots of supernatants were separated and used for biochemical and cellular estimations.

Estimation of oxidative-nitrosative stress markers

Lipid peroxidation (LPO). The extent of lipid peroxidation was determined quantitatively by performing the method as described by Wills (1966). The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The values were calculated using the molar extinction co-efficient of chromophore ($1.56 \times 10 \text{ M}^{-1} \text{ cm}^{-1}$).

Nitrite. The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay with Greiss reagent (0.1% *N*-(1-napththyl) ethylene diamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid) (Green et al., 1982). Equal volumes of the supernatant and the Greiss

reagent were mixed and the mixture was incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve.

Reduced glutathione (GSH). Reduced GSH in the brain parts was estimated according to the method of Ellman et al. (1961). Homogenate (1 ml) was precipitated with 1.0 ml of 4% sulfosalicylic acid and the samples were immediately centrifuged at 1200*g* for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer of pH 8.0 and 0.2 ml of 0.01 M dithiobisnitrobenzoic acid (DTNB). The yellow color developed was read immediately at 412 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of reduced GSH per milligram of protein.

Superoxide dismutase. Superoxide dismutase (SOD) activity was assayed by the method of Kono (1978) where in the reduction of nitro blue tetrazolium (NBT) was inhibited by the superoxide dismutase and is measured. The assay system consists of EDTA 0.1 mM, sodium carbonate 50 and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of the above mixture, 0.05 ml of hydroxylamine and 0.05 ml of the supernatant was added and auto-oxidation of hydroxylamine was measured for 2 min at 30-s intervals by measuring absorbance at 560 nm using Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, USA).

Catalase. Catalase activity was determined by the method of Luck (1971), wherein the breakdown of hydrogen peroxide (H_2O_2) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 , phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm using Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of H_2O_2 decomposed per milligram of protein/min.

Protein. The protein content was estimated by the biuret method (Gornall et al., 1949) using bovine serum albumin as standard.

Serum corticosterone (CORT) estimations

Preparation of serum. Blood was collected (1.0 ml) between 8.00–9.00 AM through retro orbital bleeding in the test tube and allowed to clot at room temperature. The tubes were then centrifuged at 2000 rpm for 10 min. The straw colored serum was separated and stored frozen at -20 °C.

CORT assessment. For extraction of CORT the method of Silber et al. (1958) was modified as described. 0.1-0.2 ml of serum was treated with 0.2 ml of freshly prepared chloroform:methanol mixture (2:1, v/

v), followed by 3 ml of chloroform instead of dichloromethane used in the procedure of Silber and its group. The step of treatment of petroleum ether was omitted. The samples were vortexed for 30 s and centrifuged at 2000 rpm for 10 min. The chloroform layer was carefully removed with the help of a syringe with a long 16 gauge needle attached to it and was transferred to a fresh tube. The chloroform extract was then treated with 0.1 N NaOH by vortexing rapidly and the NaOH layer was rapidly removed. The sample was then treated with 3 ml of 30 N H₂SO₄ by vortexing vigorously. After phase separation, chloroform layer on top was removed using a syringe as described above and discarded. The tubes containing H₂SO₄ were kept in the dark for 30-60 min and thereafter fluorescence measurements carried out in fluorescence spectrophotometer (make Hitachi, model F-2500) with excitation and emission wavelength set at 472 and 523.2 nm respectively. The standard curve depicting the fluorescence yield versus CORT concentration was used for result analysis.

Molecular estimations

Estimation of pro-inflammatory cytokines. The quantifications of TNF- α and IL-6 were done by rat TNF- α and IL-6 immunoassay kit (R&D Systems, Minneapolis, MN, USA). The Quantikine rat TNF- α and IL-6 immunoassay is a 4.5-h solid phase ELISA designed to measure rat TNF- α and IL-6 levels. The assay employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-a or IL-6 has been pre-coated in the microplate. Standards control and samples are pipetted into the wells and any rat TNF- α or IL-6 present is bound by the immobilized antibody. After washing away any unbound substance, an enzyme-linked polyclonal antibody specific for rat TNF- α or IL-6 is added to the wells. Following a wash to remove any unbound antibodyenzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of rat TNF- α or IL-6 bound in the initial steps. The sample values are then read off the standard curve.

Estimation of apoptotic factor (caspase-3). Caspase-3, also known as CPP-32 is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspasespecific peptide that is conjugated to the color reporter molecule p-nitroaniline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate/homogenate is directly proportional to the color reaction. The enzymatic reaction for caspase activity was carried out using R&D Systems caspase-3 colorimetric kit.

Histopathology of brain tissues

Tissue sections preparation. Remaining animals were deeply anaesthetized and perfused transcardially via the ascending aorta with cold phosphate-buffered saline (0.1 M, pH 7.4) followed by fixative solution containing 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered solution (PBS) solution (pH 7.4). The cerebral cortex and hippocampal regions of the brain were dissected out and fixed overnight at 4 °C in the same buffer containing 4% (w/v) paraformaldehyde. The brain parts were then washed with 0.1 M PBS (pH 7.4) for 1 h, dehydrated in alcohol, and then embedded in paraffin wax. Serial coronal sections (5- μ m thickness) of brain parts were then obtained.

Hematoxylin and eosin (H&E) staining. The paraffin sections of brain parts (thickness 5 μ m) were dewaxed and rehydrated with alcohol for hematoxylin–eosin (H&E) staining. The neurons in hippocampus and cortex were examined under electron microscopy and photomicrographs were prepared.

Statistical analysis

Data are expressed as mean \pm SEM. The data was analyzed by One-way analysis of variance (ANOVA) followed by the Tukey's test. p < 0.05 was considered as statistically significant. All statistical procedures were carried out using sigma stat Graph Pad Prism (Graph Pad Software, version 5, San Diego, CA, USA).

RESULTS

Behavioral observations

Effects of quercetin, minocycline and their combination on open field performance task. OBX animals exhibited a characteristic hyperactivity in open field arena as depicted by increased ambulation, rearing, defecation (number of fecal pellets) and reduced grooming/licking episodes which were significant as compared to sham animals. Sham treatment did not show any significant effect as compared to naïve animals. Quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment significantly improved ambulation, rearing, defecation and grooming/ licking episodes as compared to OBX group. However, lower doses of guercetin (20 mg/kg) and minocycline (25 mg/kg) did not show any significant improvement in the open field task. Further, co-administration of sub effective doses of quercetin (20, 40 mg/kg) with sub effective dose of minocycline (25 mg/kg) significantly potentiated their open field performance as seen by improvement ambulation [F(10, 119) = 124.82]in (p < 0.01)], rearing [F(10, 119) = 134.22 (p < 0.05)], aroomina [F(10, 119) = 190.45](p < 0.05)] and defecation [F(10, 119) = 203.44 (p < 0.05)] parameters as compared to their effects alone in OBX-treated animals (Table 1).

Treatment (mg/kg)	Open field behavior (counts per 5 min)				
	Ambulation	Rearing	Grooming/licking episodes	Number of fecal pellets	
Naive	63.1 ± 5.12	12.2 ± 2.58	11.8 ± 0.77	1.1 ± 0.27	
Sham	64.5 ± 4.45	12.5 ± 2.42	11.2 ± 0.46	1.4 ± 0.32	
OBX	187.4 ± 5.21^{a}	43.5 ± 3.56^{a}	2.3 ± 0.28^{a}	8.5 ± 0.25^{a}	
OBX + Q(20)	172.4 ± 9.34	41.4 ± 3.45	3.0 ± 0.38	8.2 ± 0.68	
OBX + Q(40)	$132.7 \pm 8.35^{b,c}$	33.1 ± 4.56^{b}	$6.1 \pm 0.86^{b,c}$	$6.1 \pm 0.20^{b,c}$	
OBX + Q(80)	$95.6 \pm 5.32^{c,d}$	$21.2 \pm 2.45^{c,d}$	$9.4 \pm 0.34^{c,d}$	$3.2 \pm 0.65^{c,d}$	
OBX + M(25)	169.6 ± 7.50	39.1 ± 2.45	3.2 ± 0.68	7.3 ± 0.36	
OBX + M(50)	$114.6 \pm 9.32^{b,e}$	$23.3 \pm 4.12^{b,e}$	$7.4 \pm 1.12^{b,e}$	$5.2 \pm 0.56^{b,e}$	
OBX + Q(20) + M(25)	122.6 ± 5.87 ^{c,e}	$25.4 \pm 3.12^{c,e}$	$6.6 \pm 0.54^{c,e}$	6.6 ± 0.42^{c}	
OBX + Q(40) + M(25)	$90.3 \pm 8.43^{d,e}$	$21.2 \pm 1.23^{d,e}$	$9.2 \pm 0.32^{d,e}$	$3.2 \pm 0.18^{d,e}$	

Table 1. Effect of quercetin and its interaction with minocycline on open field behavior

Values are expressed as mean \pm SEM (n = 12). For statistical significance, ^ap < 0.05 as compared to Sham group; ^bp < 0.05 as compared to OBX; ^cp < 0.05 as compared to OBX + Q(20); ^dp < 0.05 as compared to OBX + Q(40); ^ep < 0.05 as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.



Fig. 1. Diagrammatic representation of the study protocol.



Fig. 2. Effect of quercetin and its interaction with minocycline on immobility period. Values are expressed as mean \pm SEM (n = 12). For statistical significance, ^ap < 0.05 as compared to Sham group; ^bp < 0.05 as compared to OBX; ^cp < 0.05 as compared to OBX + Q(20); ^dp < 0.05 as compared to OBX + Q(40); ^ep < 0.05 as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.

Effects of guercetin, minocycline and their combination on immobility period. There was a significantly increase in the immobility time of OBX rats as compared to the sham group. Sham treatment did not show any significant effect as compared to naïve animals. quercetin (40, 80 mg/kg) Treatment with and minocycline (50 mg/kg) significantly shortened the immobility time as compared to OBX group. The combination of sub effective doses of guercetin (20, 40 mg/kg) and minocycline (25 mg/kg) significantly potentiated their protective effects (shortened immobility period) as compared to their effects per se in OBX-treated animals. F(10, 119) = 73.32 (p < 0.01)] (Fig. 2).

Biochemical observations

Effects of quercetin, minocycline and their combination on oxidative-nitrosative stress markers. Ablation of olfactory bulbs caused significant oxidative-nitrosative damage as evidenced by rise in lipid peroxidation (MDA) and nitrite concentration, depletion of reduced GSH, SOD and catalase levels in both cerebral cortex and hippocampus as compared to the sham group. Sham treatment did not show any significant effect as compared to naïve animals. Quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment significantly attenuated the oxidative-nitrosative stress markers (reduced MDA, nitrite levels, restored reduced GSH, SOD and catalase levels) as compared to OBX control. However, lower dose of guercetin (20 mg/kg) and minocycline (25 mg/kg) showed no significant improvement. Further, combination of sub effective doses of quercetin (20, 40 mg/kg) and minocycline (25 mg/kg) potentiated their anti-oxidant effect and showed significant effect on LPO [F(10.59) = 63.21](p < 0.01)], GSH [F(10, 59) = 102.34 (p < 0.05)], Nitrite [F(10,59) = 83.23 (p < 0.05)], SOD [F(10,59) = 123.40](p < 0.05)] and Catalase [F(10, 59) = 64.34 (p < 0.05)]activity as compared to their effects alone in OBXtreated rats (Table 2).

Table 2. Effect of quercetin and its interaction with minocycline on oxidative-nitrosative stress markers

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Treatment (mg/kg)	LPO (mol of MDA/ mg pr)	GSH (µmol of GSH/ mg pr)	Nitrite (µg/ml)	Catalase (µmol of H ₂ O ₂ hydrolyzed/ min/mg pr)	SOD (U/ mg pr)
Naïve					
Cerebral cortex	0.169 ± 0.03	0.085 ± 0.003	344.5 ± 10.2	0.88 ± 0.06	1.79 ± 0.45
Hippocampus	0.109 ± 0.06	0.061 ± 0.002	257.2 ± 12.4	0.59 ± 0.04	1.36 ± 0.29
Sham					
Cerebral cortex	0.178 ± 0.05	0.083 ± 0.004	3535 ± 115	0.85 ± 0.04	1.74 ± 0.36
Hippocampus	0.112 ± 0.08	0.059 ± 0.006	263.5 ± 9.3	0.58 ± 0.02	1.26 ± 0.54
Cerebral cortex	0.612 ± 0.00^{a}	0.021 ± 0.005^{a}	$7/25 \pm 162^{a}$	0.16 ± 0.01^{a}	0.48 ± 0.01^{a}
Hippocampus	0.012 ± 0.000 0.410 + 0.06 ^a	0.021 ± 0.003^{a}	604.3 ± 14.6^{a}	$0.10 \pm 0.01^{\circ}$ 0.11 + 0.03°	0.40 ± 0.01 0.29 + 0.03 ^a
	0.110 ± 0.00	0.010 ± 0.000	001.0 ± 11.0	0.11 ± 0.00	0.20 ± 0.00
OBX + Q(20)	0.504 + 0.05	0.000 + 0.004	704.0 + 40.5		0.54 + 0.00
	0.594 ± 0.05	0.033 ± 0.004	724.0 ± 13.5	0.23 ± 0.05	0.54 ± 0.03
hippocarripus	0.391 ± 0.00	0.010 ± 0.000	501.0 ± 12.0	0.18 ± 0.07	0.34 ± 0.05
OBX + Q(40)					
Cerebral cortex	$0.421 \pm 0.06^{b,c}$	$0.043 \pm 0.006^{\circ}$	$583.1 \pm 13.4^{b,c}$	$0.45 \pm 0.06^{\text{b,c}}$	$0.87 \pm 0.05^{b,c}$
Hippocampus	$0.252 \pm 0.05^{0.0}$	$0.037 \pm 0.004^{\text{b,c}}$	$482.5 \pm 10.4^{0,0}$	$0.34 \pm 0.04^{0,c}$	$0.66 \pm 0.08^{0.00}$
OBX + Q(80)					
Cerebral cortex	$0.282 \pm 0.08^{c,d}$	$0.074 \pm 0.005^{c,d}$	$424.5 \pm 12.4^{c,d}$	$0.72 \pm 0.02^{c,d}$	$1.05 \pm 0.08^{b,c}$
Hippocampus	$0.153 \pm 0.06^{c,d}$	$0.054 \pm 0.004^{c,d}$	$340.0 \pm 8.3^{c,d}$	$0.49 \pm 0.01^{c,d}$	$0.95 \pm 0.06^{c,d}$
OBX + M(25)					
Cerebral cortex	0.573 ± 0.06	0.028 ± 0.005	764.4 ± 12.1	0.25 ± 0.03	0.59 ± 0.04
Hippocampus	0.382 ± 0.06	0.018 ± 0.004	523.4 ± 11.6	0.22 ± 0.04	0.38 ± 0.05
OBX + M(50)					
Cerebral cortex	$0.341 \pm 0.03^{b,e}$	$0.055 \pm 0.006^{b,e}$	$502.3 \pm 14.5^{b,e}$	$0.59 \pm 0.05^{b,e}$	0.87 ± 0.04^{b}
Hippocampus	$0.183 \pm 0.09^{b,e}$	$0.047 \pm 0.003^{b,e}$	399.5 ± 11.6 ^{b,e}	0.38 ± 0.02^{b}	$0.83 \pm 0.05^{b,e}$
OPX + O(20) + M(25)					
OBA + Q(20) + W(20)	0 403 ± 0 05 ^{c,e}	0.048 ± 0.004 ^{c,e}	5/3 / ⊥ 13 / ^{c,e}	$0.48 \pm 0.03^{c,e}$	0 02 ± 0 07 ^{c,e}
Hippocampus	0.403 ± 0.03	0.040 ± 0.004 0.039 ± 0.003 ^{c,e}	343.4 ± 13.4 $161.3 \pm 11.1^{c,e}$	0.46 ± 0.05	0.32 ± 0.07 0.71 + 0.08 ^{c,e}
inppotattipus	0.240 ± 0.04	0.000 ± 0.000		0.00 ± 0.07	0.71 ± 0.00
OBX + Q(40) + M(25)			440.4 × 40.0de	0.74 - 0.04de	1 00 1 0 00 ^d 0
Cerebral cortex	$0.203 \pm 0.04^{\text{d},\text{e}}$	$0.078 \pm 0.006^{\circ,e}$	$413.4 \pm 10.6^{u,e}$	$0.74 \pm 0.04^{0.6}$	$1.09 \pm 0.08^{d,e}$
nippocampus	0.142 ± 0.05^{-10}	0.059 ± 0.003^{-30}	$332.3 \pm 14.1^{4.0}$	0.51 ± 0.04^{-7}	$0.98 \pm 0.03^{0,0}$

Values are expressed as mean \pm SEM (n = 6). For statistical significance, ${}^{a}p < 0.05$ as compared to Sham group; ${}^{b}p < 0.05$ as compared to OBX; ${}^{c}p < 0.05$ as compared to OBX + Q(20); ${}^{d}p < 0.05$ as compared to OBX + Q(40); ${}^{e}p < 0.05$ as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.

Effects of quercetin, minocycline and their combination on serum CORT levels. OBX caused a significant rise in serum CORT level as compared to the sham group. Sham treatment did not show any significant effect as compared to naïve animals. Two weeks treatment with quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) significantly attenuated the serum CORT levels as compared to OBX rats. In-addition, lower doses of quercetin (20 mg/kg) and minocycline (25 mg/kg) showed no significant improvement in serum CORT levels. Further, combination of sub effective doses of quercetin (20, 40 mg/kg) and minocycline (25 mg/kg) showed a significant amelioration in serum CORT levels as compared to their effects alone in OBX-treated animals. [F(10, 119) = 114.34 (p < 0.01)] (Fig. 3).

Molecular observations

Effects of quercetin, minocycline and their combination on the activity of pro-inflammatory cytokines – Brain TNF- α and IL-6. OBX caused a significant increase in the TNF- α and IL-6 activity in both cerebral cortex and hippocampus as compared to the sham group. Sham treatment did not show any significant effect as compared to naïve animals. Quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) treatment significantly attenuated TNF- α and IL-6 levels as compared to OBX. However, lower dose of guercetin (20 mg/kg) and minocycline (25 mg/kg) did not show any significant improvement as compared to the control. Further, combination of subeffective doses of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) potentiated their protective effects on TNF- α [*F*(10, 59) = 184.82 (p < 0.05)] (Fig. 4) and IL-6 [F(10, 59) = 124.60](p < 0.05)] (Fig. 5) activity as compared to their effects alone in OBX-treated rats.

Effects of quercetin, minocycline and their combination on the apoptotic factor activity (caspase-3). There was a significant increase in the activity of caspase-3 in both



Fig. 3. Effect of quercetin and its interaction with minocycline on serum corticosterone levels. Values are expressed as mean \pm SEM (n = 12). For statistical significance, ^ap < 0.05 as compared to Sham group; ^bp < 0.05 as compared to OBX; ^cp < 0.05 as compared to OBX + Q(20); ^dp < 0.05 as compared to OBX + Q(40); ^ep < 0.05 as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.



Fig. 4. Effect of quercetin and its interaction with minocycline on TNF- α levels. Values are expressed as mean \pm SEM (n = 6). For statistical significance, ^ap < 0.05 as compared to Sham group; ^bp < 0.05 as compared to OBX; ^cp < 0.05 as compared to OBX + Q(20); ^dp < 0.05 as compared to OBX + Q(40); ^ep < 0.05 as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.

cerebral cortex and hippocampus of OBX animals as compared to the sham group (p < 0.01). Sham treatment did not show any significant effect as compared to naïve animals. Chronic treatment with quercetin (40, 80 mg/kg) and minocycline (50 mg/kg) significantly attenuated the increased activity of caspase-3 as compared to the control. However, lower dose of quercetin (20 mg/kg) and minocycline (25 mg/kg) did not show any significant improvement on increased apoptotic factor activity as compared to OBX. Further, combination of subeffective doses of quercetin (20, 40 mg/kg) with minocycline (25 mg/kg) potentiated their protective effects as compared to their effects alone in OBX-treated animals [F(10, 59) = 97.40 (p < 0.01)] (Fig. 6).

Histopathological observations

Effects of quercetin, minocycline and their combination on histopathological alterations in cerebral cortex and hippocampal brain region. Brain sections of both sham and naïve groups showed normal histopathological structures of neurons in cerebral cortex and hippocampal regions. Olfactory bulb ablation caused a significant increase in the number of microglial (neuroinflammatory) and apoptotic cells which further resulted in reduced cell density in the cerebral cortex and hippocampal region as compared to the sham group. Treatment with guercetin (40, 80 mg/kg) could significantly attenuate the increased levels of microglial cells resulting in cell death (apoptosis) as compared to OBX control. However, treatment with lower dose of quercetin (20 mg/kg) and minocycline (25 mg/kg) did not attenuate these histological abnormalities and was similar to OBX control (not shown). Further, combination of subeffective dose of minocycline (25 mg/kg) with quercetin (20, 40 mg/kg) potentiated their attenuation effects on neuroinflammatory cells as compared to quercetin (20, 40 mg/kg) alone in OBX-treated rats (Fig. 7).



Fig. 5. Effect of quercetin and its interaction with minocycline on IL-6 activity. Values are expressed as mean \pm SEM (n = 6). For statistical significance, ^ap < 0.05 as compared to Sham group; ^bp < 0.05 as compared to OBX; ^cp < 0.05 as compared to OBX + Q(20); ^dp < 0.05 as compared to OBX + Q(40); ^ep < 0.05 as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.



Fig. 6. Effect of quercetin and its interaction with minocycline on caspase-3 activity. Values are expressed as mean \pm SEM (n = 6). For statistical significance, ^ap < 0.05 as compared to Sham group; ^bp < 0.05 as compared to OBX; ^cp < 0.05 as compared to OBX + Q(20); ^dp < 0.05 as compared to OBX + Q(40); ^ep < 0.05 as compared to OBX + M(25) (One-way ANOVA followed by the Tukey's test). OBX, olfactory bulbectomy; Q, quercetin; M, minocycline.



Fig. 7. Representative photomicrographs of cerebral cortex (A) and hippocampal CA1 region (B) of rat brain sections. Sections (n = 6) were stained with Hematoxylin and Eosin. Black arrows indicate neuroinflammation along with apoptotic cells. (1) Sham control: neurons are preserved. (2) OBX: large number of inflammatory and apoptotic cells. (3) Quercetin (20 mg/kg): mild to moderate inflammation of neurons with apoptotic cells. (4) Quercetin (40 mg/kg): mild inflammation of neurons with less apoptotic cells. (5) Quercetin (20 mg/kg) + minocycline (25 mg/kg): less inflammation of neurons. (6) Quercetin (40 mg/kg) + minocycline (25 mg/kg): neurons are intact (Scale bar = 50 μ m).



Fig. 7 (continued)

DISCUSSION

The results of the present study are consistent with the notion of an additive interaction between quercetin; a dietary flavonol and minocycline; a known microglial inhibitor involved in suppression of neuroinflammation (microglial activation) pathway and providing antidepressant effects associated with rat model of OBX.

The bilateral destruction of the olfactory bulbs creates an altered state of brain with complex changes in behavioral and neurobiological correlates, many of which resembles human depression (Song and Leonard, 2005). In the present study, OBX rats exhibited an increased locomotor activity in a novel 'open field' environment, forecasting symptoms associated with model of agitated depression. The open field scores were counted by two experimenters (blind to treatment groups) in the presence of an expert veterinarian. The 'open field' exposure is associated with increased stress and/or anxiety, which is a crucial determinant of OBXrelated hyperactivity (Kelly et al., 1997). Increased locomotor/exploratory behavior is an important trait of the OBX syndrome (Song and Leonard, 2005), examined when the animal is exposed to a new location. OBX model is recommended to possess good face validity which mimics with human depression (Kelly et al., 1997). However, others argue that the restoration of the behavioral abnormality in OBX rats following chronic antidepressant treatment is more likely to be a predictive screening for antidepressants rather than to study depression pathophysiology. Hyperactivity associated with OBX model differs from other stressbased models of depression in modeling agitated rather

than retarded behavioral activity (Willner, 1990). Therefore, OBX has also been suggested to model a subgroup of depressed patients with psychomotor agitation (Willner, 1990). Another behavioral outcome of this surgery consists of a reproducible increase in immobility time in FST, which is yet another important hallmark of depression-like state (Song and Leonard, 2005). The OBX-induced open field hyperactivity and increased immobility period were normalized on chronic treatment with guercetin, minocycline and their combination. Further, there were no reports of motor in-coordination in high dose guercetin/minocyclinetreated rats and animals could explore easily in the field. Our data relating to beneficial effects of quercetin in both these behavioral tests (Open field and FST) coincide with those presented in previous reports (Bhutada et al., 2010; Selvakumar et al., 2013). Minocycline, a broad-spectrum antibiotic is known to exhibit long-term neuroprotective effects via suppression of direct activation of microglial cells and inhibition of successive pro-inflammatory mediators (Kitayama et al., 2011). Further, recent reports from O'Connor et al. (2009) revealed that minocycline produced antidepressant-like effects in an animal model of lipopolysaccharide-induced inflammation. Taken together, we propose that in the present study quercetin may have involved analogous pathway as the microglial inhibitor, minocycline and both have additive effect in attenuating depression-like behavior associated with OBX.

Regulation of HPA axis gets altered in a number of clinical conditions including depression (Pariante and Lightman, 2008). Ablation of olfactory bulbs in rodents has been associated with dysregulation of HPA axis and increase in serum CORT levels (Cairncross et al.,

1977). In the current study, we found a significant rise in serum CORT levels in OBX rats indicating hyperactivity of the HPA axis which is further supported by a recent report (Machado et al., 2012). Treatment with quercetin, minocycline and their combination restored the increased levels of serum CORT in OBX rats. These results are in agreement with reports from Bhutada et al. (2010), which suggest a reciprocating role of lowering CORT auercetin in levels against corticotrophin-releasing factor (CRF)-induced anxiogenic and depressant-like effects. CORT treatment is known enhance neuroinflammatory and neurotoxic to responses via activation of microglia (Kelly et al., 2012). Therefore, guercetin along with minocycline in the present study suppressed microglial neuroinflammation cascade and exerts their beneficial effects in lowering HPA axis activity and CORT secretion.

Oxidative stress is a state which results in the production of reactive oxygen species and reduces antioxidants level in a particular cell. Oxidative stress has been implicated in the pathogenesis of several disorders and may constitute a common pathogenic mechanism for several psychiatric disorders (Gladkevich et al., 2007). OBX results in the depletion of the antioxidant system, as evidenced by lower antioxidant enzyme activities and total antioxidant status (Tasset et al., 2010). Clinical reports on patients suffering from depression showed a reduced lipid peroxidation and antioxidant enzymes levels which returned to normal after treatment with chronic antidepressants (Bilici et al., 2001). Similarly, in the present study OBX reversed the oxidative status, characterized by an increase in lipid peroxidation products and reduction in levels of reduced GSH, catalase and superoxide dismutase in both cerebral cortex and hippocampal region. This was followed by enhanced level of brain nitrite (peroxynitrite) which results due to reaction between superoxide and nitric oxide, and leading to oxidative modification of proteins and ultimately causing neuronal death (Tasset et al., 2010). Earlier, clinical studies on depressed patients showed an increased level of plasma nitrate (Suzuki et al., 2001). In the present study, we observed that the combination of quercetin with microglial inhibitor, minocycline proved as an effective antioxidant which reversed OBX-induced oxidative damage and regulated the levels of brain nitrite. These observations are in accordance with the recent studies which report potent antioxidant effects of quercetin owing to its multiple actions viz superoxide and hydroxyl radicals scavenging activity and the ability to inhibit various oxidase's (Selvakumar et al., 2013). Earlier, quercetin enhanced the reduced GSH levels and increased the levels of SOD and catalase in ethanol-induced oxidative stress model (Molina et al., 2003). Studies from Kukongviriyapan et al. (2012) evidenced the beneficial effect of quercetin on the restoration of eNOS expression and elevated nitrite levels in a mouse model of lipopolysaccharide-induced oxidative stress. Moreover. these beneficial effects of quercetin against both oxidative and nitrosative stress are in line with the recent findings from our laboratory (Rinwa and Kumar, 2013).

On the other hand, microglial inhibitors including minocycline are known to exert their anti-oxidative potential as a result of direct scavenging of free radicals, adding to their neuroprotective effects (Kraus et al., 2005).

Besides the involvement of oxidative and nitrosative stress, neuroinflammation is also reported to influence neuronal functions in the pathophysiology of depression (Maes. 2008). Recent clinical studies describe that patients observed with depression behavior were found to have significantly higher levels of inflammatory markers (Krogh et al., 2013; Lindqvist et al., 2013). Another study confirms that neuropathic pain-related depression symptoms were accompanied by changes in the expression of neuroinflammatory genes in brain regions (Burke et al., 2013). Inflammatory cytokines might alter metabolic processes, neurotrophic factors, glutamatergic excitotoxicity and apoptosis (Hayley et al., 2005; Maes, 2008). The pro-inflammatory cytokines are produced by microglial cells, astrocytes, neutrophils and macrophages and augment both inflammation and subsequent immune responses. In our present study, we found elevated levels of both IL-6 and TNF- α in cerebral cortex and hippocampal brain regions following OBX, suggesting inflammatory reaction accompanied by neuronal damage (Song et al., 2009). Further, inflammatory cytokines are known to exacerbate cell death. impair neurogenesis and causes neurodegeneration (Patel et al., 2006). Similarly, in our current study, apart from increased neuroinflammation. we also observed a significant elevation in the levels of caspase-3, a key mediator of apoptotic cell death, suggesting a role of neuroinflammation-induced apoptotic pathway in OBX rats. Earlier, OBX was accounted to cause neuronal cell death (apoptosis) in different brain regions (Hall and Macrides, 1983). Further, studies from Nesterova et al. (2008) reported a significant degeneration in temporal cortex and hippocampal neurons after bulbectomy. We found that treatment with quercetin and minocycline attenuated IL-6, TNF- α and caspase-3 levels which attributes to their potent anti-inflammatory and anti-apoptotic properties. Previously, quercetin blocked inflammationmediated apoptotic cell death in combination with resveratrol (Bureau et al., 2008). On the other side, minocycline administration may have obstructed OBXinduced neuroinflammation and apoptotic degeneration moderately by inhibiting activated microglia and accordingly decreasing the secretion of excitotoxic agents (Borre et al., 2012). Activated microglias are known to generate cytokines, chemokines, and other neurotoxic agents (Streit et al., 2004) and play a role in the onset of the neurodegenerative processes. These results are further supported from histopathological reports which illustrate the presence of a large number of inflammatory and apoptotic cells leading to severe neurodegeneration in OBX rats. However, quercetin and minocycline combination treatment considerably improved histopathology of neurons in both cerebral cortex and hippocampal regions of OBX animals thereby again showing their neuroprotective effects. Minocycline, most likely via its inhibitory effects on microglia has confined protective effects in OBX-induced neuroinflammation.

In summary, our data suggest that both oxidativenitrosative stress and neuroinflammation (activated microglia) may have contributed to neurodegenerative process, increasing the neuronal apoptosis and reducing the neurogenesis, events that together might have contributed to the phenotype of depression in rat model of OBX. However, there is no direct evidence to support that the neuroinflammatory mediators produced the behavioral changes in OBX rat. On the other hand, combination of guercetin and minocycline showed an additive anti-inflammatory-like effect, suggesting that quercetin might have followed the similar microalial suppression pathway as minocycline. However, further studies are required to fullv elucidate the neuroprotective mechanism of quercetin/minocycline and to establish the association between their antiinflammatory and antidepressant-like effects and to prove their clinical effectiveness in patients suffering from depression associated with neuroinflammation.

CONFLICT OF INTEREST

There is no conflict of interest between any of the authors.

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