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Research report

Moderate treadmill exercise prevents oxidative stress-induced anxiety-like behavior in rats

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ABSTRACT

Recent work has suggested correlation of oxidative stress with anxiety-like behavior. There also is evidence for anxiolytic effects of physical exercise. However, a direct role of oxidative stress in anxiety is not clear and a protective role of physical exercise in oxidative stress-mediated anxiety has never been addressed. In this study, we have utilized rats to test direct involvement of oxidative stress with anxiety-like behavior and have identified oxidative stress mechanisms likely involved in anxiolytic effects of physical exercise. Intraperitoneal injections at non-toxic dose of L-buthionine-(S,R)-sulfoximine (BSO), an agent that increases oxidative stress markers, increased anxiety-like behavior of rats compared to vehicle-treated control rats. Prior 2 weeks treatment with the antioxidant, tempol attenuated BSO-induced anxiety-like behavior of rats suggesting a role of oxidative stress in this phenomenon. Moreover, moderate treadmill exercise prevented BSO-induced anxiety-like behavior of rats and also prevented BSO-mediated increase in oxidative stress markers in serum, urine and brain tissue homogenates from hippocampus, amygdala and locus coeruleus. Thus increasing oxidative stress increases anxiety-like behavior of rats. Moreover, antioxidant or treadmill exercise training both reduce oxidative stress in the rat brain regions implicated in anxiety response and prevent anxiety-like behavior of rats.

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1. Introduction

The involvement of abnormalities in traditional neurotransmitter systems including the gamma amino butyric acid (GABA) and serotonin receptor systems have long been the focus of most anxiety research [1]. A concept away from these traditional theories is the involvement of oxidative stress in anxiety [2–4]. Several recent reports support this concept. Oxidative stress induced by L-buthionine-(S,R)-sulfoximine (BSO) in hypothalamus and amygdala has been reported to occur in parallel with anxiety-like behavioral pattern in mice [4]. Consumption of high sucrose diet has been reported to increase protein oxidation in frontal cortex and induce anxiety-like behavior in rats [5]. Increased anxiety-like behavior has been found to be positively correlated with increases in reactive oxygen species in granulocytes [6]. In another study, oxidative stress in the adult rat hippocampus was reported to be anxiogenic, with decreased locomotion and exploration of the rat in an open-field test [7]. While several studies suggest a positive correlation between oxidative stress and anxiety-like behavior, Berry et al. [8] showed that deletion of gene $p66^{Shc}$, which regulates reactive oxygen species metabolism, reduced anxiety-like behavior in mice. Two human studies on social phobia must also be mentioned here that have suggested a relationship between increased antioxidant enzymes and oxidative stress [9,10].

As summarized above, while several studies suggest a correlation between oxidative stress and anxiety-like behavior, direct involvement of oxidative stress in anxiety-like behavior is unclear. Interestingly, treadmill exercise, known as a general mood elevator in humans [11,12], is reported to reduce the rise in oxidative stress in the brain [13]. Overall, a large volume of literature describes an association of physical activity and general well-being, mood and anxiety in humans [11,12]. In animals, however, data are scarce and conflicting mainly due to the use of different exercise paradigms and hence differential effects on brain and behavior [14–17]. Although benefits of exercise to improve mood and cognition have been known for a long time, this may not be true for intense exhaustive exercise. In fact, exhaustive exercise has been reported to increase reactive oxygen species, leading to oxidative damage [18–20]. While moderate exercise has been reported to cause adaptation of brain antioxidant system by increasing its resistance to oxidative stress [21,22], exhaustive exercise is reported to enhance lipid peroxidation [23,24]. Having said that, it would be fair to say that the effects of exhaustive exercise on brain oxidative stress are still conflicting [19,25].

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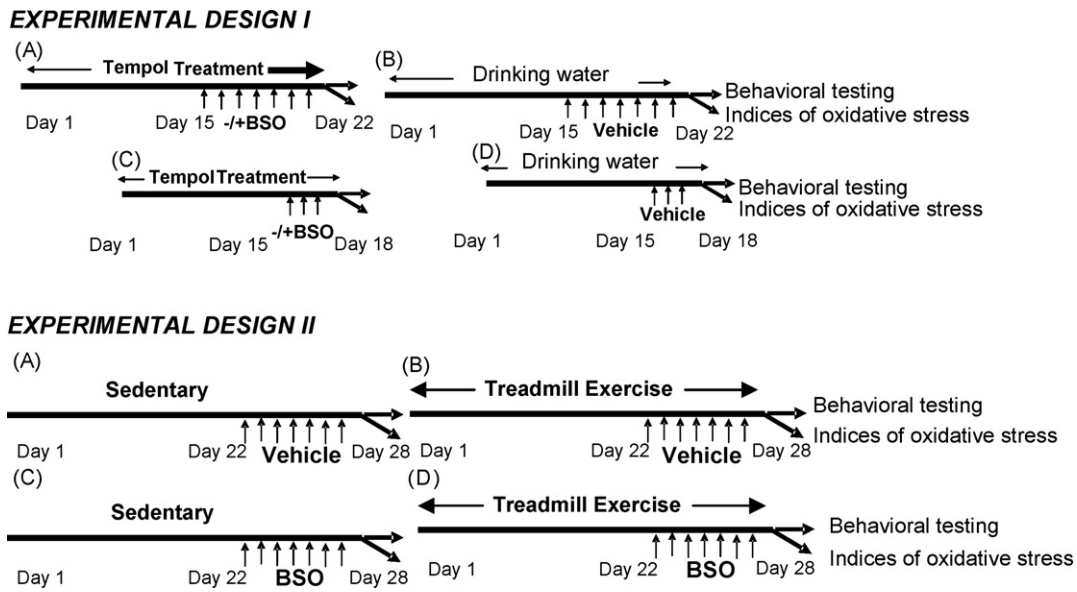

Fig. 1. Schematic representation of the experimental plan. *Experimental Design I*: Rats were provided with tempol (1 mM) in drinking water *ad libitum* for 2 weeks prior to 7 days (A) or 3 days (B) of BSO (300 mg/kg body weight) treatment in separate experiments. The control groups, tempol alone and no tempol (C and D) received vehicle injections (1% $NaHCO_3$ in saline). *Experimental Design II*: Rats in group A and C were not subjected to treadmill exercise (sedentary rats) but injected with vehicle (A) or BSO for 7 days (C). Rats in (B) and (D) were subjected to treadmill exercise for 3 weeks prior to 7 days vehicle (B) or BSO treatment (D). Exercise was continued during the BSO or vehicle treatment for a total of 4-week period. After 24 h of the last BSO/vehicle treatment, anxiety tests were conducted, brains harvested and biochemical and oxidative parameters measured.

In general, compared to the wide range of research on the positive effects of exercise in major depression, anxiety disorders have been less frequently studied. Several animal studies suggest that moderate exercise increases antioxidant enzyme activity and attenuates oxidative stress in the brain [13,26,27].

In this study, we have addressed the critical issue germane to the connection between oxidative stress and anxiety-like behaviour—can prevention of oxidative stress attenuate oxidative stress-induced anxiety-like behavior of rats? To answer this question, we have employed a rat model in which we have induced oxidative stress using BSO (an agent that produces oxidative stress) [4,28] for 3 and 7 days and assessed direct involvement of oxidative stress with anxious behavior. Using this model we tested two options to either reverse or prevent oxidative stress, *one* utilized a pharmacological intervention (antioxidant supplementation) to reverse brain oxidative stress and *second* utilized moderate treadmill exercise training to prevent oxidative stress in the brain. Our findings suggest that sub-chronic BSO treatment induces anxiety-like behavior of rats, which is reversed by antioxidant tempol supplementation and is also prevented by 4-weeks of moderate treadmill exercise in rats.

2. Materials and methods

2.1. Animal model of oxidative stress

All experiments were conducted in accordance with the IACUC guidelines using approved protocols from the University of Houston Animal Care Committee. Male Sprague–Dawley rats (200–250 g) between 7 and 8 weeks of age were acclimatized for 1 week before any treatment. L-Buthionine-(S,R)-sulfoximine (BSO) (300 mg/kg body weight i.p.) was administered to rats daily for 3 (short-term treatment) or 7 days (sub-chronic treatment) (Fig. 1: Experimental Design I). BSO is a selective inhibitor of γ-glutamyl cysteine synthase, an enzyme of glutathione biosynthetic pathway. It inhibits glutathione biosynthesis and causes depletion of cellular glutathione levels [29] Glutathione is an antioxidant molecule in its reduced form. It gets oxidized upon interaction with an oxidant and thus protects the cell from oxidant damage. The rationale for choosing this dose of BSO is based upon a published report where it caused an increase in oxidative stress in amygdala and hypothalamus and increased anxiety-like behavior in mice [4]. Rats were provided with tempol (1 mM) in drinking water *ad libitum* for 2 weeks prior to (Fig. 1: Experimental Design I A) 7 days or (Fig. 1: Experimental Design I B) 3 days of BSO treatment and were continued to be provided with tempol water through out the experiment. The control groups, tempol alone and no tempol (Fig. 1: Experimental Design I C and D) received vehicle injections (1% $NaHCO_3$ in saline). After 24 h of last BSO injection, anxiety tests were conducted, following which, rats were anesthetized with mild anesthesia (2-bromo-2-chloro-1,1,1-trifluorpethane; B4388, Sigma–Aldrich) and quickly decapitated. The abdomen was immediately opened and blood from aorta and urine from the bladder was collected. Blood serum was collected after centrifugation at 3500 rpm. Oxidative stress parameters were measured in serum, urine and brain tissue homogenates.

2.2. Moderate treadmill exercise

The rats were subjected to treadmill exercise on a motorized rodent treadmill purchased from Columbus Instruments, Columbus, OH. The apparatus consisted of a 3-lane animal exerciser utilizing single belt construction with dividing walls suspended over the tread surface. The exercising belt is made with special material that facilitates the animals' grip and is easy to clean. The overall dimensions of the treadmill are, 33 cm × 50.8 cm × 50.8 cm and each exercise lane dimensions are 43.8 cm × 12 cm × 12.7 cm. All rats were pre-trained in order for the animals to be exposed to the treadmill equipment and handling. The rats that did not run after repeated trials were excluded from the study. Overall, rats were able to run on the treadmill with minimal requirement for external stimuli or manual prodding. Rats (12/group) were randomly selected in four groups: (A) sedentary control, (B) exercise control, (C) sedentary with 7 days BSO treatment and (D) exercise plus 7 days BSO treatment (Fig. 1: Experimental Design II). Groups B and D were subjected to treadmill exercise training at zero inclination for 4 weeks. Groups A and C were not exercised. The rats were subjected to treadmill exercise protocol for a total of 4 weeks: 30 min daily for 1 week and then 60 min of exercise for 3 additional weeks at a speed of 15 m/min [30]. The rats were given a rest period of 5 min after 15 min of exercise the first week and then after 30 min in the last 3 weeks. All groups of rats had free access to standard rodent chow and water. After every run, the treadmill was cleaned with 70% ethanol solution, wiped and air dried before the next set of three rats were put on the treadmill. Groups C (sedentary, no exercise, on day 22) and D (after 3 weeks of exercise, on day 22) were injected with BSO (300 mg/kg, i.p.) once daily for 7 days. On day 22, groups A and B were injected with vehicle (1% $NaHCO_3$ in saline).

2.3. General body parameters

Body weight, food and water intake were recorded manually every day. Fifty grams of rodent chow (diet pellets) and 50 ml of tap water per rat was provided to the rats every day. The amount of food and water consumed was measured by weighing the left-over diet pellets and drinking water 24 h later. Data collected were averaged for each treatment group of rats on the last day of the experiment before behavior testing. Rectal temperature was determined in rats that had been gently restrained by wrapping in a towel using an animal rectal probe for adult rats (Physitemp, Clifton, NJ). The probe was held in place until the meter reading

Table 1
BSO or tempol treatment has no adverse effect on body weight, food or water intake and body temperature. Fifty grams of rodent chow (diet pellets) and 50 ml of tap water per rat was provided to the rats every day. The amount of food and water consumed was measured by weighing the left-over diet pellets and drinking water 24 h later. Data collected were averaged for each treatment group of rats on the last day of the experiment before behavior testing. Rectal temperature was manually recorded using animal rectal probe every day after 30 min of BSO/vehicle injections. $N = 8–12$ rats.

General parameters	Control	Tempol	BSO (3 days)	BSO + tempol (3 days)
Body weight (g)	220 ± 2.0	221 ± 2.1	224 ± 2.2	223 ± 2.1
Food intake (g)	20.1 ± 3.0	21.2 ± 3.1	21.0 ± 3.0	20.8 ± 3.3
Water intake (ml)	22 ± 2.1	21 ± 2.0	23 ± 2.5	24 ± 2.6
Body temperature (°C)	36.84 ± 0.33	36.77 ± 0.30	36.02 ± 0.11	37 ± 0.22

equilibrated which took less than 10 s. An initial temperature was taken on all rats to allow them to acclimate to having their temperature taken. This initial temperature reading was not used in the data analysis. Temperature was manually recorded every day after 30 min of BSO/vehicle injections.

2.4. Anxiety behavior tests

Animals were housed in groups of three to five in plastic cages (14 in. × 18 in. × 18 in.) and kept in a room on a 12-h light/dark schedule (lights on at 6:00 a.m.) at 23 °C, 60% humidity, with food and tap water available *ad libitum*. Upon arrival at the animal housing facility, male Sprague–Dawley rats (200–250 g; 7–8 weeks of age) were randomly selected, grouped in cages and acclimatized for 1 week before any treatment. On the day of behavior testing, the rats were brought to the behavior room at least an hour before testing time and left undisturbed in a quiet setting. After each behavior test, the apparatus was cleaned with 70% ethanol wiped with hand towels and allowed to air dry in between animal testing. At first open-field test was conducted following a rest period of 1 h, the same animals were tested in light-dark exploration test the same day.

2.4.1. Open-field activity

The open-field test was used to analyze exploratory behavior of rats 24 h after the last BSO injection. The open-field task was carried out in 60 cm × 40 cm open field surrounded by 50 cm high walled Plexiglas chambers in standard room lighting conditions [31]. The animals were placed at the center of the compartment and were left free to explore the arena for 15 min. Activity was quantitated using a computer-operated Opto-Varimex Micro Activity Meter v2.00 system (Optomax, Columbus Instruments; OH) that utilizes sensors containing eight infrared light emitting diodes and eight phototransistors that emit and detect modulated infrared light beams. Sensors were positioned to form two-dimensional cages each with rearing monitoring. Movement was detected by beam breaks and data from three test chambers was recorded simultaneously, one rat per chamber, collected in 3 min intervals over a 15-min test session. The program tabulated activity counts, zone entries, zone times, center time and the periphery time, distance travelled and rearing for every cage in the system. For center time analysis, an approximately 25 cm × 25 cm square in the center of the open-field arena was defined as the center zone for data analysis. The total time spent in the center of the arena and rearings were calculated for each group.

2.4.2. Light-dark exploration

The rats were subjected to light-dark exploration test. Rodents are nocturnal and prefer darker areas, so the decrease in the exploratory activity in a lighted area is believed to be indicative of increased anxiety-like behavior [7] and the time spent in the light is considered as a measure of anxiety-like behavior [32]. The light-dark box consisted of a light compartment (27 cm × 27 cm × 27 cm) and a dark compartment (black colored surrounding walls and floor, 27 cm × 18 cm × 27 cm) separated by a partition with an opening (7 cm × 7 cm) for passage from one compartment to the other. This apparatus was made at the Baylor College of Medicine machine shop, Houston, TX, using published dimensions of test chambers [33]. The apparatus was situated within a screen enclosed area of behavior core facility room with only one experimenter/observer present in the room at the time of experiment under standard lighting conditions of approximately 700 lx as previously used by others [31,34]. The observer scored the data using a Microsoft Excel software program that we designed ourselves that enabled the observer to record time by manually scoring the data by pressing computer keys "L" for light and "D" for dark on the keyboard. The experiment began by putting the rat gently at the center of the light compartment facing the opening to the dark chamber [32]. "L" was pressed immediately on the computer keyboard placed next to the apparatus but hidden from the rat by a screen. Pressing the "L" key automatically started the computer clock. Movement of the rat to the dark compartment was recorded by pressing "D" on the keyboard. This way, total time spent in the illuminated part was recorded by an observer blinded to treatment for 5 min. A rat was defined to have entered the lit or dark box when both front paws and shoulders were inside the respective compartment.

2.5. Brain dissections

Experimental and control rats were anesthetized using mild anesthesia immediately after anxiety behavior tests. The brains were quickly removed and rapidly frozen in isopentane on crushed dry ice and stored at −80 °C until analysis. The hind brain was sectioned into 200 μm coronal slices and samples of locus coeruleus (LC) were removed using a 0.5 μm tissue punch. Hippocampus and amygdala were grossly dissected using a brain slicer (Zivic Instruments, PA). The slices were made relative to bregma and brain regions were identified according to Paxinos and Watson [35]. Homogenates of the brain regions were prepared in buffer containing 20 mM Tris–HCl, 4 mM EDTA plus protease inhibitors, 100 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin [36]. The lysates were examined for protein concentration using Pierce's protein detection kit (Pierce, Rockford, IL) [37] and used for analysis of oxidative stress marker measurements.

2.6. Indices of oxidative stress

Serum and urine 8-isoprostane levels were measured using EIA kit (Cayman, Ann Arbor, MI). Brain glutathione levels were assayed using a colorimetric assay kit (OXIS, Foster City, CA). Malondialdehyde (MDA) was measured as published [38,39]. MDA requires higher protein than what is available to work with LC. Therefore, we conducted MDA in hippocampus and amygdala but not in LC. Small size of the LC tissue samples provided an average of ∼140 μg protein from each rat LC (total from both sides) while protein requirement for MDA assay was ∼1–2 mg/ml. For LC we have conducted nitrotyrosine assay. Protein-nitration was determined by Western blotting using HRP-conjugated nitrotyrosine antibody (Upstate Biotechnology, Charlottesville, VA) as reported by Koo and Vaziri [40]. First, brain homogenates were prepared from LC, amygdala and hippocampus, next, protein concentrations were determined by the Pierce protein detection kit (Pierce, Rockford, IL). The cell lysates were diluted with 4× Laemmli buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1 mg/ml bromophenol blue) and equal amounts of protein (25 μg per lane) were loaded on a 10% SDS-PAGE and subjected to immunoblotting with HRP-conjugated nitrotyrosine antibody at a dilution of 1:1000 in 2.5% BSA–TBS Tween solution. The blot was incubated for 1 h at room temperature while rocking, washed three times with 1% TBST solution for 30 min. The immunoreactive bands were developed using chemiluminescence reagent made by adding p-coumarric acid and luminol in 1 M Tris–HCl and hydrogen peroxide solution on an Alpha-Innotech imaging system and densitometric analysis was performed using Fluorochem FC8800 software. The blots were stripped and then probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a loading control and detected by probing with mouse anti-GAPDH monoclonal antibody (Millipore, MA; 1:2000 dilution). The bands corresponding to nitrotyrosine were normalized to those of GAPDH and the data was represented as a densitometric ratio of the two proteins.

2.7. Data analysis

Data are expressed as mean ± SEM. Significance was determined by one way ANOVA applying Tukey's post hoc test (GraphPad Software, Inc. San Diego, CA). A value of $P < 0.05$ was considered significant.

3. Results

Involvement of oxidative stress in anxiety-like behavior was established by directly scavenging radicals with antioxidant, tempol treatment prior to BSO treatment and the protective effect of moderate treadmill exercise on oxidative stress-mediated anxiety-like behavior was measured.

3.1. Effect of short-term BSO treatment (3 days) on general body parameters, indices of oxidative stress and anxious behavior of rats

There were no significant changes observed in body weight, body temperature, food or water intake habits in all groups of rats. All rats irrespective of treatment consumed similar amounts of rodent chow and tap water daily (Table 1).

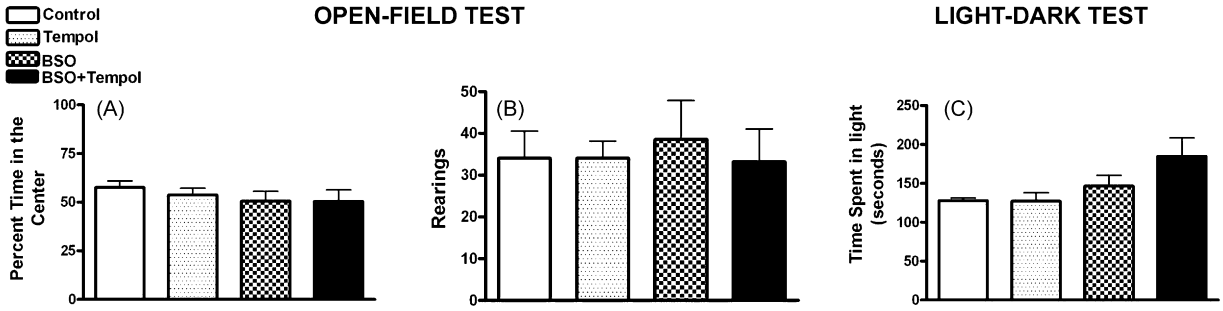

Fig. 2. BSO treatment for 3 days has no effect on anxiety-like behavior of rats as compared to control or tempol plus BSO treatment. Open-field test was used to measure percent time spent in the middle of the open-filed arena (A) and number of rearing for each group (B). On the *y*-axis, zero corresponds to 0 min and 100 correspond to 15 min. In light-dark test (C), time spent by the rats in the light box is indicated. The total light-dark test time was 5 min. $N = 12$ rats per group.

Open-field and light-dark tests were conducted to examine anxiety-like behavior. Open-field test demonstrated that 3-day BSO treatment did not result in reduced time spent in the center of the open-field test chamber or reduced rearing as compared to the control rats (Fig. 2A and B). In the light-dark test, the results suggest that 3-day BSO-treated rats spent 146 s in the light compartment and control and tempol supplemented BSO group rats spent 127 and 184 s, respectively, in the light compartment suggesting that BSO treatment did not significantly alter the time the rats spent in lit area (Fig. 2C). Vehicle-treated controls and tempol alone treated control rats spent same time in the lit area, 127 s. After behavior assessments were made, rats were sacrificed, blood (for serum preparation) and urine was collected and brains harvested to conduct oxidative stress marker measurements in serum, urine, hippocampus, amygdala and LC regions of the brain.

Treatment with BSO for 3 days caused significant decrease in a major antioxidant, glutathione, in the hippocampus (56%) and amygdala (50%) tissue homogenates. In addition, BSO-treated rats showed significant increases in markers of lipid peroxidation (MDA assay) and protein oxidation (nitrotyrosine) in hippocampus (MDA: 75%; nitrotyrosine: 124%) and amygdala (MDA: 48%; nitrotyrosine: 232%) tissues. Nitrotyrosine levels also were estimated in LC region where a significant increase was observed with 3-day BSO treatment (208%). Also, a significant increase in plasma (37.5%) and urinary (28%) 8-isoprostane was observed in BSO-treated rats (Table 2). Tempol supplementation to BSO-treated rats decreased nitrotyrosine, MDA, and 8-isoprostane levels and increased brain tissue glutathione when compared to BSO treatment alone bringing the values closer to the control rats injected with vehicle alone. No significant differences were observed in oxidative or antioxidative markers between vehicle-treated rats and rats that were treated with tempol alone (Table 2).

3.2. *Effect of sub-chronic BSO treatment (7 days) on general body parameters, indices of oxidative stress and anxious behavior of rats*

There were no significant changes observed in body weight, body temperature, food or water intake habits in all groups of rats (Table 3).

The open-field test indicated that BSO-treated rats spent significantly reduced percent time in the center of the open-field chamber than vehicle-treated control rats or tempol alone treated rats respectively. BSO-treated rats spent 27% time in the center while vehicle-treated control rats or tempol alone treated rats spent 56 and 55% time in the center respectively (Fig. 3A). Furthermore, BSO-treated rats displayed significantly reduced rearing (−66%) as compared to vehicle treated or tempol alone control groups. BSO plus tempol supplemented rats significantly increased rearing by ∼263% as compared to BSO alone group (Fig. 3B). After behavior assessments were made, rats were sacrificed, blood (for serum preparation) and urine was collected and brains harvested

Table 2
BSO treatment for 3 days increases oxidative stress and decreases brain glutathione levels. Plasma and urinary 8-isoprostane levels and glutathione levels in the hippocampus and amygdala are presented for vehicle injected control rats and BSO+/− tempol rats. Brain homogenates from hippocampus, amygdala and LC were subjected to western blotting using anti-nitrotyrosine and GAPDH (loading control) antibodies. Data are represented as densitometric ratios of nitrotyrosine normalized against GAPDH. $N = 8–12$ rats.

Indices of oxidative stress	Control	Tempol	BSO (3 days)	BSO + tempol (3 days)
8-Isoprostane urine, pg/mg creatinine	0.8 ± 0.01	0.81 ± 0.01	1.1 ± 0.02* $P < 0.05$	0.88 ± 0.03# $P < 0.05$
8-Isoprostane serum, fg/mg creatinine	38.3 ± 2.1	38.0 ± 2.2	49.0 ± 3.0* $P < 0.05$	36.8 ± 4.0# $P < 0.05$
MDA, nmol/mg of protein (hippocampus)	0.33 ± 0.01	0.34 ± 0.03	0.58 ± 0.02* $P < 0.05$	0.30 ± 0.02# $P < 0.05$
MDA, nmol/mg of protein (amygdala)	0.27 ± 0.01	0.26 ± 0.02	0.40 ± 0.01* $P < 0.05$	0.25 ± 0.02# $P < 0.05$
Glutathione, nmol/mg protein (hippocampus)	1.84 ± 0.3	1.88 ± 0.1	0.81 ± 0.1* $P < 0.05$	1.88 ± 0.2# $P < 0.05$
Glutathione, nmol/mg protein (amygdala)	1.32 ± 0.2	1.30 ± 0.2	0.66 ± 0.1* $P < 0.05$	1.29 ± 0.3# $P < 0.05$
Nitrotyrosine/GAPDH (hippocampus)	0.45 ± 0.03	0.43 ± 0.02	1.01 ± 0.02* $P < 0.05$	0.46 ± 0.03# $P < 0.05$
Nitrotyrosine/GAPDH (amygdala)	0.566 ± 0.03	0.551 ± 0.02	1.88 ± 0.03* $P < 0.05$	0.506 ± 0.03# $P < 0.05$
Nitrotyrosine/GAPDH (locus coeruleus)	0.561 ± 0.02	0.509 ± 0.01	1.73 ± 0.02* $P < 0.05$	0.568 ± 0.02# $P < 0.05$

* Significantly different from control.
\# Significantly different from BSO-treated rats, $P < 0.05$, using ANOVA Tukey's post hoc analysis.

Table 3
BSO treatment for 7 days or tempol treatment has no adverse effect on body weight, food or water intake and body temperature. Fifty grams of rodent chow (diet pellets) and 50 ml of tap water per rat was provided to the rats every day. The amount of food and water consumed was measured by weighing the left-over diet pellets and drinking water 24 h later. Data collected were averaged for each treatment group of rats on the last day of the experiment before behavior testing. Rectal temperature was manually recorded using animal rectal probe every day after 30 min of BSO/vehicle injections. $N = 8–12$ rats.

General parameters	Control	Tempol	BSO (7 days)	BSO + tempol (7 days)
Body weight (g)	225 ± 2.2	227 ± 2.2	228 ± 2.3	253 ± 2.4
Food intake (g)	19.2 ± 3.1	20.2 ± 3.0	22.0 ± 3.2	22.1 ± 3.7
Water intake (ml)	24 ± 2.2	25 ± 2.0	24 ± 2.3	25 ± 2.81
Body temperature (°C)	36.33 ± 0.32	37.03 ± 0.32	36.84 ± 0.21	37.1 ± 0.18

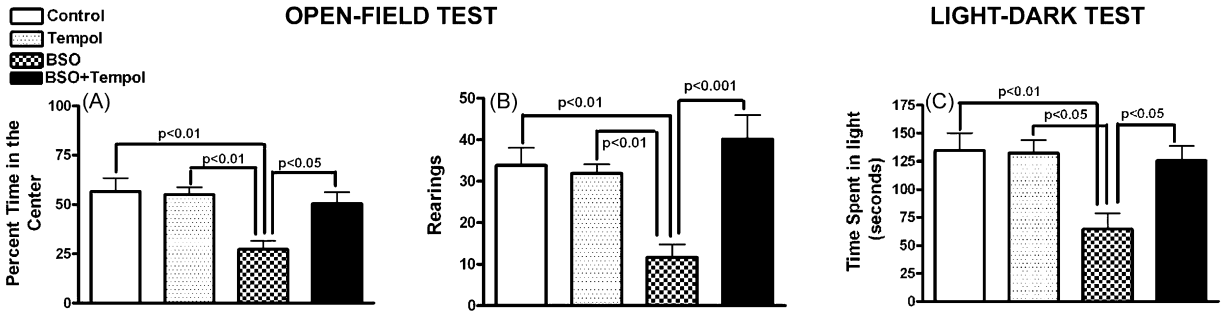

Fig. 3. BSO treatment for 7 days increases anxiety-like behavior of rats as compared to controls or tempol plus BSO treatment. Open-field test was used to measure percent time spent in the middle of the open-filed arena (A) and number of rearing for each group (B). On the *y*-axis, zero corresponds to 0 min and 100 correspond to 15 min. In light-dark test (C), time spent by the rats in the light box is indicated. The total test time in light-dark test was 5 min. Significance between groups determined with ANOVA using Tukey's post hoc analysis is indicated as *P* value. $N = 12$ rats per group.

to conduct oxidative stress markers measurements in serum, urine, hippocampus, amygdala and LC regions of the brain as before.

Light-dark exploration test demonstrates that sub-chronic BSO-treated rats (7 days) spent significantly less time in the light compartment (64 s) than vehicle-treated control (134 s) or tempol treated rats (2-week supplementation in drinking water prior to BSO injections) (125 s) (Fig. 3C). Vehicle-treated controls and tempol alone treated rats spent nearly similar time in the lit area, 134 and 132 s, respectively.

Overall, the degree of oxidative stress was greater with 7-day BSO treatment than with 3-day BSO treatment. Treatment with BSO for 7 days caused significant decrease in glutathione levels in hippocampus (66%) and amygdala (55%) tissue homogenates. In addition, BSO-treated rats showed significant increases in lipid peroxidation (MDA assay) and protein oxidation (nitrotyrosine) in hippocampus (MDA: 114%; nitrotyrosine: 195%) and amygdala (MDA: 150%; nitrotyrosine: 328%) tissues. Nitrotyrosine levels also were estimated in LC region where a significant increase was observed with 7-day BSO treatment (312%). Also, a significant increase in plasma (73%) and urinary (55%) 8-isoprostane was observed in BSO-treated rats (Table 4). Tempol supplementation to BSO-treated rats decreased nitrotyrosine, malondialdehyde, and 8-isoprostane levels and increased brain tissue glutathione when compared to BSO treatment alone bringing the values closer to the control rats injected with vehicle alone. No significant differences were observed in oxidative or antioxidative markers between vehicle-treated rats and rats that were treated with tempol alone (Table 4).

Table 4
BSO treatment for 7 days increases oxidative stress and decreases brain glutathione levels. Plasma and urinary 8-isoprostane levels and glutathione levels in the hippocampus and amygdala are presented for vehicle injected control rats and BSO+/− tempol rats. Brain homogenates from hippocampus, amygdala and LC were subjected to western blotting using anti-nitrotyrosine and GAPDH (loading control) antibodies. Data are represented as densitometric ratios of nitrotyrosine normalized against GAPDH.

Indices of oxidative stress	Control	Tempol	BSO (7 days)	BSO + tempol (7 days)
8-Isoprostane urine, pg/mg creatinine	0.82 ± 0.02	0.88 ± 0.01	1.42 ± 0.02* $P < 0.05$	0.91 ± 0.04# $P < 0.05$
8-Isoprostane serum, fg/mg creatinine	40.2 ± 3.1	41.3 ± 3.0	62.0 ± 4.0* $P < 0.05$	42.1 ± 5.0# $P < 0.05$
MDA, nmol/mg of protein (hippocampus)	0.28 ± 0.01	0.29 ± 0.02	0.60 ± 0.02* $P < 0.05$	0.3 ± 0.01# $P < 0.05$
MDA, nmol/mg of protein (amygdala)	0.20 ± 0.02	0.24 ± 0.01	0.501 ± 0.01* $P < 0.05$	0.22 ± 0.01# $P < 0.05$
Glutathione, nmol/mg protein (hippocampus)	1.82 ± 0.3	1.90 ± 0.3	0.61 ± 0.1* $P < 0.05$	1.38 ± 0.2# $P < 0.05$
Glutathione, nmol/mg protein (amygdala)	1.22 ± 0.3	1.20 ± 0.22	0.54 ± 0.1* $P < 0.05$	0.97 ± 0.2# $P < 0.05$
Nitrotyrosine/GAPDH (hippocampus)	0.44 ± 0.03	0.53 ± 0.03	1.3 ± 0.02* $P < 0.05$	0.521 ± 0.03# $P < 0.05$
Nitrotyrosine/GAPDH (amygdala)	0.614 ± 0.04	0.587 ± 0.02	2.63 ± 0.04* $P < 0.05$	0.72 ± 0.01# $P < 0.05$
Nitrotyrosine/GAPDH (locus coeruleus)	0.468 ± 0.02	0.561 ± 0.01	1.93 ± 0.02* $P < 0.05$	0.417 ± 0.02# $P < 0.05$

* Significantly different from control.
\# Significantly different from BSO-treated rats, $P < 0.05$, using ANOVA Tukey's post hoc analysis, $N = 8–12$ rats.

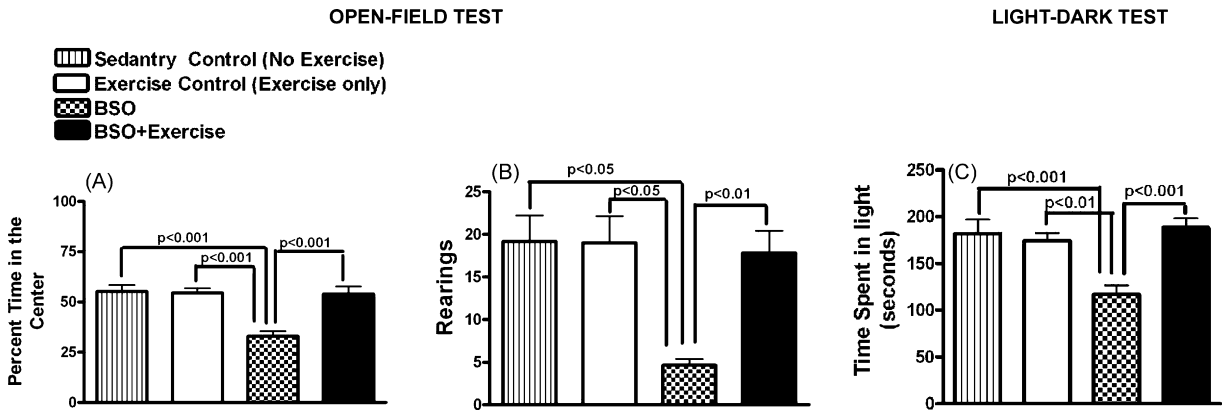

Fig. 4. Four weeks of treadmill exercise reversed BSO-induced anxiety-like behavior in rats. Open-field test was used to measure percent time spent in the middle of the open-filed arena (A) and number of rearing for each group (B). On the *y*-axis, zero corresponds to 0 min and 100 correspond to 15 min. In light-dark test (C), time spent by the rats in the light box is indicated (A). The total test time was 5 min. Significance between groups determined with ANOVA using Tukey's post hoc analysis is indicated as *P* value. *N* = 12 rats per group.

3.3. Effect of 4 weeks of treadmill exercise on indices of oxidative stress and anxiety-like behavior of rats

Body weight, food and water intake and body temperature were measured as before (Tables 1 and 3). As observed earlier, no significant changes were seen in body weight, body temperature, food or water intake habits in all groups of rats as a result of exercise (data not shown). Seven-day BSO-treated rats displayed significantly reduced percent center time (32%) as compared to the sedentary or exercise alone rats that spent 55 and 54% time, respectively. BSO plus exercise group rats significantly increased their center time, 65% as compared to BSO alone group 32% and spent as much time in the center as the sedentary control (55%) or exercise alone (54%) group of rats (Fig. 4A). Furthermore, BSO-treated rats displayed significantly reduced rearing (−79%) as compared to exercise alone or sedentary control groups. BSO plus exercise rats significantly increased rearing by ~325% as compared to BSO alone group (Fig. 4B).

Light-dark test suggest that 7-day BSO-treated rats spent significantly less time in the light compartment (117 s) than sedentary control (rats subjected to 4 weeks of treadmill exercise only) (182 s) or exercise only control rats (174 s). Sedentary or exercise only rats, considered as controls, spent nearly similar time in the lit area, 182 and 174 s, respectively. BSO plus exercise group spent significantly more time (188.7 s) in the lit area as compared to BSO alone group (117 s) (Fig. 4C).

In agreement with previous observations shown in Tables 2 and 4, treatment with BSO for 7 days caused significant decrease in glutathione levels in hippocampus (−59%) and amygdala (−68%) tissue homogenates as compared to exercise alone or sedentary rats. In addition, BSO-treated rats showed significant increases in lipid peroxidation (MDA assay) and protein oxidation (nitrotyrosine) in hippocampus (MDA: +110%; nitrotyrosine: +191%) and amygdala (MDA: 83%; nitrotyrosine: 200%) tissues respectively. Nitrotyrosine levels also were estimated in LC region where a significant increase was observed with 7-day BSO treatment (+213%). Also, a significant increase in plasma (+134%) and urinary (+41%) 8-isoprostane was observed in BSO-treated rats (Table 5). Exercise plus BSO-treated rats decreased nitrotyrosine, malondialdehyde, and 8-isoprostane levels and increased brain tissue glutathione when compared to BSO treatment alone bringing the values closer to the control rats. No significant differences were observed in oxidative or antioxidative markers between sedentary or exercise only control rats (Table 5).

Table 5
Treadmill exercise reduces BSO-induced oxidative stress. Plasma and urinary 8-isoprostane levels and glutathione levels in the hippocampus and amygdala are presented for vehicle injected control rats and BSO+/− tempol rats. Brain homogenates from hippocampus, amygdala and LC were subjected to western blotting using anti-nitrotyrosine and GAPDH (loading control) antibodies. Data are represented as densitometric ratios of nitrotyrosine normalized against GAPDH.

Indices of oxidative stress	Control (sedentary)	Control (exercise)	BSO (7 days)	Exercise + BSO
8-Isoprostane urine, pg/mg creatinine	0.900 ± 0.02	0.901 ± 0.02	2.11 ± 0.02* $P<0.05$	1.01 ± 0.04# $P<0.05$
8-Isoprostane serum, fg/mg creatinine	40.2 ± 1.1	44.2 ± 1.1	62.4 ± 2.0* $P<0.05$	43.2 ± 4.0# $P<0.05$
MDA, nmol/mg of protein (hippocampus)	0.22 ± 0.01	0.20 ± 0.01	0.42 ± 0.02* $P<0.05$	0.22 ± 0.01# $P<0.05$
MDA, nmol/mg of protein (amygdala)	0.20 ± 0.01	0.18 ± 0.01	0.33 ± 0.02* $P<0.05$	0.20 ± 0.01# $P<0.05$
Glutathione, nmol/mg protein (hippocampus)	2.22 ± 0.2	2.02 ± 0.2	0.82 ± 0.1* $P<0.05$	2.2 ± 0.2# $P<0.05$
Glutathione, nmol/mg protein (amygdala)	1.31 ± 0.2	1.41 ± 0.2	0.44 ± 0.1* $P<0.05$	1.3 ± 0.2# $P<0.05$
Nitrotyrosine/GAPDH (hippocampus)	0.55 ± 0.02	0.45 ± 0.02	1.31 ± 0.02* $P<0.05$	0.55 ± 0.02# $P<0.05$
Nitrotyrosine/GAPDH (amygdala)	0.56 ± 0.01	0.66 ± 0.03	1.98 ± 0.03* $P<0.05$	0.70 ± 0.03# $P<0.05$
Nitrotyrosine/GAPDH (locus coeruleus)	0.66 ± 0.02	0.60 ± 0.01	1.88 ± 0.02* $P<0.05$	0.63 ± 0.02# $P<0.05$

* Significantly different from control.
\# Significantly different from BSO-treated rats, $P<0.05$, using ANOVA Tukey's post hoc analysis, $N = 8–12$ rats.

4. Discussion

Accumulating evidence suggests an association between oxidative stress and anxiety-like behavior [2–7] and the beneficial effects of exercise in anxiety and cognition [11,12]. In this study we have investigated the direct link between oxidative stress, anxiety-like behavior and the beneficial role of moderate treadmill exercise in oxidative stress-mediated anxiety-like behavior. Our results suggest that short and sub-chronic oxidative stress results in different consequences on anxiety-like behavior in rats. Sub-chronic oxidative stress in the brain (7 days BSO treatment) results in anxiety-like behavior while short-term oxidative stress (3 days BSO treatment) does not. This difference in anxiety-like behavior between short and sub-chronic BSO treatment most likely is a consequence of differing levels of oxidative stress occurring in the brain in the two situations, with sub-chronic BSO treatment causing more oxidative stress than acute treatment. Prolonged oxidative stress as a result of sub-chronic BSO treatment most likely overwhelms the antioxidant homeostasis limiting its ability for adaption which consequently increases anxiety-like behavior. Moreover, the increase in anxiety-like behavior is reversed by antioxidant tempol treatment, suggesting direct involvement of oxidative stress in mediating anxiety-like behavior of rats. Tempol is a water soluble and cell membrane permeable molecule [41], with demonstrated antioxidant activity [42] in various biological systems [43,44] and is known to cross blood brain barrier [45,46].

Earlier studies had used BSO injections for 2 days and reported an induction of oxidative stress in mice [4]. In our studies, rats did not develop anxiety-like behavior or oxidative stress in LC, amygdala, hippocampus or hypothalamus regions of the brain at 2 days of BSO injections (data not shown). However, 3 days BSO treatment increased oxidative stress in LC, amygdala and hippocampus but did not cause anxiety-like behavior. These differing results most likely are a consequence of different pharmacokinetics of BSO in mice versus rats. In general, our results are in agreement with previous studies that have suggested a positive correlation of oxidative stress with anxiety-like behavior [4,7,47–50] but our studies provide more direct link between oxidative stress and anxiety-like behavior.

Display of increased anxiety-like behavior indicative from reduced time spent by the rats in the center versus the periphery of the open-field arena and avoidance of lit area was not a result of sickness behavior that might be argued to have resulted from BSO or tempol treatments as the body weight, food and water intake and body temperature of all groups of rats were similar. Fever, reduction of body weight and loss of appetite are common sickness responses to infection and inflammation [51,52]. Avoidance of lit areas by the rats was also not due to any sickness or eye inflammation as the eye reflexes were checked everyday by placing a cotton swab close to ~1 in. of the eyes. The rats blinked in response to the cotton swab and quickly moved aside. The alertness and muscular reflexes of the rats also was monitored on a daily basis and all rats responded to sounds and startle indicating that they were alert throughout the experiment. The fact that all groups of rats ran on treadmill without major avoidance suggests that they did not have any motor deficits that impair their movement and tendency to explore in anxiety tests. Based upon these facts, we are confident that our anxiety measurements truly suggest anxious behavior and not sickness behavior.

As the second part of this study, we investigated the effect of moderate treadmill exercise on BSO-induced oxidative stress and anxious behavior of rats. Our interest in this model is based upon several recent reports that have suggested beneficial effects of moderate treadmill exercise on anxiety [11,12] and also that moderate exercise increases antioxidant enzyme activity and attenuates oxidative stress in the brain [13,26,27]. Body weight, temperature, food and water intake was measured on a daily basis as before. These parameters were tested to rule out any sickness behavior in rats due to BSO or tempol treatments and these parameters remained unaltered in all groups. While BSO treatment resulted in increased anxiety-like behavior, prior treadmill exercise prevented rats from exhibiting anxiety-like behavior. Furthermore, while BSO treatment increased oxidative stress in serum, urine and brain tissues from hippocampus, amygdala and LC (regions implicated in anxiety response) [53], exercise prevented BSO-mediated increase in oxidative stress from occurring.

In summary, our results clearly demonstrate that oxidative stress is involved in anxiety-like behavior and that antioxidant and moderate treadmill training both are able to reverse and prevent anxiety-like behaviour, respectively. These observations clearly indicate that there is a need to identify oxidative stress mechanisms that result in anxiety-like behavior. Such information is important because it ultimately will help in the development of novel pharmacotherapy for anxiety disorders. The involvement of antioxidant enzymes is likely to contribute to the pathophysiology of anxiety-like behavior generated via oxidative stress mechanisms. In particular, glyoxalase (Glo) 1 and glutathione reductase (Gsr) 1, genes involved in antioxidant enzymatic defenses are potential players in this process. Using genetically inbred anxious strains of mice, Hovatta et al. [2] found that in mouse brain, overexpression of Glo1 and Gsr1 increased while inhibition of Glo1 expression by siRNA decreased the level of anxiety-like behavior of mice. However, several studies have suggested a negative relationship between anxiety-like behavior and Glo1 protein expression. Particularly, the selection studies of Krömer et al. [47] with outbred Swiss CD1 strain high-anxiety-related behavior (HAB-M) or low-anxiety-related behavior (LAB-M) mice established that Glo 1 is expressed to a higher extent in LAB-M than in HAB-M mice in several brain areas including hypothalamus, amygdala, and motor cortex. Williams et al. [54] recently suggested that the differences in Glo1 expression in the selected lines of mice between the aforementioned studies with those of Hovatta et al. [2] is most likely due to the duplication; a combination of genetic drift and inbreeding. Regardless of different outcome of results due to genetic variation or differences in models, the regulation of the antioxidant protein pool is more than likely one of the many important elements of anxiety. Hence, mechanisms that regulate the antioxidant expression should be known.

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