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Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress

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ABSTRACT

Reactive oxygen species (ROS) have been shown to play a role in the pathophysiology of depression. Taking into account that experimental chronic unpredictable stress (CUS) induces depressive-like behavior and that ascorbic acid has antidepressant-like effect in animals, the objective of this study was to investigate the influence of ascorbic acid on depressive-like behavior induced by CUS paradigm, serum corticosterone levels and markers of oxidative stress in cerebral cortex and hippocampus of mice. Animals were submitted to CUS procedure during 14 days. From the 8th to the 14th day mice received ascorbic acid (10 mg/kg) or fluoxetine (10 mg/kg, conventional antidepressant, positive control) once a day by oral route. On 15th day behavioral and biochemical parameters were analyzed. CUS exposure caused a depressive-like behavior evidenced by the increased immobility time in the tail suspension test and decreased time in which mice spent grooming in the splash test. Depressive-like behavior induced by CUS was accompanied by a significant increased lipid peroxidation (cerebral cortex and hippocampus), decreased catalase (CAT) (cerebral cortex and hippocampus) and glutathione reductase (GR) (hippocampus) activities and reduced levels of glutathione (cerebral cortex). Repeated ascorbic acid or fluoxetine administration significantly reversed CUS-induced depressive-like behavior and oxidative damage. No alteration was observed in locomotor activity, corticosterone levels and glutathione peroxidase (GPx) activity. These findings indicate a rapid and robust effect of ascorbic acid in reversing behavioral and biochemical alterations induced by CUS in mice, suggesting that this vitamin may be an alternative approach for the management of depressive symptoms.

1. Introduction

Major depressive disorder is a common, recurrent and incapacitating psychiatric illnesses associated with significant morbidity and mortality (Nemeroff, 2007). The neurobiology of this condition and detailed knowledge of its etiology is not yet well established, however, it is known that major depression might originate from both environmental and genetic risk factors (Nestler et al., 2002). It has been reported that stressful life events have a considerable causal association with depression, and there is now compelling evidence that adverse early life experience and the effects of chronic stress on neurobiological systems are associated with the pathophysiology of this disorder (Charney and Manji, 2004; Heim et al., 2008).

The chronic unpredictable stress (CUS) model of depression was developed in an attempt to mimic some of the environmental factors contributing to the induction of depressive disorders in humans. The model has good face validity (almost all demonstrable symptoms of depression have been reported), construct validity (chronic mild stress reproduces pathophysiological characteristics of the human condition) and predictive validity (behavioral changes are reversed by chronic treatment with antidepressants) (Bondi et al., 2008; Kumar et al., 2011; Larsen et al., 2010; Lu et al., 2006).

A growing body of data has suggested that oxidative stress, characterized by the imbalance between production of free radicals and the antioxidant capacity of organism, may contribute to the neuropathology of neurological and psychiatric diseases, including major depression (Ng et al., 2008). There are several reports showing that chronic stress can have a substantial impact on reactive oxygen species generation (ROS) in the brain. It has been shown that repeated and unpredictable stress situations increase ROS production in the rat brain (Fontella et al., 2005; Lucca et al., 2009; Madrigal et al., 2001), which in turn results in oxidative damage in the central nervous system. Similarly, studies which investigated the oxidative stress profile in depressed patients found

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impairments in the blood levels of the antioxidants enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and higher products of lipid peroxidation than healthy controls (Bilici et al., 2001; Khanzode et al., 2003; Ozcan et al., 2004), strongly suggesting that major depressive disorder is accompanied by disturbances in the balance between pro- and anti-oxidative processes.

Drug resistance and treatment failures are frequent with existing therapies to treat depression, adding urgency to the need for better understanding of the pathophysiology of this disorder and to conceive novel compounds in order to improve the clinical management of this condition. Ascorbic acid, a water-soluble vitamin with neuroprotective and antioxidant properties (Rice, 2000), is emerging as a novel putative compound to assist in the treatment of depression. In clinical studies, it was reported that the administration of ascorbic acid relieved ACTH-induced depression in a child (Cocchi et al., 1980) and decreased scores in a Beck Depression Inventory in healthy young adults (Brody, 2002), indicating mood improvement. Moreover, recently our group demonstrated that administration of ascorbic acid in mice produced an antidepressant-like effect in the tail suspension test (TST), a property that is dependent on the interaction of this vitamin with the monoaminergic systems (Binfaré et al., 2009) and with NMDA receptors and L-arginine–NO–cGMP pathway (Moretti et al., 2011). In addition, it was shown that ascorbic acid caused a synergistic antidepressant-like effect with the conventional antidepressants fluoxetine, imipramine and bupropion in the TST in mice (Binfaré et al., 2009).

Considering the findings mentioned above, the aims of this study were to examine the influence of a CUS paradigm on depressive-like behavior, serum corticosterone levels and on markers of oxidative stress (levels of lipid peroxidation, CAT activity and glutathione antioxidant defense system) in the mouse cerebral cortex and hippocampus, which are structures closely implicated in the pathophysiology of depression (Nestler et al., 2002). Moreover, in view of the reported antidepressant-like effect of ascorbic acid, we sought to investigate the effect of repeated administration of this vitamin on behavioral and biochemical alterations induced by CUS, comparing its effect to the one produced by the widely clinically used antidepressant fluoxetine.

2. Materials and methods

2.1. Animals

The behavioral experiments were conducted using female Swiss mice (30–40 g), maintained at 20–22 °C with free access to water and food, under a 12:12 h light/dark cycle, with lights on at 7:00 a.m. The animals were caged in groups of 15 in a 41 × 34 × 16 cm cage. All behavioral tests were carried out between 9.00 a.m. and 04.00 p.m. The animals were used according to the NIH Guide for the Care and Use of Laboratory Animals and the experiments were performed after approval of the protocol by the Ethics Committee of the Institution. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

2.2. Drugs and treatment

Ascorbic acid and fluoxetine (obtained from Sigma Chemical Co., St. Louis, U.S.A) were dissolved in distilled water and administered orally (p.o.) in a dose of 10 mg/kg once a day during the last 7 days of the CUS procedure. Ascorbic acid and fluoxetine solutions were freshly prepared before administration and administered in a volume of 1 ml/kg. To develop this study mice were divided into six groups, as follows: (1) non-stressed + vehicle; (2) non-stressed + fluoxetine; (3) non-stressed + ascorbic acid; (4) stressed + vehicle; (5) stressed + fluoxetine; (6) stressed + ascorbic acid.

2.3. Experimental procedure

CUS protocol, adapted from the procedure described by Lu et al. (2006), consisted of a variety of stressors applied randomly and at varying times of day for 14 days (Table 1). This paradigm was designed in order to minimize the prediction. Animals were divided into two groups: control (non-stressed) and CUS (stressed) mice. Control and stressed animal were maintained in separate room due to stress odor. During the fourteen days of procedure, control mice were kept in their home cages according to conditions previously reported. Control and stressed mice were weighed once a week. On 15th day, 24 h after the last administration of ascorbic acid, fluoxetine or vehicle, animals were submitted to TST, open-field test or splash test. Mice were euthanized by decapitation immediately after the behavioral tests and trunk blood, cerebral cortex and hippocampus were collected for biochemical analysis.

Forced swimming was carried out by placing the animal in an open cylindrical container (diameter 10 cm, height 25 cm) containing 19 cm of water at 25 ± 1 °C or 15 ± 1 °C, as shown in Table 1.

The footshock was applied in an apparatus consisting of a 50 × 25 × 25 cm plastic box with a front glass wall, whose floor had parallel 10-mm bronze bars. The mice were gently placed on the grid and received a scrambled 0.7-mA, 0.5 s/min footshock during 3 min.

Tail pinch was performed by applying a clothespin placed at 1 cm from the base of the tail, as previously described (Lu et al., 2006).

2.4. Behavioral tests

2.4.1. Tail suspension test

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Mice were considered immobile only when they hung passively and completely motionless. Immobility time was manually recorded during a 6-min period by an experienced observer. The observer was in the room where experiments were performed and was blind to the animal condition.

2.4.2. Open-field test

Ten minutes after the tail suspension test, the locomotor activity was assessed in an open-field test as described previously (Moretti et al., 2011). The apparatus consisted of a wooden box measuring

Table 1
Schedule of stressor agents used in the 14-day of chronic stressful stimuli.

Day	Stressor	Duration	Time of day
1	Restraint	1.5 h	2:00 p.m.
2	Cold swim (15 °C)	10 min	9:30 a.m.
3	Wet wood shavings/box housing tilted (45°)	16 h	10:30 a.m.
4	Inversion of the light/dark cycle	16 h	6:00 p.m.
5	Tail pinch	10 min	12:30 p.m.
6	Swim (25 °C)	6 min	2:00 p.m.
7	Restraint	1.5 h	9:30 p.m.
8	Food and water deprivation	16 h	8:30 a.m.
9	Cold swim (15 °C)	10 min	4: 30 p.m.
10	Wet wood shavings/box housing tilted (45°)	24 h	12:30 p.m.
11	Inescapable shock (0.7 mA, 0.5 s/min)	3 min	5:30 p.m.
12	Inversion of the light/dark cycle	16 h	6:00 a.m.
13	Tail pinch	10 min	2:30 p.m.
14	Inescapable shock (0.7 mA, 3 s/min)	3 min	9:00 a.m.

40 × 60 × 50 cm high. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossings) was manually counted in a 6-min session. The light was maintained at minimum to avoid anxiety behavior. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.4.3. Splash test

Ten minutes after the open-field test the splash test was carried out. This test was carried out as described by Isingrini et al. (2010), with minor modifications and consists of squirting a 10% sucrose solution on the dorsal coat of a mouse placed individually in clear Plexiglas boxes (9 × 7 × 11 cm). Because of its viscosity, the sucrose solution dirties the mouse fur and animals initiate grooming behavior. After applying sucrose solution, the time spent grooming was manually recorded for a period of 5 min as an index of self-care and motivational behavior, considered to be parallel with some symptoms of depression such as apathetic behavior (Willner, 2005). The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.5. Biochemical analysis

2.5.1. Tissue preparation

The animals were killed by decapitation and the cortices and hippocampi were removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at 16,000 × *g*, at 4 °C for 20 min and the supernatants obtained were used for the determination of enzymatic activities and for the quantification of the levels of GSH and thiobarbituric acid reactive substances (TBARS). Whole blood was centrifuged at 3000 × *g*, at room temperature for 10 min and the obtained serum was used to measure corticosterone levels.

2.5.2. Corticosterone circulating levels

Corticosterone levels were determined using commercially available enzyme immunoassay Kit (Assay Design, Inc. MI, USA), according to the manufacturer instructions.

2.5.3. Activity of antioxidant enzymes

Hippocampal and cortical glutathione reductase (GR) activity was determined based on the protocol developed by Carlberg and Mannervik (1985). Briefly, GR reduces GSSG to GSH at the expense of NADPH, the disappearance of which can be followed at 340 nm. Hippocampal and cortical glutathione peroxidase (GPx) activity was determined based on the protocol developed by Wendel (1981) by indirectly measuring the consumption of NADPH at 340 nm. The GPx uses GSH to reduce the *tert*-butyl hydroperoxide, producing GSSG, which is readily reduced to GSH by GR using NADPH as a reducing equivalent donor. Catalase activity was measured by the method of Aebi (1984). The reaction was started by the addition of freshly prepared 30 mM H_2O_2. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm.

2.5.4. Glutathione levels

Hippocampal and cortical GSH levels were measured as non-protein thiols based on the protocol developed by Ellman (1959). Hippocampal and cortical homogenates were precipitated in cooled trichloroacetic acid 10% and centrifuged at 15,000 × *g* for 2 min, and the supernatant was incubated with DTNB in a 1 M phosphate buffer, pH 7.0. Absorbances were measured at 412 nm. A standard curve of reduced glutathione was used to calculate GSH levels.

2.5.5. Thiobarbituric acid reactive species (TBARS) formation

TBARS were determined in the hippocampal and cortical homogenates using the method described by Ohkawa et al. (1979), in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid to form a colored complex. The samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.67% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as standard.

2.5.6. Determination of protein

The protein content was quantified by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.5.7. Statistical analysis

All data are presented as mean ± SEM Differences among experimental groups were determined by two-way ANOVA followed by Duncan's post hoc test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Body weight

At the beginning of the experiment, there were no significant differences ($p > 0.05$) in body weights between groups: 35.2 ± 1.7, 33.7 ± 0.8, 34.5 ± 1.4, 31.3 ± 0.8, 33.6 ± 0.8, and 34.3 ± 1.2 for groups 1–6 (mean ± SEM), respectively. The body weights at the end of treatments were also not significant different between groups ($p > 0.05$): 39.6 ± 1.4, 37.2 ± 0.7, 38.4 ± 1.2, 34.0 ± 1.0, 35.9 ± 0.8, and 36.9 ± 0.7 for groups 1–6 (mean ± SEM), respectively. Therefore, the results suggest an absence of systemic toxicity after ascorbic acid administration and unpredictable stress.

3.2. Behavioral observations

3.2.1. Immobility time in TST

Fig. 1A shows the influence of treatment of mice with ascorbic acid and fluoxetine on the depressive-like behavior elicited by CUS procedure. The two-way ANOVA revealed significant differences for CUS procedure [$F(1,41) = 17.88$, $p < 0.01$], treatment [$F(2,41) = 75.04$, $p < 0.01$] and CUS procedure × treatment interaction [$F(2,41) = 3.36$, $p < 0.05$]. Post hoc analyses indicated that exposure to different stressors significantly increased the immobility time in the TSR, as compared to control mice. Repeated ascorbic acid administration significantly reversed the increase in immobility time in stressed mice, effect which was comparable to that elicited by fluoxetine. Ascorbic acid and fluoxetine administration in non-stressed mice also decrease the immobility time in the TST in comparison with control mice.

3.2.2. Locomotor activity

The open-field test was used for assessing locomotor activity in mice after 14-day of chronic stressful stimuli and no alteration in spontaneous locomotion was observed in mice, as showed in Fig. 1B. The two-way ANOVA revealed no differences for CUS procedure [$F(1,44) = 2.99$, $p > 0.05$], treatment [$F(2,44) = 0.02$, $p > 0.05$] and CUS procedure × treatment interaction [$F(2,44) = 0.81$, $p > 0.05$].

3.2.3. Splash test

Regarding the time spent grooming, the two-way ANOVA revealed no significant main effects for CUS procedure [$F(1,49) = 0.20$, $p > 0.05$] and treatment [$F(2,49) = 3.10$, $p > 0.05$], but revealed significant differences for CUS procedure × treatment interaction [$F(2,49) = 6.74$, $p < 0.01$]. Post hoc analyses indicated that CUS procedure decreased significantly the time spent grooming in mice treated with vehicle and the treatment with ascorbic

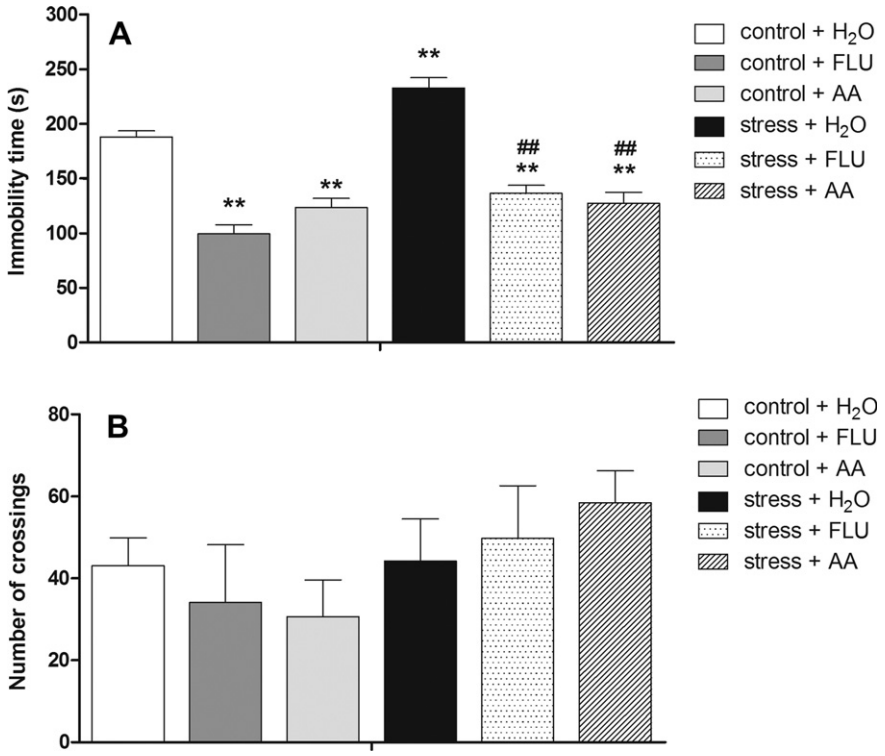

Fig. 1. Effect of repeated treatment with ascorbic acid and fluoxetine on immobility time in the TST (panel A) and on locomotor activity (panel B) in mice submitted to CUS procedure. Bars represent means ± SEM of 8–10 mice. $^{**}p < 0.01$ vs. control mice, $\#\#p < 0.01$ vs. CUS + vehicle group, according to two-way ANOVA followed by the Duncan's post hoc test.

acid and fluoxetine restored the total grooming time to the non-stressed level, as illustrated in Fig. 2.

3.3. Biochemical observations

3.3.1. Corticosterone measurements

As depicted in Fig. 3, neither CUS procedure nor treatments with ascorbic acid and fluoxetine altered corticosterone levels. The two-way ANOVA revealed no differences for CUS procedure [$F(1,36) = 1.43$, $p > 0.05$], treatment [$F(2,36) = 1.53$, $p > 0.05$] and CUS procedure × treatment interaction [$F(2,36) = 0.46$, $p > 0.05$].

3.3.2. Lipid peroxidation

As shown in Fig. 4, TBARS levels were significantly increased in the cerebral cortex and hippocampus of stressed mice as compared to non-stressed mice. Repeated treatment with ascorbic acid produced a significant reduction in TBARS levels only in the hippocampus of stressed mice. Conversely, fluoxetine treatment significantly reduced TBARS levels in the cerebral cortex of mice submitted to CUS procedure. Ascorbic acid and fluoxetine administration to non-stressed mice did not show any significant effect on TBARS levels (cerebral cortex: CUS procedure [$F(1,24) = 36.38$, $p < 0.01$], treatment [$F(2,24) = 1.55$, $p > 0.05$], CUS procedure × treatment interaction [$F(2,24) = 7.19$, $p < 0.01$]; hippocampus: CUS procedure [$F(1,24) = 15.98$, $p < 0.01$], treatment [$F(2,24) = 4.12$, $p < 0.05$], CUS procedure × treatment interaction [$F(2,24) = 3.46$, $p < 0.05$]).

3.3.3. Antioxidant profile

As can be observed in Fig. 5A, in cerebral cortex, stressed mice displayed lower glutathione levels than the control group. This reduction was significantly reversed by the repeated treatment with ascorbic acid and fluoxetine. The administration of ascorbic

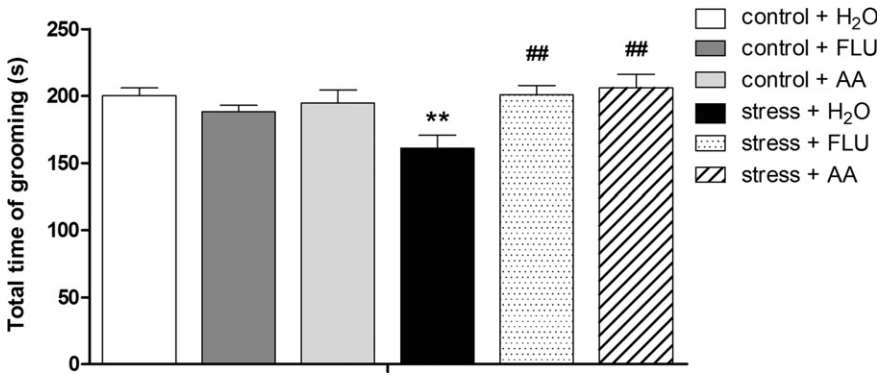

Fig. 2. Effect of repeated treatment with ascorbic acid and fluoxetine on time spent grooming in mice submitted to CUS procedure. Bars represent means ± SEM of 8–10 mice. $^{**}p < 0.01$ vs. control mice, $\#\#p < 0.01$ vs. CUS + vehicle group, according to two-way ANOVA followed by the Duncan's post hoc test.

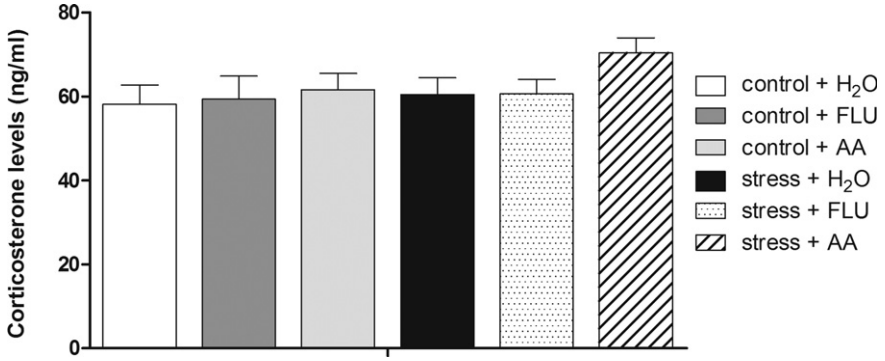

Fig. 3. Effect of repeated treatment with ascorbic acid and fluoxetine on serum corticosterone levels of mice submitted to CUS procedure. Bars represent means ± SEM of 7 mice. Two-way ANOVA showed no effect.

and fluoxetine did not affect the levels of glutathione in non-stressed group in cerebral cortex (CUS procedure [$F(1,32) = 0.56$, $p > 0.05$], treatment [$F(2,32) = 4.06$, $p < 0.05$], CUS procedure × treatment interaction [$F(2,32) = 5.76$, $p < 0.01$]). In hippocampus, no significant change in GR activity was observed, independently from the stress condition and treatment (CUS procedure [$F(1,33) = 0.43$, $p > 0.05$], treatment [$F(2,33) = 0.68$, $p > 0.05$], CUS procedure × treatment interaction [$F(2,33) = 0.21$, $p > 0.05$]).

As depicted in Fig. 5B, CAT activity was significantly decreased in cerebral cortex and hippocampus of stressed mice as compared to control mice. This reduction induced by CUS was significantly blunted by the treatment with ascorbic acid in both cerebral cortex and hippocampus. Fluoxetine treatment reversed the CUS-induced decrease in CAT activity only in the hippocampus. In non-stressed mice group, the treatment with ascorbic increased CAT activity in the hippocampus, but fluoxetine administration did not produce any significant effect in CAT activity (cerebral cortex: CUS procedure [$F(1,25) = 2.75$, $p > 0.05$], treatment [$F(2,25) = 0.28$, $p > 0.05$], CUS procedure × treatment interaction [$F(2,25) = 3.60$, $p < 0.05$]; hippocampus: CUS procedure [$F(1,24) = 0.72$, $p > 0.05$], treatment [$F(2,24) = 29.35$, $p < 0.01$], CUS procedure × treatment interaction [$F(2,24) = 7.38$, $p < 0.01$]).

Fig. 6A shows that no significant statistical alterations were observed in GPx activity in cerebral cortex and hippocampus of stressed mice compared to non-stressed animals both injected with vehicle. Also, repeated treatment with ascorbic acid and fluoxetine did not alter GPx activity in the evaluated structures, independent of stress condition (cerebral cortex: CUS procedure [$F(1,35) = 0.46$, $p > 0.05$], treatment [$F(2,35) = 1.35$, $p > 0.05$], CUS procedure × treatment interaction [$F(2,35) = 1.19$, $p > 0.05$]; hippocampus: CUS procedure [$F(1,25) = 0.75$, $p > 0.05$], treatment [$F(2,25) = 1.10$, $p > 0.05$], CUS procedure × treatment interaction [$F(2,25) = 1.10$, $p > 0.05$]).

GR activity was reduced in the hippocampus of stressed mice, as illustrated in Fig. 6B. This reduction was significantly abolished by the treatment with ascorbic acid in stressed mice. The efficacy of ascorbic acid was comparable to that of fluoxetine, which in hippocampus, also significantly increased GR activity in stressed mice. The administration of ascorbic and fluoxetine to non-stressed mice caused no significant effects on hippocampal GR activity (CUS procedure [$F(1,27) = 0.22$, $p > 0.05$], treatment [$F(2,27) = 4.35$, $p < 0.05$], CUS procedure × treatment interaction [$F(2,27) = 4.30$, $p < 0.05$]). In the cerebral cortex, no significant alterations were observed in GR activity, independently from the stress condition and treatment (CUS procedure [$F(1,35) = 0.06$, $p > 0.05$], treatment [$F(2,35) = 0.49$, $p > 0.05$], CUS procedure × treatment interaction [$F(2,35) = 0.35$, $p > 0.05$]).

4. Discussion

The main findings of the present study can be summarized as follows: (a) CUS procedure in vehicle-treated mice caused a depressive-like behavior, as can be observed in the TST and splash test results; (b) significant reduction in depressive-like behavior was evident in stressed mice treated with ascorbic acid or fluoxetine; (c) CUS procedure induced oxidative damage by increasing lipid peroxidation and decreasing antioxidant defenses in cerebral

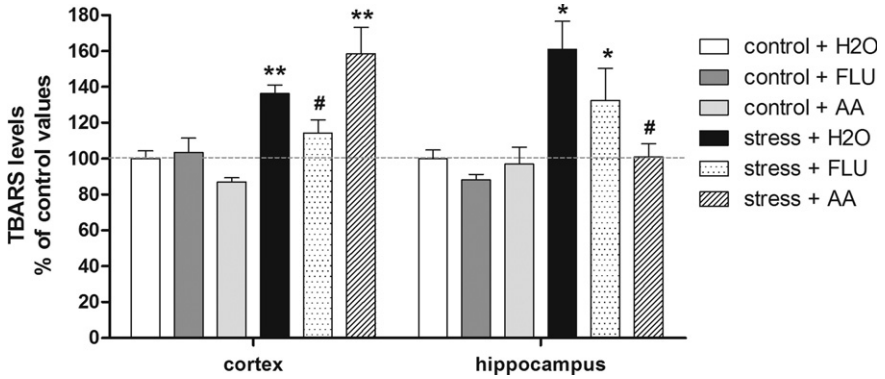

Fig. 4. Effect of repeated treatment with ascorbic acid and fluoxetine on thiobarbituric acid reactive substances (TBARS) in cerebral cortex and hippocampus of mice submitted to CUS procedure. Bars represent means ± SEM of 5 mice. Mean ± SEM for 100% values correspond to 7.48 ± 0.32 nmol MDA equivalent/mg protein (cerebral cortex) and 8.06 ± 0.38 MDA equivalent/mg protein (hippocampus) .*$p < 0.05$ vs. control mice, #$p < 0.05$ vs. CUS + vehicle group, ##$p < 0.01$ vs. CUS + vehicle group according to two-way ANOVA followed by the Duncan's post hoc test.

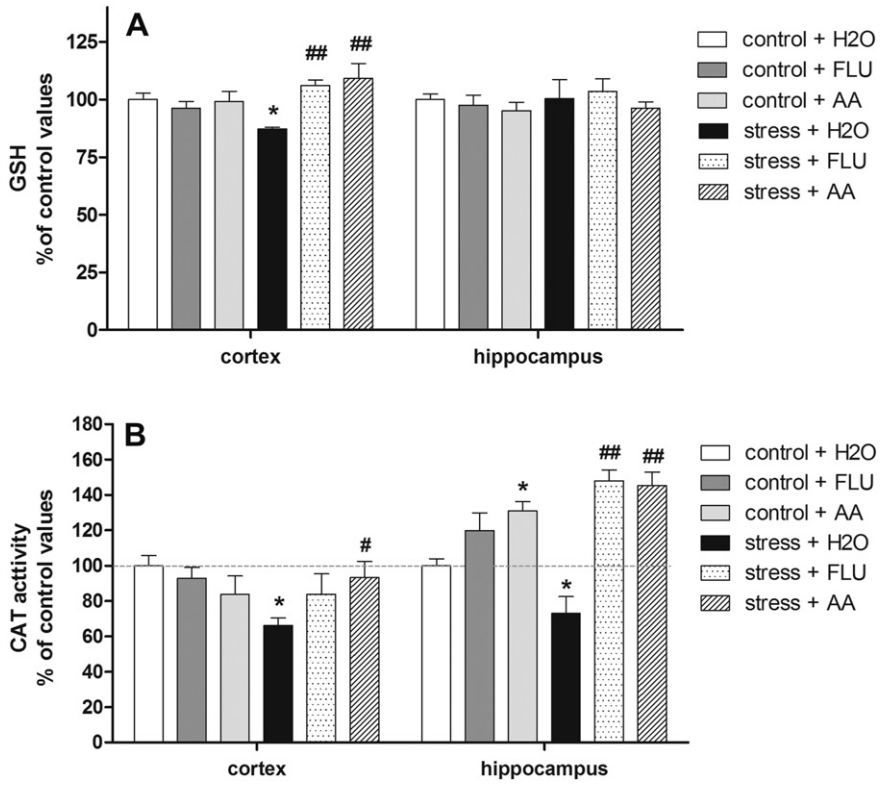

Fig. 5. Effect of repeated treatment with ascorbic acid and fluoxetine on glutathione levels (panel A) and on CAT activity (panel B) in cerebral cortex and hippocampus of mice submitted to CUS procedure. Bars represent means ± SEM of 5–7 mice. In the panel A mean ± SEM for 100% values correspond to 28.28 ± 0.79 nmol GSH/mg protein for cerebral cortex and 33.93 ± 0.80 nmol GSH/mg protein for hippocampus; in the panel B mean ± SEM for 100% values correspond to 0.75 ± 0.04 μmol H_2O_2 consumed/min/mg protein for cerebral cortex and 0.39 ± 0.01 μmol H_2O_2 consumed/min/mg protein for hippocampus. $^*p < 0.05$ vs. control mice, $\#p < 0.05$ vs. CUS + vehicle group, according to two-way ANOVA followed by the Duncan's post hoc test.

cortex and hippocampus of vehicle-treated mice; (d) ascorbic acid and fluoxetine promoted beneficial effects against CUS-induced changes in oxidative stress-related parameters in the mouse brain.

A substantial number of studies have reported that mice or rats exposed to chronic stress exhibit depressive-like behavior, evidenced by increased immobility period in behavioral despair tests, particularly in the TST and forced swimming test (Kumar et al., 2011; Lu et al., 2006; Nirmal et al., 2008). In addition, this model evokes lower sucrose consumption (sweet food) (Garcia et al., 2009; Li et al., 2011; Willner et al., 1987) and decreases self-care and motivational behavior (Isingrini et al., 2010), postulated to reflect anhedonia (the decreased capacity to experience pleasure of any sort) in animals, one of the two core symptoms required for diagnosis of a major depressive episode in humans (American Psychiatric Association, 1994). Therefore, the present data are in agreement with previous findings that showed consistent depressive-like behavior induced by repeated and unpredictable stress in rodents. Importantly, the long-term administration of different uncontrollable stressors in an unpredictable way is a well-recognized animal model for the preclinical evaluation of antidepressants. Considering face and construct validities, chronic stress models appear more suitable for the experimental investigation of depression compared to tests predictive of antidepressant activity (Willner, 2005).

In the present study, at the first time, we provide evidence that CUS-induced depressive-like behaviors in mice can be reversed by the repeated administration of ascorbic acid which presented efficacy comparable to that of the clinically active antidepressant fluoxetine. This ability of ascorbic acid in reversing the CUS-induced anhedonia-like behavior and in decreasing the immobility time in the TST supports the idea that it has antidepressant potential. Besides, it is important to note that neither ascorbic acid nor fluoxetine treatment affected the spontaneous locomotion, excluding the hypothesis that psychostimulant effects might be responsible for their behavioral effects in the TST.

This reported antidepressant-like effect of ascorbic acid may be dependent on different properties of this vitamin, such as its neuromodulatory and antioxidant actions. Studies describe that in the central nervous system (particularly in neurons) ascorbic acid is maintained at elevated concentrations and may act as a neuromodulator, facilitating the release of some neurotransmitters and inhibiting neurotransmitter binding to receptors, including responses mediated by glutamatergic system (Majewska and Bell, 1990; Majewska et al., 1990; Rosa et al., 2005), which is proposed to represent a key role in the pathophysiology of depression (Sanacora et al., 2008, 2012). In this same scenario, ascorbate transport to the extracellular milieu has been linked to glutamate uptake in a process termed "ascorbate-glutamate heteroexchange" (Rebec and Pierce, 1994), which decreases extracellular glutamate levels and, in turn, reduces excitotoxicity and pro-oxidative damage. Recently, it was showed that the mechanism by which ascorbic acid exerts its anti-immobility effect in mice is dependent, at least in part, on the NMDA receptor inhibition (Moretti et al., 2011). Consistent with this notion, previous results provided direct evidence that NMDA receptor antagonists, such as ketamine and Ro 25-6981 (a selective NR2B antagonist), rapidly reverse the behavioral, morphological and physiological deficits resulting from chronic stress (Garcia et al., 2009; Li et al., 2011). In addition, it was reported that CUS procedure stimulates the brain monoamine oxidase (MAO-A and MAO-B) enzyme activity (Kumar et al., 2011; Lin et al., 2005), which may result in depleted brain monoamine levels, an interesting finding considering the reported capacity of

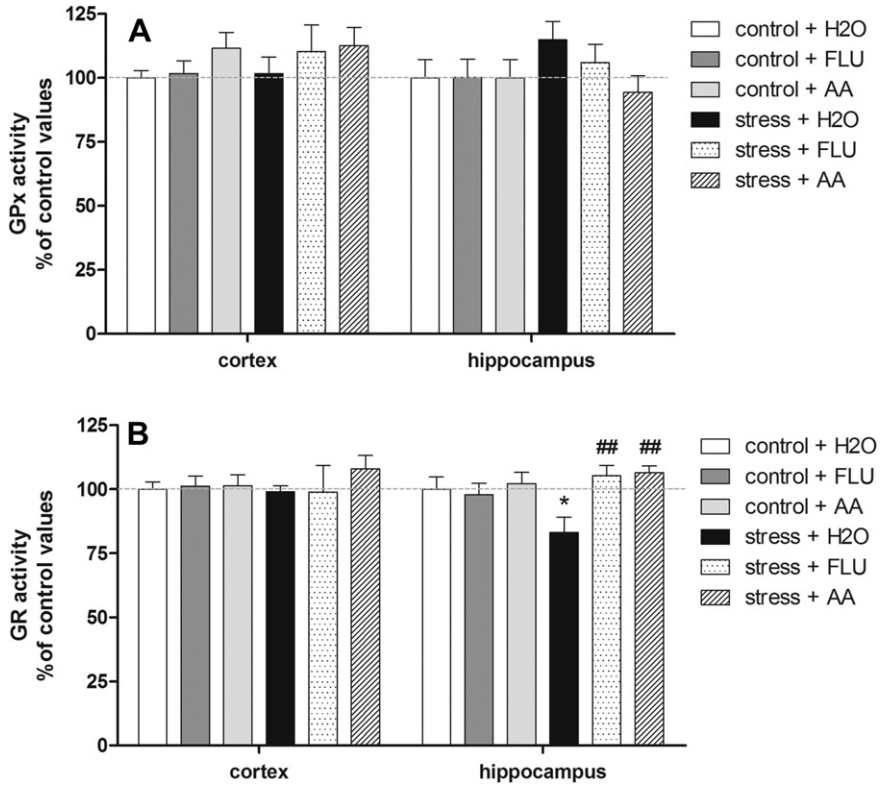

Fig. 6. Effect of repeated treatment with ascorbic acid and fluoxetine on GPx activity (panel A) and on GR activity (panel B) in cerebral cortex and hippocampus of mice submitted to CUS procedure. Bars represent means ± SEM of 5–7 mice. In the panel A mean ± SEM for 100% values correspond to 5 ± 0.13 nmol NADPH oxidized/min/mg protein for cerebral cortex and 4.69 ± 0.30 nmol NADPH oxidized/min/mg protein for hippocampus; in the panel B mean ± SEM for 100% values correspond to 14.64 ± 0.41 nmol NADPH oxidized/min/mg protein for cerebral cortex and 17.74 ± 0.83 nmol NADPH oxidized/min/mg protein for hippocampus. $^{*}p < 0.05$ vs. control mice, $\#\#p < 0.01$ vs. CUS + vehicle group, according to two-way ANOVA followed by the Duncan's post hoc test.

ascorbic acid to interact with the monoaminergic systems (Binfaré et al., 2009).

Our results showed no alteration in corticosterone levels in our experimental groups. Several studies have demonstrated that CUS exposure increases plasma/serum corticosterone levels (Chen et al., 2007; Garcia et al., 2009; Lu et al., 2006); on the other hand, no change in corticosterone levels after CUS procedure was also reported (Marin et al., 2007; Noschang et al., 2009). Literature data suggests that the adaptations of HPA axis depend on type, severity and duration of a stress regime (Aguilera, 1998; Blanchard et al., 1998; Laucher et al., 1994; Pitman et al., 1990). Moreover, the adaptations of the corticosterone secretion also seem to be dependent on stress intensity (Pitman et al., 1990) and rodent strain (Bielajew et al., 2002), which may explain, at least in part, the diversity of results regarding corticosterone level after chronic stress protocols.

In this study, we also investigated the influence of CUS paradigm on oxidative stress-related parameters in mice brain, such as markers of oxidative damage to lipids and antioxidant capacity. Our results showed that depressive-like behavior induced by CUS was paralleled by a significant lipid peroxidation, as evidenced by increasing amount of TBARS in cerebral cortex and hippocampus of mice. A previous study has already shown increased hippocampal lipid peroxidation in rats subjected to restraint stress (Fontella et al., 2005). Additionally, 21 days of exposure to different stressors resulted in increased lipid peroxidation in mice brain (Kumar et al., 2011). Besides, in agreement with our results and consistent with the idea that different animal models of depression stress-induced is associated with increased amount of TBARS, Lucca et al. (2009) demonstrated an increase in lipid peroxidation in cerebellum and striatum of rats after a 40-day chronic mild stress protocol. Accordingly, in humans, serum MDA was found to be increased in plasma and serum of patients with major depression as compared to control subjects (Bilici et al., 2001; Khanzode et al., 2003). Although the aforementioned experimental studies (Fontella et al., 2005; Kumar et al., 2011; Lucca et al., 2009) suggest a potential relationship between increased lipid peroxidation in specific encephalic structures and depressive-like behavior in stress-based depression models, the molecular mechanisms mediating stress-induced lipid peroxidation and the relationship between oxidative stress and depressive-like behavior are not completely understood. Our results shed light on this theme by showing that repeated administration of ascorbic acid (an antioxidant compound), which prevented CUS-induced depressive-like behavior, also restored the stress-induced lipid peroxidation, suggesting a potential relationship between both events. However, it is noteworthy that the effects of ascorbic acid on lipid peroxidation were region-specific: decreased TBARS levels were observed only in the hippocampus. Conversely, treatment with fluoxetine restored the lipid peroxidation in stressed mice only in the cerebral cortex. Consistent with our findings regarding the antioxidant effect of ascorbic acid, a study performed by Santos et al. (2009) showed that lipid peroxidation in the hippocampus during experimental seizures can be ameliorated with the administration of ascorbic acid. Moreover, ascorbate addition to cultured cells, brain slices, and brain microsomes prevents lipid peroxidation induced by different oxidizing agents (Kovachich and Mishra, 1983; Seregi et al., 1978), especially when combined with α-tocopherol, the most active and abundant form of vitamin E (Bano and Parihar, 1997; Sato et al., 1993). Anyway, the current literature does not

permit to state whether the antioxidant properties of ascorbic acid, which seem to be responsible for reversing CUS-induced hippocampal lipid peroxidation, are enough to elicit the observed antidepressant-like effects observed in our study.

The primary antioxidant defense system, which involves coordinated effects of superoxide dismutase, CAT and glutathione, has consistently been studied in depression (Ng et al., 2008). The present study found a decrease in CAT (cerebral cortex and hippocampus) and GR (hippocampus) activities and reduced levels of glutathione (cerebral cortex) in stressed mice, indicating an alteration in antioxidant brain defenses in CUS-induced depressive-like behavior. A decreased activity of CAT is associated with a large amount of H_2O_2 available to react with transition metals and to generate the radical hydroxyl (the most harmful radical), resulting in increased lipid peroxidation and, as consequence, neuronal damage (Kelner et al., 1995; Matés and Sánchez-Jiménez, 1999). In addition, since glutathione represents a main cellular non-protein antioxidant and redox regulator in protecting nervous tissue against reactive oxygen species (Meister and Anderson, 1983), the observed reduction in glutathione levels and GR activity further reinforces the notion that CUS paradigm induces increased oxidative damage in mice brain. Based on these evidences and on our own data, one could suppose that the significant oxidative damage observed in our study, which was herein evidenced by increased hippocampal and cerebro-cortical lipid peroxidation, is resultant of the decreased activities of CAT (hippocampus and cerebral cortex) and GR (hippocampus), as well as of the decreased glutathione levels (cerebral cortex). Interestingly, ascorbic acid or fluoxetine treatments, which prevented CUS-induced depressant-like behavior and hippocampal lipid peroxidation, also prevented CUS-induced decrease of CAT activity. These results are relevant because render catalase an important protein that might mediate the depressant-like effects and lipid peroxidation induced in the CUS model, as well as the beneficial effects of ascorbic acid and fluoxetine. These results are particularly important taking into account the recent findings pointing to CAT as an important enzyme whose levels are changed in the course of depression in humans (Galecki et al., 2009). Our findings on oxidative stress-related parameters are also in agreement with basic and clinical results which have reported that depression is accompanied by significantly lower antioxidant defenses against lipid peroxidation (Bilici et al., 2001; Eren et al., 2007a,b; Khanzode et al., 2003; Lucca et al., 2009; Ozcan et al., 2004).

Supporting our findings, consistent evidence for the antioxidant effects of antidepressants have been shown, since oxidative damage evaluated in plasma, serum and blood cells was reported to be reversed after treatment with classical antidepressants (Bilici et al., 2001; Herken et al., 2007; Khanzode et al., 2003), suggesting that antioxidant properties may contribute to their clinical effects. Furthermore, the treatment with N-acetyl cysteine, a GSH precursor, attenuates the depressive symptoms in patients (Berk et al., 2008) and treatment of human monocytes with fluoxetine increase the mRNA levels of − glutamylcysteine synthetase, the rate-limiting enzyme for the GSH synthesis (Schmidt et al., 2008). Additionally, it was demonstrated that micronutrients with antidepressant properties such as folic acid and zinc (Rosa et al., 2003; Brocardo et al., 2008) may be useful agents in the neuroprotection against brain damage caused by oxidative stress in rodents (Brocardo et al., 2010, 2007).

Our results showed no alterations in GPx activity in cerebral cortex and hippocampus of mice, independent of stress and treatment condition, data that are in agreement with a recent study performed by Tagliari et al. (2010), which found no changes in GPx activity in the brain of rats submitted to different mild stressors for 40 days. The absence of a pattern for changes in antioxidant enzyme activities as a result of stress and the drug treatments support the notion that cellular defense mechanisms to counteract oxidative stress do not always involve a coordinated regulation of all antioxidant enzymes and that their activities may be regulated by different mechanisms (Röhrdanz et al., 2000; Wilson and Johnson, 2000).

It is important to note that additional mechanisms might modulate the depressive-like and/or antidepressant behavior observed in our study. In fact, although monoamines' deficiency represents an important aspect involved in the pathophysiology of depression (Heuther et al., 1997; Pandey et al., 1992), the increased metabolism of monoamines can represent a cause of the increased ROS generation in depression (Fridovich, 1983). Additionally, increased glutamatergic transmission is characteristic of depression (Sanacora et al., 2012). Pathologically high levels of glutamate can cause excitotoxicity by allowing high levels of calcium ions to enter the cell, which, if present in excess, stimulate the production of ROS (Reynolds and Hastings, 1995; Savolainen et al., 1998). The aforementioned processes may cause direct damage to cellular proteins, DNA and lipids, and consequently lead to the loss of cell membrane fluidity and abnormalities of monoaminergic receptor function (Valko et al., 2007; Van-der-Vliet and Bast, 1992). Considering the ability of ascorbic acid to modulate monoaminergic and glutamatergic system (Binfaré et al., 2009; Moretti et al., 2011) and to impose a limitation on free radical reactions and concentration of their products by scavenging/neutralizing an array of reactive oxygen species (Traber and Stevens, 2011), one could suppose the involvement of the aforementioned events in its antidepressant-like effects. However, additional studies are necessary to confirm such hypothesis from a mechanistic point of view.

Importantly, the evaluated brain areas in this study represent different cell types and have, at least in part, heterogeneity in terms of physiological and metabolic characteristics, including differences in the antioxidant capacity (Prediger et al., 2004), cellular oxidative metabolism, production of compounds which can lead to enhanced formation of free radicals (Gamaro et al., 2003; Graumann et al., 2002), or heterogeneity in the distribution of iron in the mice brain (Benkovic and Connor, 1993). Thus, the analyzed brain structures may respond distinctly, which may explain some of the observed regional differences in the vulnerability to stress effects and treatments, a fact also reported by other authors (de Vasconcellos et al., 2006; Manoli et al., 2000; Prediger et al., 2004).

Finally, it is noteworthy that this study was performed using female mice, since several studies have shown that stress-related psychiatric disorders (e.g., depression, post-traumatic stress disorder) are twice as prevalent in women compared to men (Wong and Licinio, 2001). This higher prevalence of depression and other stress-related disorders in females implies an increased sensitivity of stress-related systems or substrates, as evidenced in previous studies which documented an increased responsiveness of the hypothalamic–pituitary–adrenal axis in female vs. male rats (Critchlow et al., 1963; Kitay, 1961; Le Mevel et al., 1979; Seale et al., 2004) and a higher sensitivity of noradrenergic system to certain stressors in brain of female rats (Curtis et al., 2006).

Taken together, the findings presented herein point to a genuine antidepressant-like effect for ascorbic acid, since it overcomes two parameters of depressive-like behavior induced by CUS model: the increase in the immobility time in the TST and the decreased grooming in the splash test. In addition, this vitamin improves the brain antioxidant system in parallel with behavioral changes, thus reinforcing the notion that oxidative stress plays a role in the pathogenesis and treatment of CUS-induced depression. Noteworthy, the behavioral and biochemical results obtained with ascorbic acid treatment are comparable to those obtained with

fluoxetine. These findings are especially interesting since CUS procedure have been suggested to meet criteria needed to be a valid animal model of depression. Thus, taking into account that the effects of CUS is closely related with depressive symptoms in humans and considering that many patients do not tolerate or respond adequately to the available drugs for treating depression, ascorbic acid may be further investigated as a new promising agent to improve the therapeutics for depression.

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Contributors

Ana Lúcia S. Rodrigues, Marcelo Farina and Morgana Moretti designed the study and wrote the protocol. Morgana Moretti wrote the first draft of the manuscript and undertook the statistical analysis. Morgana Moretti, André Colla and Grasiela O Balen performed the stress protocol and the behavioral tests. Danúbia B dos Santos, Andiara E de Freitas and Josiane Budni performed the biochemical analysis. All authors contributed to and have approved the final manuscript.

Disclosure/conflict of interest

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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