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ORIGINAL ARTICLE

Attenuation of acute restraint stress-induced depressive like behavior and hippocampal alterations with protocatechuic acid treatment in mice

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Abstract Protocatechuic acid ethyl ester (PCA), a phenolic compound, exhibits neuroprotective effects through improving endogenous antioxidant enzymatic and nonezymatic system. Based on the role of oxidative stress in modulating depressive disorders and the relationship between neuroprotective and antioxidant potential of PCA, we studied if its antidepressant like effect is associated by modulation of cerebral cortex and hippocampal antioxidant alterations. Acute restraint stress (ARS) is known to induce depressive like behavior by neuronal oxidative damage in mice. Swiss albino mice subjected to ARS exhibited an increased immobility time in forced swim test, elevated serum corticosterone and produced oxidative stress dependent alterations in cerebral cortex and hippocampus mainly increased thiobarbituric acid reactive substances and reduced catalase (CAT), superoxide dismutase (SOD) activity. Treatment with PCA was able to prevent stress induced immobility time in forced swim test without altering locomotor activity in mice. Further, PCA treatment attenuated the elevation of serum corticosterone, lipid peroxidation and restored enzymatic antioxidants in cerebral cortex and hippocampus in ARS mice. Altogether, the experimental findings demonstrate the notion that PCA exhibit antidepressant like activity might be related, at least in part, to its capability of modulating antioxidant defense system and oxidative damage induced by ARS in cerebral cortex and hippocampus in mice and thus maintain the pro−/anti-oxidative homeostasis.

Keywords Acute restraint stress · Serum corticosterone · Depressive like-behavior · Hippocampal antioxidants · Protocatechuic acid ethyl ester

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Introduction

Stress is a state of vulnerable homeostasis which leads to altered physiological and behavioral responses. The alterations depend on severity of stress, type and duration of stressful events and also an attempt to re-establish body homeostasis subsequently results into dysfunctioning the central nervous system (CNS) (Burri et al. 2013; Chrousos 2009; Jaggi et al. 2011). These CNS alterations may be accountable in the development of psychiatric disorders like depression, associated with cognitive alterations (Calabrese et al. 2011). Similar relationship among stressful life events and occurrence of pathological aspects of depressive disorders (especially in genetically predisposed individuals) were previously documented (Hammen et al. 2009; Mazure 1998). Generation of excessive free radicals due to oxidative stress and subsequently defective antioxidant defense along with enhanced lipid peroxidation are implicated in depressive disorders (Thakare and Patel 2015). In addition, impaired hypothalamic-pituitary-adrenal axis (HPA-axis) is known to participate in the induction of depressive like behavior (Gold et al. 1988; Morris et al. 2012). The disproportionate elevation of serum corticosterone level in response to impaired HPA axis is responsible for neuronal damage which is manifested to hippocampal atrophy in depressed patients (Lee et al. 2002; McKinnon et al. 2009, Thakare et al. 2016).

The ARS stress is frequently used to induce depressive like behavior in experimental animals (Capra et al. 2010; Zafir et al. 2009; Christiansen et al. 2011; Freitas et al. 2014). It has been reported that variety of stress, like restraint stress, show imbalance of brain antioxidant system (Budni et al. 2013; de Balk et al. 2010; Enache et al. 2008; Freitas et al. 2014; Kumar and Goyal 2008), and clinically used antidepressant drugs reverse such changes (Kumar et al. 2009; Zafir and Banu 2007). ARS, an acute model of depression, mainly constitute both emotional and physical components associated with dysfunctional endogenous antioxidant defense system (Buynitsky and Mostofsky 2009). In this model, immobilization of animals for 7 h exhibited unavoidable physical and mental stress which is difficult to adapt (Jaggi et al. 2011) This subsequently manifested to depressive like behavior, evidence by increased immobility time in FST, enhanced lipid peroxidation and reduced antioxidant enzymes in brain (Capra et al. 2010; Freitas et al. 2014; Budni et al. 2013; Bettio et al. 2014).

Protocatechuic acid ethyl ester (Ethyl 3,4-dihydroxybenzoate, PCA; see Fig. 1) is a phenolic compound of *Hibiscus sabdariffa* and *Eucommia ulmoides* (Lin et al. 2003; Pacheco-Palencia et al. 2008). It exhibited neuroprotective effects probably through reducing free radical formation via promoting the endogenous antioxidant enzymatic activity in hydrogen peroxide (H_2O_2) induced oxidative damage (Shi et al. 2006) and is also beneficial in Parkinson's disease (An et al. 2006; Guan et al. 2006).

Previous studies from our laboratory have demonstrated that PCA treatment attenuated the malondialdehyde (MDA) formation, and restored the depleted antioxidants in cerebral ischemic rats and thus improved behavioral and biochemical alterations (Muley et al. 2013; Muley et al. 2012). Further, PCA pre-treatment results in modulation of cellular redox status with the restoration of endogenous antioxidant enzymes, and decreased MDA formation (Zhang et al. 2015). Although, PCA exerts neuroprotective by modulating antioxidant system, its effects on depressive behavior are scientifically unknown. Therefore, the objective of present research studies is to investigate if PCA treatment could attenuate ARS induced behavioral and biochemical alterations in mice and thus induce antidepressant like activity.

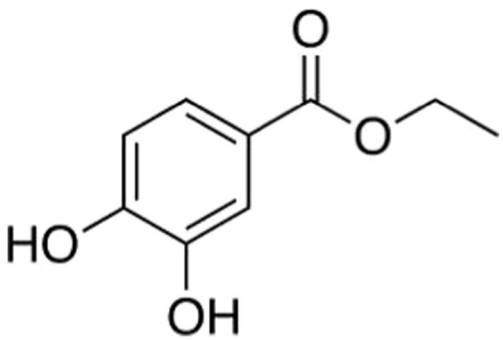

Fig. 1 Protocatechuic acid ethyl ester (PCA)

Materials and methods

Experimental animals

Swiss albino mice of either sex weighing 35–40 g (80–90 days old) were procured from Serum Institute of India, Pune, India. They were housed separately (10 mice/ per cage) in a polycarbonate cage (cage size 29 cm × 22 cm × 14 cm) under standard laboratory conditions with Food and water were available ad libitum, and lights were on from 07:00 to 19:00 h. All the mice were acclimatize to laboratory condition for 1 week prior to experiment. The protocol was approved by the Institutional Animal Ethics Committee with approval no. SIPS/IAEC/2014–15/01 and conformed to the Indian National Science Academy guidelines for the use and care of experimental animals in research. The present study was conducted at 7.30 h in the morning of the experimental day.

Drugs

PCA was purchased from Sigma Co (St. Louis USA) was prepared in 1 % (*w*/*v*) carboxy methyl cellulose (CMC) and administered orally (po) by gavage at a dose of 100 and 200 mg/kg 1 h prior to ARS procedure. The fluoxetine (Sigma Co (St. Louis USA) as standard drug for the present study was selected as per the documented reports of Jindal et al. (2013), prepared in CMC solution and administered at 20 mg/kg (po). The doses of PCA were selected from conducting pilot experiment (dose finding studies) and our earlier documented findings (Muley et al. 2012; Muley et al. 2013). PCA and fluoxetine solution were freshly prepared and administered in a volume of 10 ml/kg. Corticosterone ELISA kit was purchased from Arbor Assays, USA.

Acute restraint stress (ARS)

The mice were divided into six groups ($n = 10$) and they were treated in following way. Group I-non-stressed with received 1 % (*w*/*v*) carboxy methyl cellulose (CMC) vehicle (10 ml/kg, p.o.). Group II, III-non stressed with PCA 100 mg/kg and 200 mg/kg per oral (p.o.) respectively, Group IV-non stressed with fluoxetine (20 mg/kg., p.o.), Group V stressed with CMC vehicle, Group VI, VII- stressed with PCA 100 mg/kg and 200 mg/kg, p.o. respectively and group VIII stressed with fluoxetine (20 mg/kg., p.o.). ARS was performed in mice as per method described earlier (Freitas et al. 2014; Kumar and Goyal 2008). Briefly, the mice were subjected to immobilization for 7 h (duration of restraint stress) using an individual rodent restraint device made of Plexiglas fenestrate,

restraining all physical movement causing minimum pain. The animals were deprived of food and water during the ARS experiment. The PCA (100 and 200 mg/kg) and fluoxetine (20 mg/kg) were administered 1 h prior to ARS procedure. After 7 h, independent group of mice were released from their closure and 40 min post-release they were evaluated for behavioral changes by forced swim and open field test (i.e. the forced swim and Open-field test were performed 8 h 40 min after PCA and fluoxetine administration).

Behavioral assessment

Forced swim test (FST) in ARS and non stressed mice

Immobility time in nonstressed and ARS subjected mice was evaluated as per the method previously described by Porsolt et al. (1977). Briefly, mice were forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; and the immobility time was recorded by video tracking system (VJ Instruments, India). Individual animal was judged to be immobile when it ceased struggling and remained floating motionless in the water, showing only those movements essential to keep its head above water. A reduction in immobility time was considered as indication of an antidepressant-like activity.

Open-field test (OFT) in ARS and non stressed mice

OFT was performed as per the method described by Freitas et al. (2014) and Rodrigues et al. (1996). The number of squares crossed with all paws (crossings) of mouse and time spent in the centre of open field was counted in a 6 min session. The number of line crosses is usually used as measures of locomotor activity, but are also measures of exploration and anxiety. The apparatus was cleaned with 10 % ethanol between tests in order to hide animal clues.

Biochemical studies

After behavioral studies, mice were anaesthetized with mild ether anaesthesia, blood was withdrawn from retro-orbital plexus, and serum was separated by centrifugation (REMI, USA) and used for estimation of corticosterone.

After blood withdrawal, mice were sacrificed by decapitation, the cerebral cortex and hippocampi were removed and homogenized (1:10 *w*/*v*) in phosphate buffer solution and centrifuged at 16,000×g, at 4 °C for 20 min. The resultant supernatant solutions were used for determination of enzymatic antioxidant, GSH, and MDA levels.

Determination of serum corticosterone level in ARS and non stressed mice

Serum corticosterone levels were estimated by ELISA technique as per the procedure described by manufacturer's instructions. Briefly, 75 μl of assay buffer was added into non-specific binding (NSB) wells;50 μl of assay buffer were added into wells to act as maximum binding wells (0 pg/ml); 50 μl serum samples or standard were added into wells in the plate, and then 25 μl of corticosterone conjugate followed by addition of 25 μl corticosterone antibodies to each well except NSB wells. The plate was tapped for adequate mixing of the reagents, plate was sealed and shaken and incubated for 1 h. After one hour, the plate was washed 4 times with 300 μl of wash buffer solution. The plate was dried on clean absorbent towels. To each well, 100 μl of 3,3′,5,5′-Tetramethylbenzidine(TMB) substrate was added and plate was incubated for 30 min. Then, 50 μl of the stop solution was added to each well and absorbance was read at 450 nm by using ELISA reader (Biotek, Germany). The results were expressed as ng/ml of corticosterone level.

Assay of lipid peroxidation (MDA formation) in hippocampus and cerebral cortex of ARS and non stressed mice

The lipid peroxidation process was assayed by estimation of MDA formation in hippocampus and cerebral cortex as per the procedure described by Ohkawa and Nobuko (1979)). Briefly, the samples were incubated for 1 h at 100 °C in 0.45 % sodium dodecyl sulfate and 0.67 % thiobarbituric acid. The resultant mixture was centrifuged and absorbance was taken at 532 nm on spectrophotometer (Shimadzu 1700 UV). The amount of MDA formed was expressed as nM of MDA/mg of protein.

Assay of enzymatic and non enzymatic antioxidants in ARS and non stressed mice

The SOD activity was performed as per the procedure described by Mishra and Fridovich (1972) in the homogenates of cerebral cortex and hippocampus. Briefly, 0.5 aliquots mixed with 0.5 ml of distilled water, to this mixture 0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform were added, the resultant mixture was centrifuged (2000×g for 10 min at −4 0C), and supernatant was separated. To the aliquot of 0.5 ml supernatant, 1.5 ml of carbonate buffer (pH 10.2) and 0.5 ml of EDTA were added. At the end, 0.4 ml of epinephrine (3 μM) was added and the optical density/min at 480 nm was measured on the Shimadzu 1700 UV spectrophotometer. The SOD activity was expressed as U/g of wet tissue.

The CAT activity was performed as per the method described Aebi (1984). Briefly, the homogenates of cerebral cortex and hippocampus was diluted 20 times using phosphate buffer, 0.2 ml of this diluted homogenate was taken, 1 ml of hydrogen peroxide (8.5 μl of H_2O_2 in 2.5 ml phosphate buffer) was added. The change in absorbance was read at 240 nm on the Shimadzu 1700 UV spectrophotometer. The CAT activity was expressed as mM of H_2O_2 formed/min/g of wet tissue.

The GSH levels were determined as non-protein thiols (NPSH), as per the method described by Ellman (1959). Briefly, hippocampal and cerebral cortex homogenates were precipitated in 10 % cold trichloroacetic acid, centrifuged at 5000×g for 10 min, and the supernatant was incubated with DTNB 5,5′-dithiobis(2-nitrobenzoic acid) in a 1 M phosphate buffer (pH 7.0). The absorbance was measured at 412 nm on the Shimadzu 1700 UV spectrophotometer. The results were expressed as nM of nonprotein thiol/mg of protein.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. A value of $P < 0.05$ was considered to be statistically significant. The statistical analysis was carried out by using the software GraphPad Prism trial version 7 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effects of PCA on immobility time in ARS and non stressed mice

Two-way ANOVA revealed significant differences for ARS stress [F (1, 40) =348.8, $p = 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =38.3, $p < 0.0001$] and stress × treatments interaction [F (3, 40) =13.61, $p < 0.0001$] in the immobility time in FST. Post hoc analysis showed that mice treated with PCA and fluoxetine significantly attenuated the immobility time in ARS mice compared to vehicle control ARS mice (Fig. 2). However, we did not observe any significant differences between PCA, fluoxetine and vehicle treatment in non stressed animals.

Effects of PCA on locomotor activity in ARS and non stressed mice

Two-way ANOVA revealed non significant changes for stress [F (1, 40) =1.615, $p = 0.2112$], treatments (PCA or fluoxetine) [F (3, 40) =0.2911, $p = 0.8316$] and stress ×treatments interaction [F (3, 40) = 0.1117, $p = 0.9528$] on crossing numbers (Fig. 3a) and [F (1, 40) =1.008, $p = 0.321$], treatments (PCA or fluoxetine) [F (3, 40) =0.7466, $p = 0.5308$] and stress ×treatments interaction [F (3, 40) = 0.8277, $p = 0.4865$] on time spent in centre in OFT (Fig. 3b). Post hoc data suggest ARS mice did not induce any significant changes compared to non stressed mice. Further, PCA and fluoxetine treatment too did not showed any significant changes in both nonstressed and ARS.

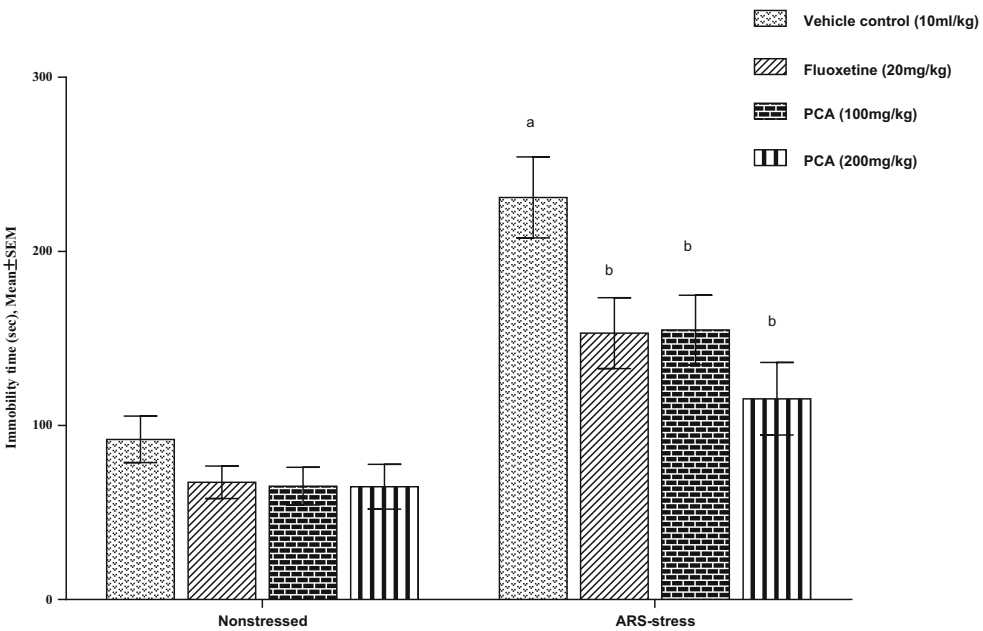

Fig. 2 Effect of PCA, fluoxetine or vehicle on immobility time in FST in nonstressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, $^{a}P < 0.0001$ when compared to nonstressed vehicle group, *(*two-way ANOVA followed by Bonferroni post hoc test.)

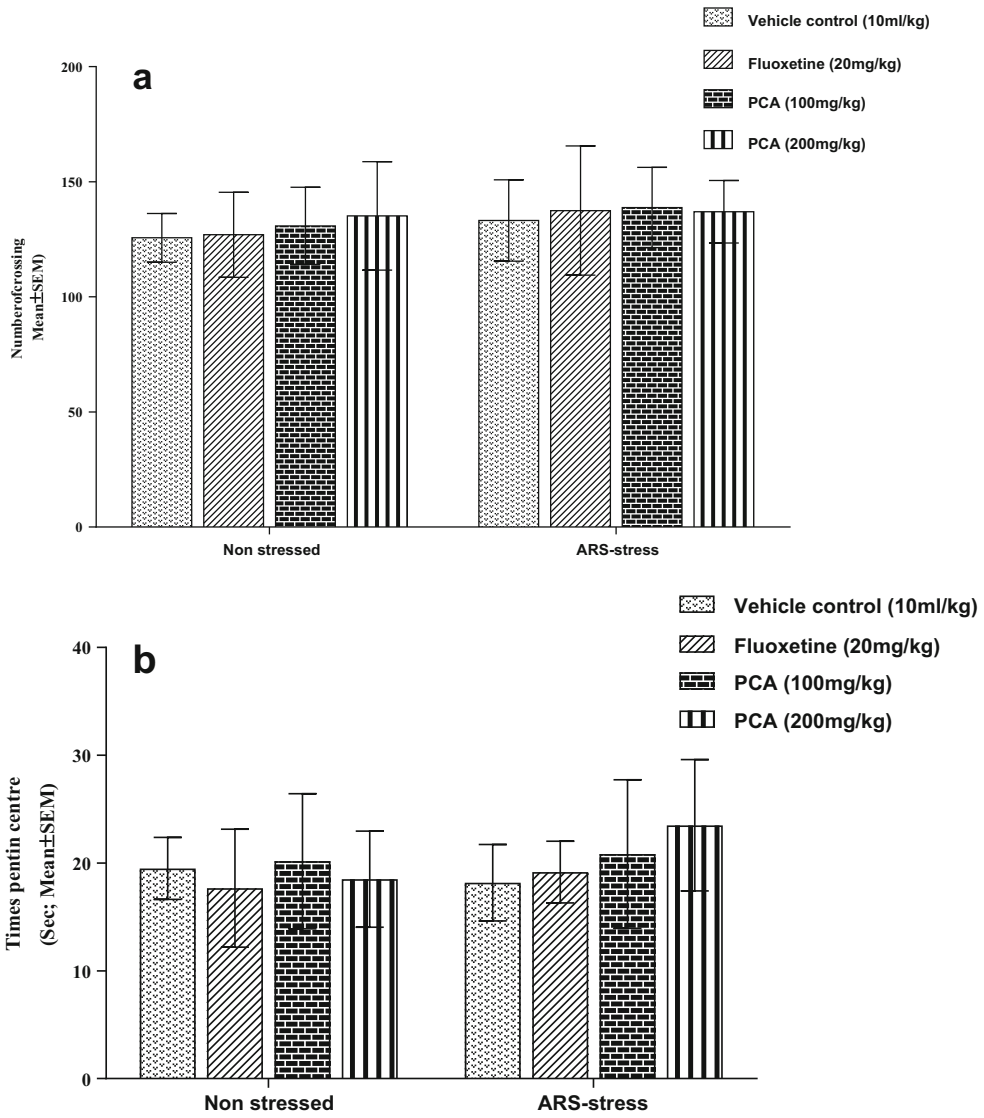

Fig. 3 Effect of PCA, fluoxetine or vehicle on number of crossings behavior **a** and time spent in centre **b** by OFT in nonstressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, (two-way ANOVA followed by Bonferroni post hoc test.)

Effects of PCA on serum corticosterone in ARS and non stressed mice

In Fig. 4 depicted the effects of PCA on serum corticosterone in stressed and non stressed mice. Two-way ANOVA demonstrated a significant effect for ARS stress [F (1, 40) =836.2, $p < 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =20.25, $p < 0.0001$] and stress × treatments interaction [F (3, 40) = 20.29, $p < 0.0001$] in serum corticosterone level in mice. Post hoc results enumerates mice exposed to ARS showed increased serum corticosterone compared to the nonstressed + vehicle group and subsequently PCA and fluoxetine treatment reduced serum corticosterone due to ARS stress.

Effects of PCA on MDA formation in hippocampus and cerebral cortex in ARS and non stressed mice

Two-way ANOVA showed a significant effect for ARS stress [F (1, 40) =267.8, $p < 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =13.59, $p < 0.0001$] and stress × treatments interaction [F (3, 40) = 10.51, $p < 0.0001$] in MDA formation in hippocampus. Post hoc results indicated that mice subjected to ARS presented a significant elevation of MDA formation in hippocampus compared to nonstressed + vehicle group. PCA and fluoxetine treatment significantly attenuated MDA formation in hippocampus as compared to ARS+ vehicle control group (Fig. 5a).

Two-way ANOVA demonstrated significant effect for stress [F (1, 40) =938.1, $p < 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =61.15, $p < 0.0001$and stress × treatments interaction [F (3, 40) = 49.36, $p < 0.0001$] in MDA formation in cerebral cortex. Post hoc results indicated that mice subjected to ARS presented a significant elevation of MDA formation in cerebral cortex compared to nonstressed + vehicle group. Administration of PCA significantly reduced MDA formation in cerebral cortex (Fig. 5b). However, fluoxetine treatment did not produce any significant changes in

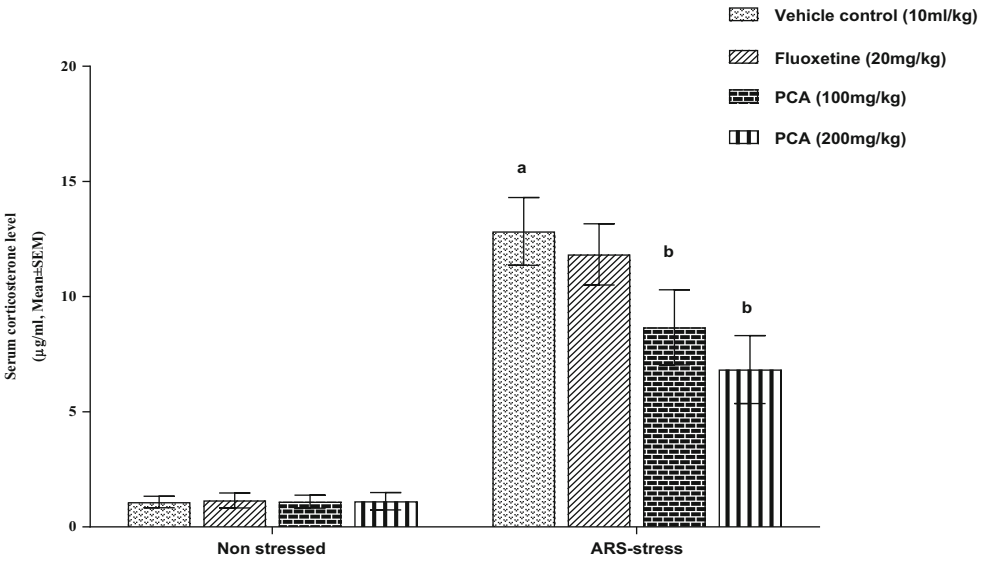

Fig. 4 Effects of PCA, fluoxetine or vehicle on serum corticosterone level in nonstressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, $^{a}P < 0.0001$ when compared to nonstressed vehicle group, (two-way ANOVA followed by Bonferroni post hoc test.)

MDA formation in cerebral cortex compared to vehicle treated ARS mice.

Effects of PCA on SOD, CAT activity and GSH levels in hippocampus and cerebral cortex in ARS and non stressed mice

Two-way ANOVA exhibited significant effect for stress [F (1, 40) =460.8, $p < 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =25.5, $p < 0.0001$] and stress × treatments interaction [F (3, 40) = 23.14, $p < 0.0001$] in SOD activity, [F (1, 40) =481, $p < 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =6.664, $p = 0.0010$] and stress × treatments interaction [F (3, 40) = 12.54, $p = 0.0030$] in CAT activity, and non significant changes for stress [F (1, 40) = 1.439, $p = 0.2374$], treatments (PCA or fluoxetine) [F(3,40) = 0.2734, $p = 0.2374$] and stress × treatments interaction [F (3,40) = 0.8873, $p = 0.4560$] in NPSH content in the hippocampus of mice. Post hoc results suggested that mice subjected to ARS induced significant reduction of SOD and CAT activities in hippocampus compared to nonstressed + vehicle control group. Treatment with PCA restored the SOD and CAT activities in hippocampus as compared to ARS+ vehicle control group (Figs. 6a and 7a). However, non significant alterations were observed in NPSH contents either in ARS subjected or drug treated groups compared to non stressed group (Fig. 8a).

Two-way ANOVA exhibited significant effect for stress [F (1, 40) =801.3, $p < 0.0001$], treatments (PCA or fluoxetine) [F (3, 40) =101.8, $p < 0.0001$] and stress × treatments interaction [F (3, 40) = 60.23, $p < 0.0001$] in SOD activity, [F (1, 40) =709.9, $p < 0.0001$], treatments (fluoxetine or PCA) [F (3, 40) =3.869, $p = 0.0161$] and stress × treatments interaction [F (3, 40) = 10.15, $p < 0.0001$] in CAT activity, and non significant changes for stress [F (1, 40) = 0.4286, $p = 0.5164$], treatments (fluoxetine or PCA) [F(3,40) = 1.208, $P = 0.3194$] and stress × treatments interaction [F (3,40) = 2.404, $p = 0.0816$] in NPSH content in the cerebral cortex of mice. Post hoc results suggested that mice subjected to ARS induced significant decrement in of SOD and CAT activities in cerebral cortex compared to nonstressed + vehicle control group. Treatment with PCA significantly restored the SOD and CAT activities in cerebral cortex of mice compared to ARS+ vehicle control group (Figs. 6b and 7b). For hippocampus too, we did not observed any significant changes on NPSH contents either in ARS subjected or drug treated groups compared to non stressed group (Fig. 8b).

Discussion

Previous research report documented that; PCA can penetrate the blood brain barrier and induced neuroprotection (Zhang et al. 2011). Increase in immobility time by FST is considered as passive behavior that disengages the mice from active forms of coping with applied stressful stimulus (Moretti et al. 2013). In the present experiment, we noticed that mice subjected to ARS exhibited significant increase in immobility time compared to nonstressed mice in FST model and subsequently attenuated with PCA treatment suggesting the attenuation of depressive behavior. These effects are in support of findings of Kim et al. (2012) in which they demonstrated protocatechuic acid isolated from *Gardenia jasminoides* might induced antidepressant property by inhibiting the activities of both monoamine oxidase-A (MAO-A) and MAO-B along with increased 5-HT in the brain tissues in rats (Kim et al. 2012). However, we did not observed any significant changes on immobility time in nonstressed mice with either PCA (100 and 200 mg/kg) or fluoxetine (20 mg/kg), it might possible that, PCA or fluoxetine elicits their inhibitory effects on immobility time mainly in stressed

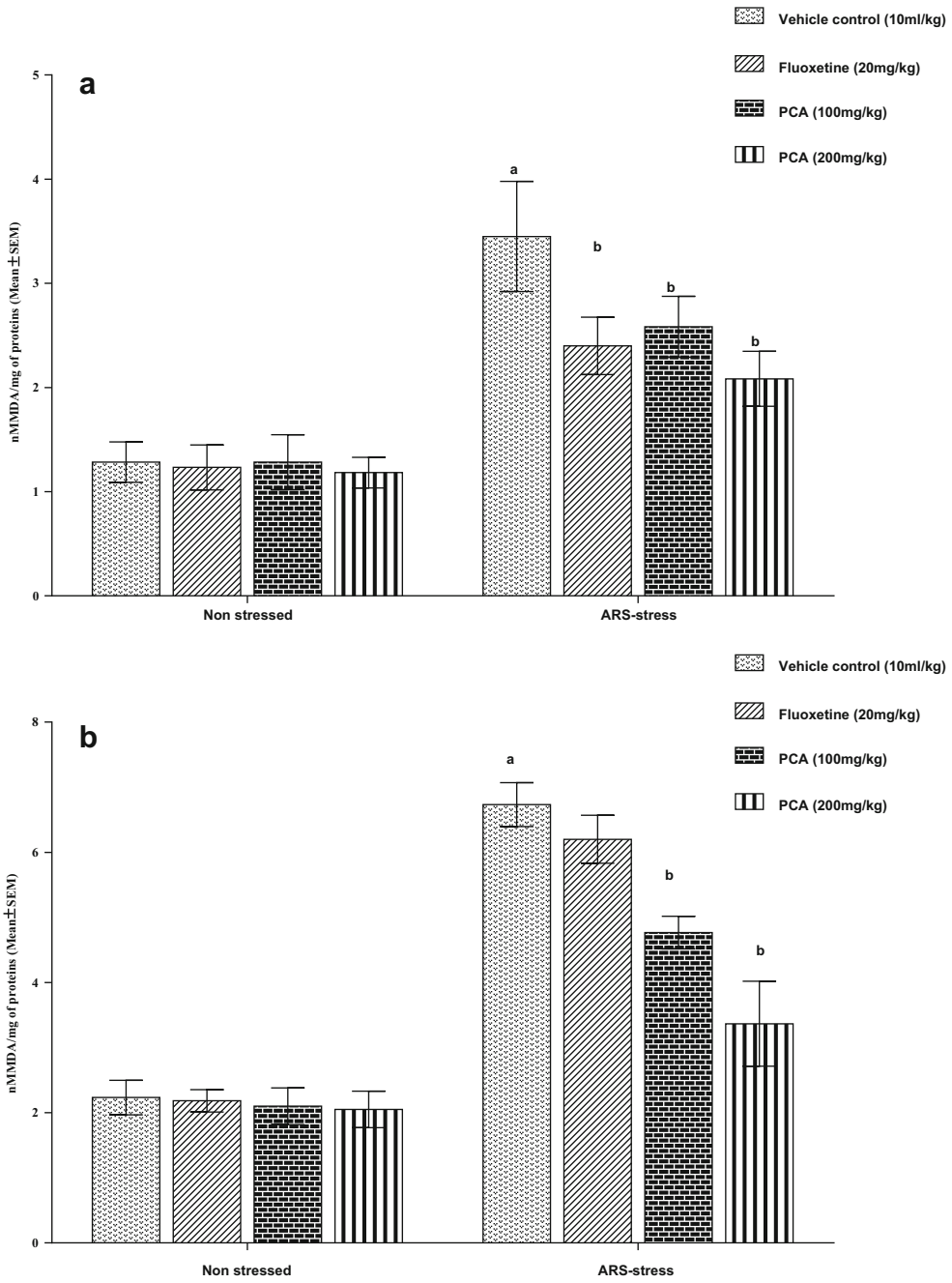

Fig. 5 Effects of PCA, fluoxetine or vehicle on MDA formation in hippocampus **a** and cerebral cortex **b** in nonstressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, $^{a}P < 0.0001$ when compared to nonstressed vehicle group, (two-way ANOVA followed by Bonferroni post hoc test.)

condition (ARS). Although, in our present finding we did not observed any significant changes in nonstressed mice in FST, the reason behind could be that, fluoxetine exhibits its effects mainly in stressed condition which are contrary to findings of Ostadhadi et al. (2016). The possible reason behind the lack of antidepressant effects in non stressed group might be due to fact that they PCA and fluoxetine were administered in a single dose at 8 h 40 min prior to FST. The extended time period was not adequate to induce reduction in immobility time probably because the anti-immobility effects of PCA and fluoxetine are related to changes which occur only after acute treatments. Further, we evaluated the mice for locomotor activity by OFT test to predict the improvement of depressive behavior due to locomotor property. Since, depressive behaviors are often associated with anxiety that share several overlapping symptoms including fatigue, impaired concentration, irritability (Ressler and Nemeroff 2000). We determined, the crossing behavior and time spent in centre of the OFT test. Time spent in centre of OFT was analyzed in order to study the anxiety like behavior in the nonstressed and ARS mice. Duration of time spent in the centre of open field is measures of exploratory behavior and anxiety. A high frequency/duration of these behaviors indicates high exploratory behavior and low anxiety levels. Herein, we found that PCA treatment did not

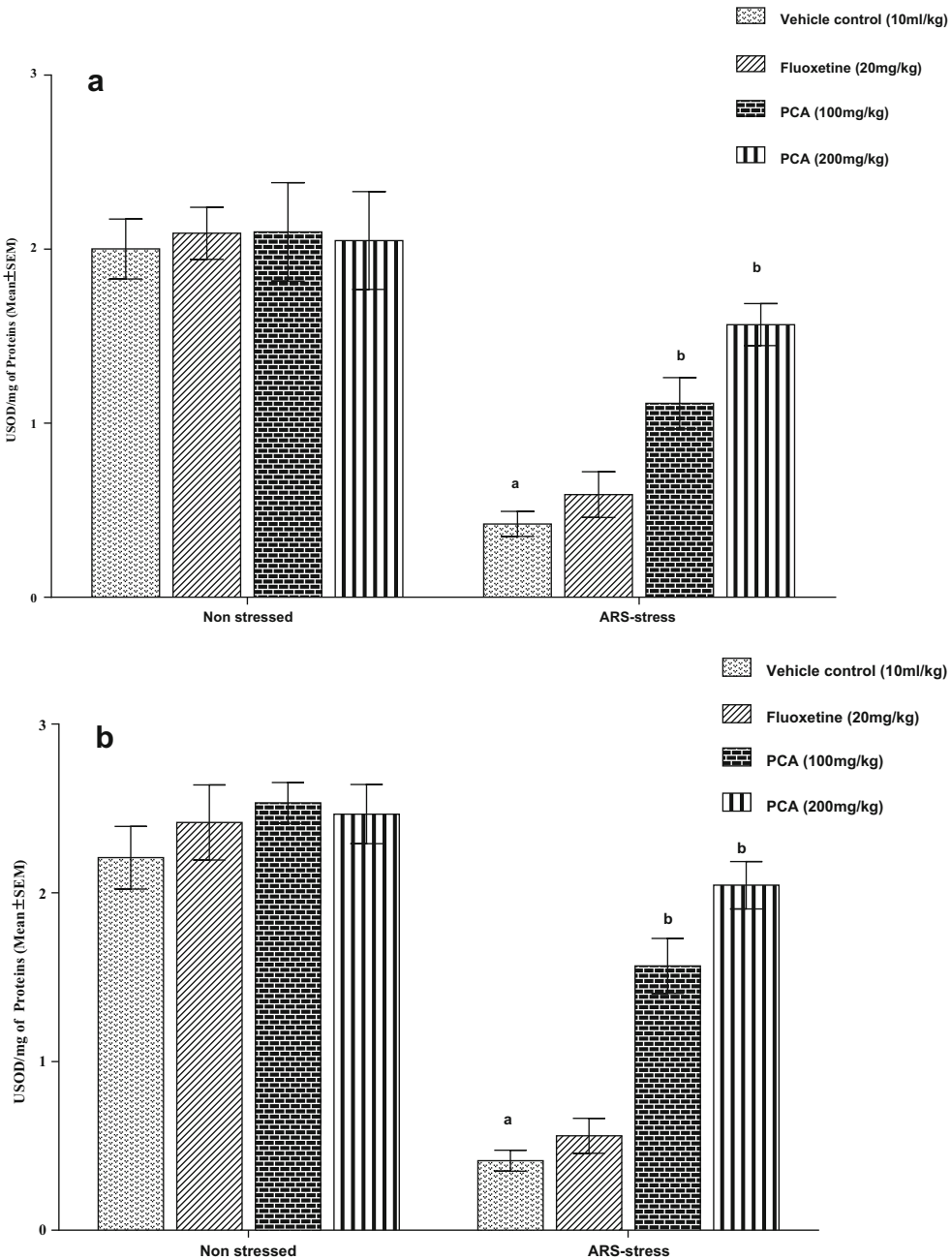

Fig. 6 Effects of PCA, fluoxetine or vehicle on SOD activity in hippocampus **a** and cerebral cortex **b** in nonstressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, $^{a}P < 0.0001$ when compared to nonstressed vehicle group, (two-way ANOVA followed by Bonferroni post hoc test.)

exhibit any significant changes on crossing behavior and time spent in centre in OFT indicating that locomotor activity and anxiety does not participate in the antidepressant like activity PCA in the present study. It might possible that acute stress does not induce anxiety and exploration (locomotor activity) and only possible during sub-chronic or chronic stress conditions. Therefore, crossing behavior and time spent in centre of open field in OFT did not affected with PCA or fluoxetine in nonstressed and ARS mice., The finding are in agreement with reports of Budni et al. (2013) where in nonstressed and ARS mice did not showed any alteration in locomotor activity in OFT.

Increased corticosterone in rodents is an indicator of HPA axis hyperactivity in response to stress and is considered to be "final common pathway" in the pathogenesis of depression and reversal of serum corticosterone level with antidepressant therapy stabilizes the HPA axis in stressed mice (Jin et al. 2015). Further, oxidative stress is known to induce hypersecretion of corticosterone indicating HPA axis dysfunction (Kobayashi et al. 2009). Moreover, elevated corticosterone levels have been implicated in the induction of behavioral, neurochemical alterations consequently resulting into depressive-like symptoms (Zhao et al. 2009; Lee et al. 2009; Crupi et al. 2010; Mao et al. 2014; Thakare et al. 2016). In the

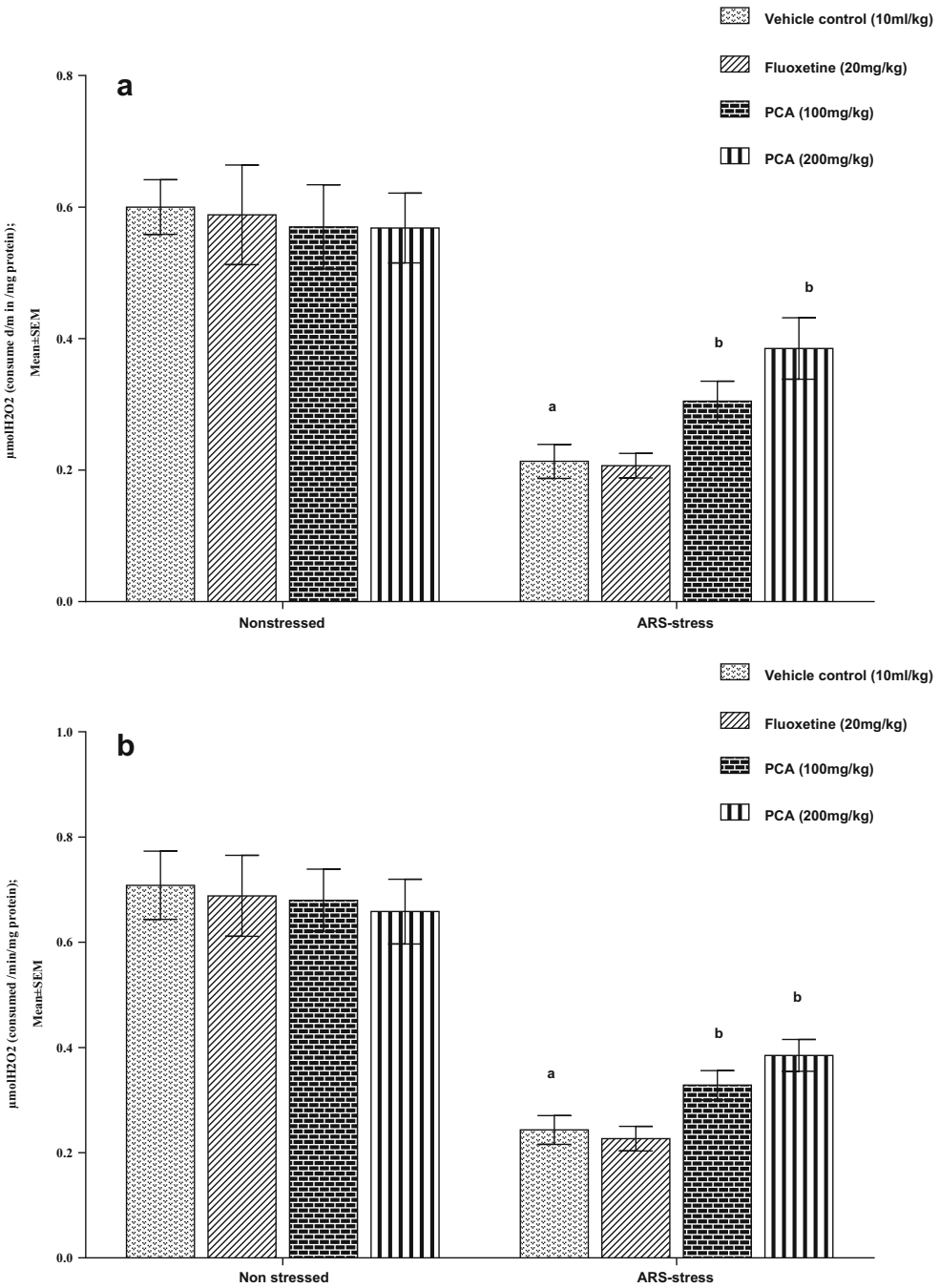

Fig. 7 Effects of PCA, fluoxetine or vehicle on CAT activity in hippocampus **a** and cerebral cortex **b** in nonstressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, $^{a}P < 0.0001$ when compared to nonstressed vehicle group, $^{b}P < 0.0001$ when compared to ARS vehicle group (two-way ANOVA followed by Bonferroni post hoc test.)

present experiment too, we found increased serum corticosterone in stressed mice and which was subsequently attenuated with PCA or fluoxetine treatment suggesting its antidepressant potential in acute stress condition.

Due to high rate of oxygen consumption, the CNS is more vulnerable to free radicals formation and consequently neuronal damage has been implicated in depression (Zafir et al. 2009). Oxidative stress in brain occurs due to several factors viz. presence of iron in brain, low endogenous antioxidant enzymatic activity, production of free radicals, and presence of high content of polyunsaturated fatty acids, an additional factor the large dependence of brain on oxidative phosphorylation for energy making the brain more susceptible to peroxidation process (Muley et al. 2012). The patients with depressive episodes are found to exhibit elevated plasma peroxide levels and higher MDA formation due to oxidative stress (Sarandol et al. 2007). These free radicals can be neutralized and/or quenched by endogenous antioxidant enzymes viz. SOD, CAT, which are however, impaired in depression (Sarandol et al. 2007; Thakare and Patel 2015). Lipid peroxidation is an important process of injury of cells/tissues during oxidative stress. A significant amount of production of ROS

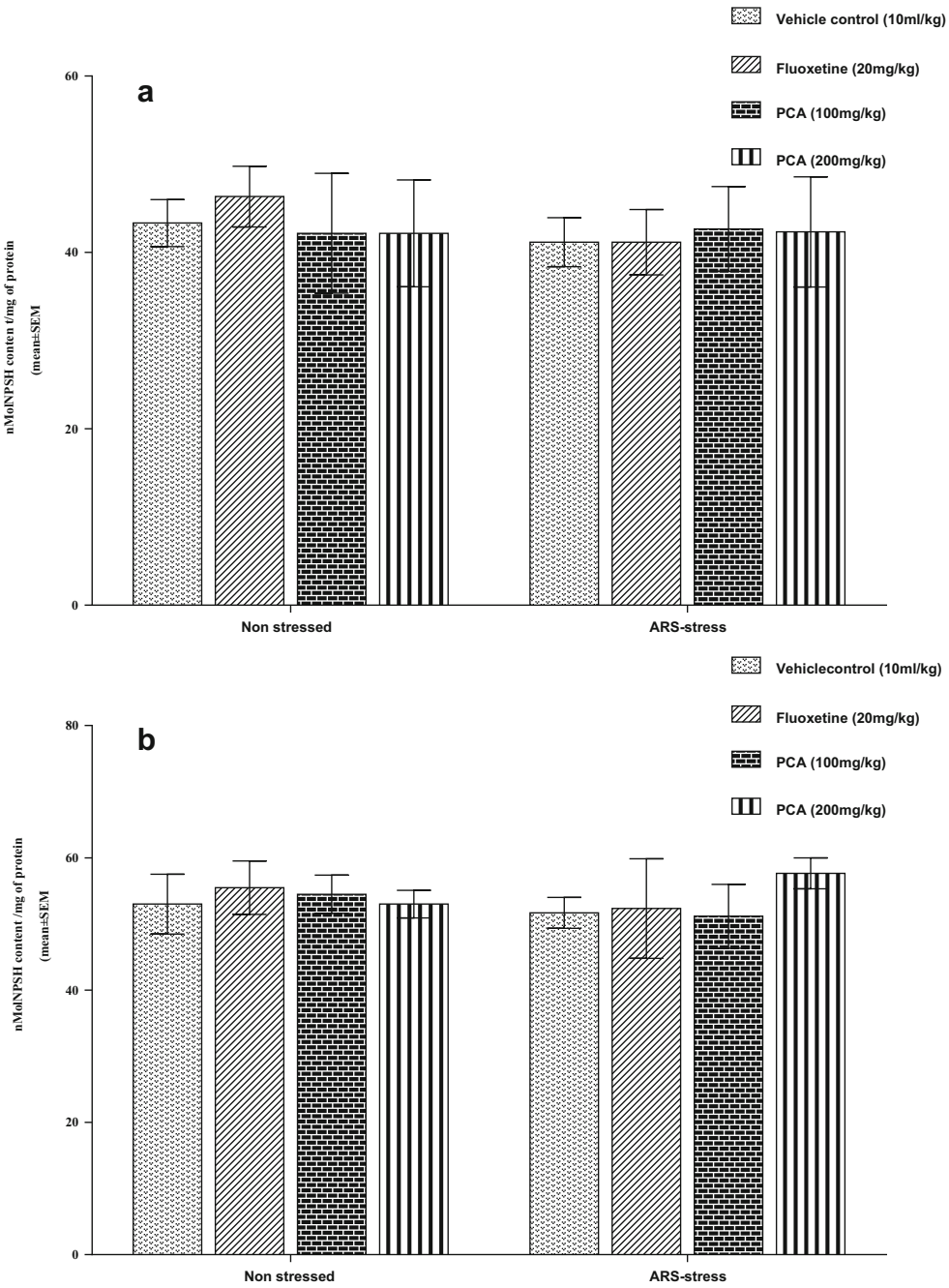

Fig. 8 Effects of PCA, fluoxetine or vehicle on GSH levels in hippocampus **a** and cerebral cortex **b** in non stressed and ARS subjected mice. PCA (100 and 200 mg/kg, p.o.), fluoxetine (20 mg/kg, p.o.) and vehicle (10 ml/kg, p.o.) were administered at 9.00 am, 1 h prior to ARS procedure. Control group received CMC 1 % (10 ml/kg, p.o.). Data are presented as mean ± SEM, $n = 10$, $^{a}P < 0.0001$ when compared to nonstressed vehicle group, (two-way ANOVA followed by Bonferroni post hoc test.)

during stress is responsible for lipid peroxidation process as demonstrated in terms of increased MDA formation an end product of lipid peroxidative process (Niki 2012; Lang and Borgwardt 2013). Moreover, experimental animals subjected to ARS exhibit elevated thiobarbituric acid reactive substances (TBARS) in hippocampus and which was abolished with drug treatments (de Balk et al. 2010; Fontella et al. 2005; Budni et al. 2013; Freitas et al. 2014). Although, there are various pro-xidants such as thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), and, possibly, hydroxyl ($OH^{\bullet}$), in the present studies, we determined only TBARS which is the limitation of the current study. In the present study, mice subjected to ARS showed reduction of SOD, CAT activities along with increased MDA formation in the hippocampus and cerebral cortex and treatment with PCA prevented the elevation of MDA formation in hippocampus and cerebral cortex, thus reduced oxidative stress. These findings are in agreement with the report of Zafir et al. (2009), and Kalueff et al. (2007) in which they demonstrated prevention of MDA formation with antidepressants treatment. Therefore, increased MDA formation has been considered to be culprit

in the hippocampal and cerebral cortex damage and might participate in the development of depressive behavior. Lipid peroxidation due to oxidative stress results into ROS formation in hippocampus and cerebral cortex in ARS procedure. ROS are the free radicals and known to be responsible for damaging hippocampus and cerebral cortex. GSH is the main non protein thiol (NPSH) of the mammalian cell known to involved in the antioxidant defense mechanism by detoxifying ROS by removal of free radicals such as H_2O_2, superoxide anions and alkoxy radicals, maintenance of membrane protein thiols and also a substrate for glutathione peroxidase and glutathione reductase (Naik et al. 2011). Impairment in GSH levels leads to development of neuropathological conditions (Lovell et al. 1998) including depression. In addition, treatment with GSH was found to exhibit significant antidepressant like effects in FST and tail suspension test in mice (Rosa et al. 2013). In the present study, however, no significant changes were observed on GSH level with PCA in hippocampus and cerebral cortex due to ARS as well as nonstressed mice. Similar findings also demonstrated and documented by Budni et al. (2013), Freitas et al. (2014), and Bettio et al. (2014) where they found both ARS and folic acid/Agmatine/Guanosine respectively caused non significant changes in GSH level in hippocampus and cerebral cortex in mice, indicating GSH levels might not directly involved in antidepressant activity, it would be interesting to investigate alterations on GSH levels in chronic state for antidepressant like activity of PCA. ARS procedure further induces reduced SOD and CAT activity in both cerebral cortex and hippocampus which were restored with PCA pretreatment. These effects corroborate with finding of Jindal et al. (2013) where in there was reduction of SOD and CAT defense system in the brain due to stress and subsequently induction of depressive like behavior which were reversed with etazolate treatment.

As a safety measure we did not observed any significant unwanted behavioral changes with the employed doses of PCA viz. excitement, convulsion, itching, skin rashes, tremors etc. which are commonly observed with tricyclic antidepressant and fluoxetine (Rang et al. 2007).

Conclusions

PCA treatment attenuate oxidative stress through improving endogenous antioxidant system (SOD and CAT activity) in hippocampus and cerebral cortex and stabilizes HPA axis by reducing corticosterone release in serum reflected in terms of improvement in behavioral changes in ARS mice.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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