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RESEARCH ARTICLE

Maternal separation induces long-term oxidative stress alterations and increases anxiety-like behavior of male Balb/cJ mice

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Abstract

Early life stress (ELS) exposure is a well-known risk factor for the development of psychiatric conditions, including anxiety disorder. Preclinical studies show that maternal separation (MS), a classical model of ELS, causes hypothalamic–pituitary–adrenal (HPA) axis alterations, a key contributor to the stress response modulation. Given that HPA axis activation has been shown to induce oxidative stress, it is possible to hypothesize that oxidative stress mediates the relationship between chronic ELS exposure and the development of several disorders. Here, we investigate the effects of MS in the oxidative status [plasma and brain reduced glutathione, catalase and thiobarbituric acid reactive substances (TBARS)], metabolism (glucose, triglycerides and cholesterol) and anxiety-like behaviors in adult Balb/cJ mice. In short, we found that MS increased anxiety-like behaviors in the open field, light/dark test but not in the elevated-plus maze. Animals also presented increased circulating cholesterol, increased TBARS in the plasma and decreased catalase in the hippocampus. Our findings suggest that MS induces long-term alterations in oxidative stress and increased anxiety-like behaviors.

Keywords Early life stress · Maternal separation · Anxiety-like behavior · Oxidative stress · Hippocampus

Introduction

The concern regarding mental health has grown in the past decades, it is alarming that ~ 4% of the global population suffer from depression or anxiety (Hannah and Max 2018). Chronic early life stress (ELS) exposure is a common risk factor associated with the development of psychiatric disorders, such as anxiety and depression (Syed and Nemeroff 2017). Spinhoven et al. (2010) observed that the frequency of traumatic childhood events critically affects the possibility of developing psychiatric conditions. It has been estimated that more than 15% of children worldwide suffer from neglect and/or inadequate maternal care (Stoltenborgh et al. 2013). Comprehending how stressful conditions during early life impact the development of this vulnerable population is essential for unraveling the mechanisms of these psychiatric conditions that occur later in life.

Preclinical studies are considered an essential tool when investigating the impact of stressful conditions in a controlled environment. The maternal separation (MS) paradigm is a classical model of ELS, in which pups are separated from their dams for a period of time during the first weeks of postnatal life that is considered a sensitive period of brain development (Andersen 2003; Lukkes et al. 2018). This manipulation during early development is believed to promote a profound impact on the physiology and behavior of the pups that continue throughout adulthood (Millstein and Holmes 2007). Several evidences have reported

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that prolonged periods of MS usually result in a disrupted maternal care, which may lead to increased anxiety-like behavior, impaired learning and memory capabilities and altered hypothalamic–pituitary–adrenal (HPA) axis response to stress (Maghami et al. 2018; Maniam et al. 2014; Romeo et al. 2003). However, the mechanisms by which this occurs are still unknown. Recently, oxidative stress has been proposed to have a possible relation with these behavior and biological alterations (Salim 2014).

The uninterrupted activation of the HPA axis has been shown to induce oxidative stress (Costantini et al. 2011; Spiers et al. 2014), which is characterized by an imbalance between the production of oxidants and antioxidant defenses that generally occur due to the excessive production of free radicals and/or the inefficiency of the antioxidant defense system (Phaniendra et al. 2015). Briefly, stress induces corticosterone which activates glucocorticoid receptors (GR). GR modulates transcriptional factors in the nucleus that increase mitochondrial membrane potential and mitochondrial oxidation (Spiers et al. 2014). This mechanism increase cellular metabolic rate promoting ATP synthesis together with spontaneous superoxide. Free radicals are a byproduct of oxidative phosphorylation, a crucial source of ATP for mitochondrial functioning. Being unpaired atoms or group of atoms, they are highly reactive substances which create chain reactions that can produce free radicals in sequence (Alkadi 2018). In excess, they promote significant damage to biological macromolecules such as DNA, protein, and lipids that can promote cell death (Evans et al. 2004; Pacifici and Davies 1991). Antioxidants are molecules that neutralize these reactive species, maintaining the adequate functioning of the cell (Lü et al. 2010). Neurons are a cellular type with high levels of metabolic activity and energy demand. In this way, oxygen is one of the main molecules necessary for the appropriate functioning of several physiological roles of the brain (Watts et al. 2018), making it susceptible to a high production of free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Wang and Michaelis 2010). The thiobarbituric acid reactive substances (TBARS) are the main markers to evaluate the cell damage originated by the oxidative degradation of lipids. Moreover, the enzymatic antioxidant defense system comprises the superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), whereas the reduced glutathione (GSH), among others, represents the non-enzymatic antioxidant system (Cui et al. 2012; Quadros et al. 2019; Singh et al. 2019).

Experimental models of early-life stress have already been associated with alterations on oxidative status and, thus, reported associations between altered mitochondrial respiratory chain, increased neuronal damage, decreased neuronal population in the hippocampus and impairments in spatial memory (Marković et al. 2017; Noschang et al. 2010; Zhu et al. 2004). These impairments can be associated with the fact that the hippocampus is a susceptible region to both behavioral early life alterations and the effects of oxidative stress (Huang 2014; Salim 2017; Wang and Michaelis 2010; Youssef et al. 2019). For example, maternal deprivation has been reported to increase levels of TBARS and SOD and reduced GPx in the infant rat hippocampus (Uysal et al. 2005). Similarly, it was observed a depressive-like behavior in association with decreased CAT activity in the hippocampus of young rats previously exposed to ELS (Réus et al. 2017). Kuloglu et al. (2002) established an association between oxidative stress and anxiety disorders, paving the way for investigating the role of oxidative metabolism. Hovatta el al. (2005) demonstrated that genes related to antioxidant activity in the brain regulate anxiety-like behaviors in multiple strains of mice. Furthermore, a correlation between oxidative status and anxiety-like behavior was also reported in a single strain of mice (Bouayed et al. 2007). These set of evidences indicate a possible mediating factor between oxidative metabolism and behavioral alterations. However, there is still a gap in the field to fully understand the mechanisms underlying the impact of early life stress on behavior and oxidative stress markers. For this reason, this study aims to provide evidences for the long-lasting impact of ELS in both behavioral and oxidative stress. To do this, we investigated the impact of a classical MS model on anxiety-like behavior and peripheral and hippocampal markers of oxidative stress of adult male Balb/cJ mice.

Methods

The present study was conducted in accordance with the guidelines of the International Council for Laboratory Animal Science (ICLAS) and was approved by the Ethics Committee on Animal Use (CEUA) of Pontifical Catholic University of Rio Grande do Sul (PUCRS), Brazil under the registration #14/00421.

Animals

Male and female BALB/cJ mice were obtained from the Center for Laboratory Animals at our university (CeMBE, PUCRS), Brazil. All animals were housed in standard plastic mouse cages (22 cm × 16 cm × 14 cm) and kept under constant room temperature (21 ± 1 °C), humidity (55 ± 5%) and ventilation. Mice were maintained in a 12 h light/dark cycle (lights on at 7 a.m.–7 p.m.) with mouse chow and water ad libitum. The breeding procedure consisted in housing two females and one male in the same cage for a 48 h mating period. The day the pups were born was defined as postnatal day (PND) 0. During the first 24 h after birth, litters were cross-fostered and culled to reach a litter size of 5–7 pups per litter. Animals were randomly assigned to undergo two

experimental conditions, MS or controls. In this study, we utilized 7 l for the MS group and 5 l for the control group. All animals were weaned at PND21, and the males were housed with two to three animals per cage. The females were housed, but used in a different experiment from our research group. Furthermore, the animals were weighed after weaned (PND21) and before the behavioral battery (PND58). To investigate the long-term effects of MS on anxiety-related traits, the animals were tested from PND58 to PND60 in the open field test (OF), elevated plus maze (EPM) and light/dark test, (LD) respectively. Thirty minutes after the LD test the animals were euthanized and the trunk blood was collected.

Maternal separation

The MS animals were deprived from maternal contact for 180 min/day from PND2 to PND15 (Bailoo et al. 2014; Wang et al. 2011). First, the dams were separated from the litter and allocated into a new cage with standard clean bedding. Dams were also transferred to another colony room to avoid ultrasonic vocalization between dams and pups (Rieger and Dougherty 2016). Then, the pups were individually removed from the nest and placed in small plastic boxes, where they stayed isolated from each other on top of a heating pad (32 °C) to prevent hypothermia. After MS the litters were returned to their home cages, followed by the dam. Dams from the control group remained undisturbed with their pups and were manipulated only during weekly cage cleaning.

Behavioral tests

Two days before the behavioral battery all animals were handled by a single researcher that performed all the animal manipulations during the following days. Animals were habituated in the room for 30 min before the tests. Only for the LD test animals remained 1 h in a dark room before the test started. All tests were performed during the end of the light cycle (4 p.m. to 7 p.m.). All apparatus were cleaned with 70% ethanol between animals to avoid any odor cue. Video was recorded using a professional camera and then analyzed in the Any-Maze Software version 4.9 (ANY-Maze, Inc., Greensburg, PA).

Open field test

The open field test was conducted to investigate the spontaneous locomotor activity and anxiety-like behavior (Walsh and Cummins 1976). Animals were placed in the center of a Plexiglas Square Box (33 × 33 cm) and allowed to freely explore the box for 5 min. The illumination of the room was set to 140 lx. The following parameters were measured: total distance covered, time in the central zone and entries in the central zone (Kovalenko et al. 2014). For the analysis, the floor of the apparatus was divided into 16 equal squares and the four central squares were considered the central zone. An entry was counted when all four paws of the animal were into the zone.

Elevated plus maze

Animals were tested in the elevated plus maze as previously described by Marchette et al. (2018). The apparatus consisted of two open arms (30 × 5 × 15 cm) and two closed arms (30 × 5 × 15 cm). The maze also had a central zone (5 × 5 cm), and was elevated (50 cm) from the floor. Animals were placed in the center of the maze facing the open arms and were allowed to explore for 5 min. The time in the open arms, number of entries in the open arms and risk assessment behaviors (stretching from center to open arms and head dipping in the open arms) were scored.

Light/dark test

The apparatus consisted of a Plexiglas Box (21 × 42 × 20 cm) divided into two chambers of equal size (light and dark compartments). The light compartment was clear, open at the top and brightly illuminated (400 lx) (Bourin and Hascoët 2003). The dark compartment had a removable black lid at the top and black walls. These compartments were connected to each other by a central open door (5 × 5 cm). Each animal was placed in the center of the light compartment and allowed to freely explore the apparatus for 5 min. The test was performed in a dark room with a direct white light (400-lx) focused in the light compartment to generate an aversive stimulus. The following parameters were measured: time in the light compartment, number of transitions and risk assessment behavior (stretching from the dark to light zone).

Biochemical analysis

Biochemical analysis was performed on whole blood samples collected in tubes with heparin. Blood was centrifuged (1000*g* for 10 min) and plasma was frozen at − 80 °C until analysis. Levels of glucose, triglyceride, and cholesterol were determined with commercial kits (Labtest Diagnóstica, Brazil), according to manufacturer's instructions, and were spectrophotometrically measured (Thermo Scientific, Spectronic GENESYS 8).

Oxidative stress

To assess oxidative stress parameters in the hippocampus, the brains were removed and the region was dissected by free hand technique, and then stored at − 80 °C. The tissue

was homogenized in 15 volumes (1:15, w/v) of sodium phosphate buffer, pH 7.4. The homogenates were centrifuged at 750*g* for 10 min at 4 °C and the supernatant was used for the analysis of TBARS, CAT, and GSH. Total protein was measured using Nanodrop Spectrophotometer (Thermo Scientific, NanoDrop Model 1000). These markers were also analyzed in the plasma.

Thiobarbituric acid reactive substance (TBARS)

TBARS is an index of lipid oxidation and measures the levels of malondialdehyde in the samples. For the test, 10 µl of the hippocampus samples and plasma were added to 10 µl of sodium dodecyl sulfate (SDS 12.4 mM) and 400 µl of thiobarbituric acid. The mixture was heated for 30 min and centrifuged at 750*g* for 10 min at 25 °C. The supernatant was collected and measured spectrophotometrically at 532 nm.

Catalase assay (CAT)

Catalase activity is based on the conversion of H_2O_2 into water and oxygen. For the test, a reaction medium containing 102 µl of H_2O_2 30% and 100 µl of Triton X-100 was pipetted with 10 µl of the hippocampus samples and plasma. The reaction was read at 240 nm immediately, and after 1 min, in a semi-automatic spectrophotometer. One CAT unit is defined as 1 µmol of H_2O_2 consumed per minute.

Reduced glutathione (GSH)

Reduced glutathione reacts with the superoxide radical (O_2), producing an increase in oxygen consumption and formation of oxidized glutathione. For the test, 250 µl of metaphosphoric was added to 10 µl of the hippocampus samples and plasma. The mixture was centrifuged at 750*g* for 10 min at 4 °C. After, 650 µl of Na_2HPO_4 and 100 µl of the color reagent (5,5′-dithiobis-2-nitrobenzoic acid) were added to 250 µl of the supernatant. The reaction was read in a semi-automatic spectrophotometer at 412 nm.

Statistical analysis

Statistical outliers with values higher or lower than two SDs from the mean were excluded in the final statistical analysis. In this sense, five animals were excluded from the experiment (3 from control group and 2 from MS group). All data are presented as mean ± standard error of the mean (SEM). Normality of the data distribution was analyzed for all variables using Shapiro–Wilk's test. A student *t* test was performed for each dependent variable. In all analysis a *p* value of < 0.05 was considered statistically significant. 17 animals for the MS group and 11 for the control group were utilized for the behavioral analysis. For the analysis of metabolic and oxidative stress markers, were used between 6 and 8 animals for the MS group and 6–9 animals for the control group. Statistical analysis was performed utilizing SPSS software v.25.0 (SPSS, Chicago, IL), and graphs were created using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA).

Results

Weight control

To evaluate the short and long-term impact of MS on weight, the animals were weighed at two time points (PND21 and PND58). At weaning (PND21 the animals exposed to MS showed a decrease in body weight when compared to control animals [$t(26) = 2.087$, $p = 0.046$, Fig. 1a]. Before the behavioral tests (PND58) there was also no significant alteration in weight when comparing MS animals to controls [$t(26) = 0.432$, $p = 0.668$, Fig. 1a].

Anxiety-like behavior evaluation

Regarding the OF test, no differences were observed in total distance travelled [$t(26) = 1.262$, $p = 0.218$, Fig. 1b]. However, mice exposed to MS spent less time in the center of the OF when compared to controls [$t(26) = 2.345$, $p = 0.026$, Fig. 1c]. No statistically significant difference was observed between groups in the number of entries in the center [$t(26) = 0.876$, $p = 0.388$, Fig. 1d]. In the EPM, no statistically significant difference was observed between groups regarding time in the open arms [$t(26) = 0.112$, $p = 0.911$, Fig. 1e], entries in the open arms [$t(26) = 1.498$, $p = 0.146$, Fig. 1f] and risk assessment frequency [$t(26) = 0.597$, $p = 0.555$, Fig. 1g]. In the LD test, mice exposed to MS spent less time in the lit compartment [$t(26) = 3.394$, $p = 0.002$, Fig. 1h]. No differences were observed in the number of transitions between compartments [$t(26) = 0.236$, $p = 0.814$, Fig. 1i]. Furthermore, animals exposed to MS made more risk assessments when compared to controls [$t(26) = 3.965$, $p < 0.001$, Fig. 1j]. Taken together, our observations suggest that mice exposed to MS showed an increased anxiety-like behavior in the OF and in the LD, but not in the EPM test.

Peripheral metabolism and oxidative stress

To investigate MS effects on overall metabolism and peripheral oxidative stress parameters, we looked at circulating glucose, triglycerides, cholesterol, as well as catalase, GSH and TBARs in the plasma. We found no differences between groups in plasma glucose or triglycerides [$t(12) = 0.581$, $p = 0.57$, Fig. 2a and $t(12) = 0.638$, $p = 0.53$, Fig. 2b, respectively]. Nevertheless, the MS group showed significantly higher circulating levels of cholesterol when compared to

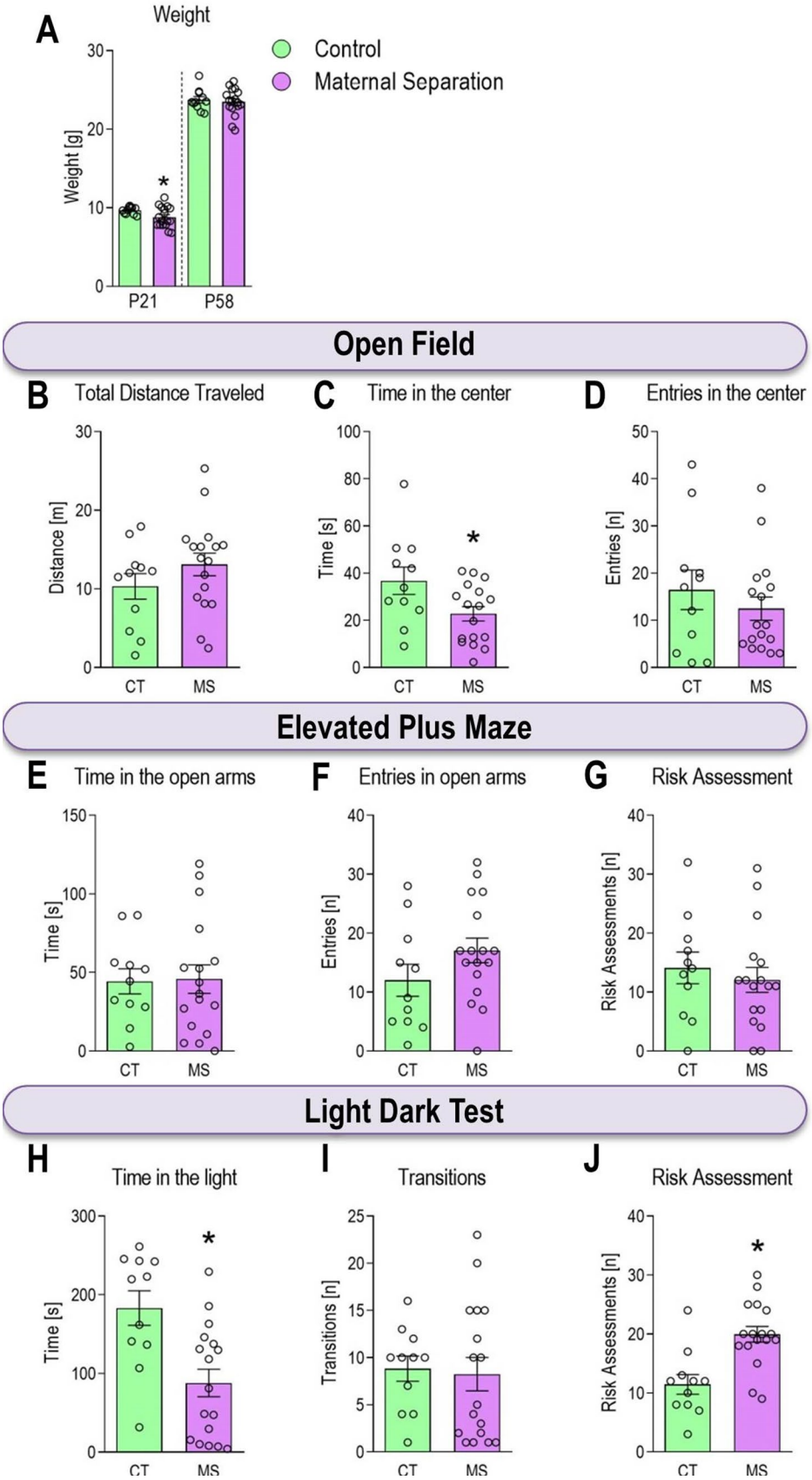

Fig. 1 Analyses of body weight and behavioral parameters; **a** body weight (g) at P21 and P58; **b** total distance traveled in the OF; **c** time in the center area of the OF; **d** entries in the center area of the OF; **e** time in the open arms of the EPM; **f** number of entries in the open arms of the EPM; **g** risk assessment behavior in the EPM (stretching from center to open arms and head dipping in the open arms); **h** time in the light zone of the L/D; **i** number of transitions between zones of the L/D; **j** risk assessment in the L/D (stretching from the dark to light zone); MS ($n = 17$), Control ($n = 11$); $^*p < 0.05$ (t test); results are expressed as the mean ± SEM

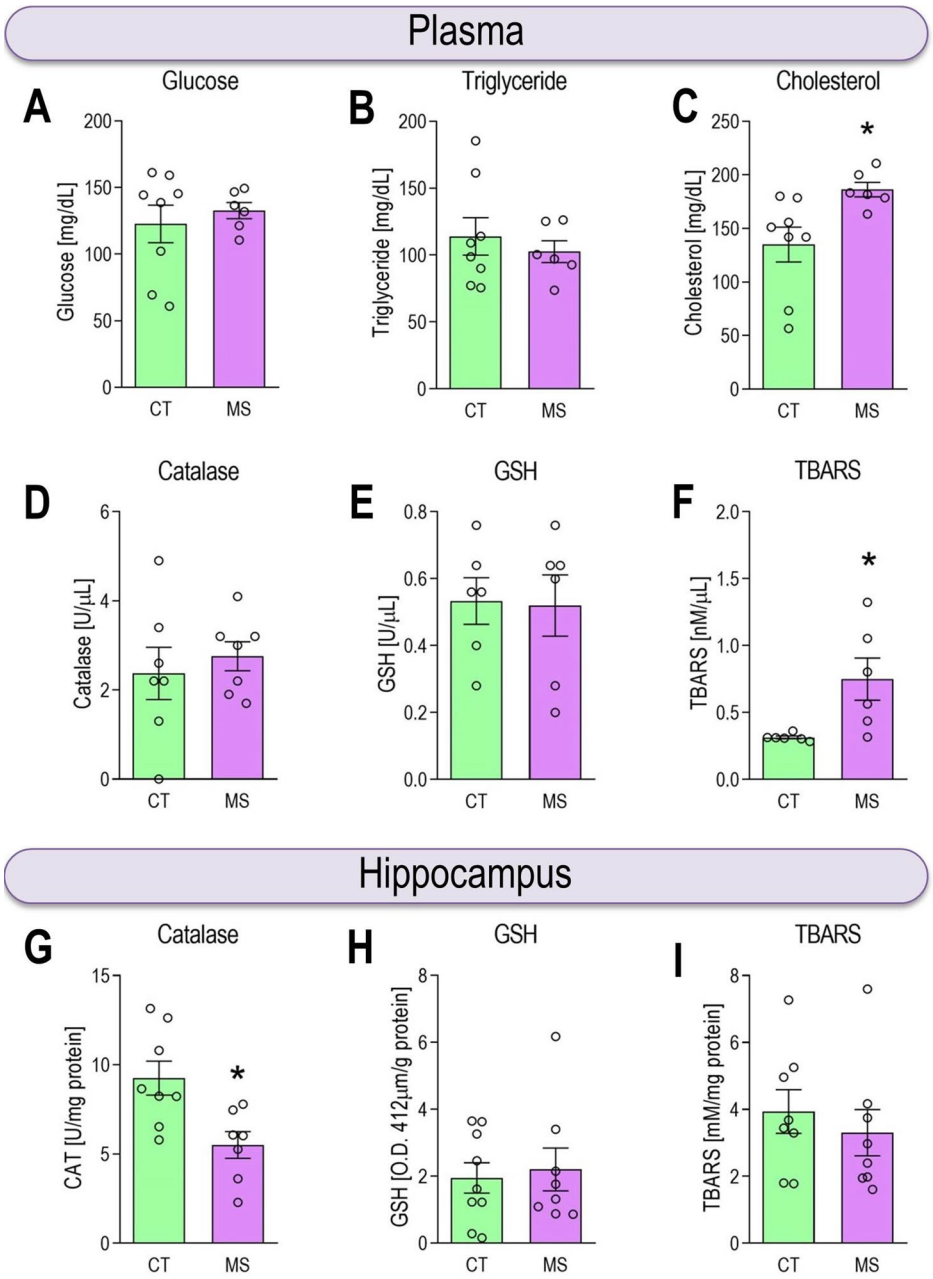

Fig. 2 Analyses of metabolic and oxidative stress markers; **a** plasma glucose levels; **b** plasma triglyceride levels; **c** plasma cholesterol levels; **d** plasma catalase; **e** plasma reduced glutathione; **f** plasma thiobarbituric and reactive substances; **g** hippocampal catalase; **h** hippocampal reduced glutathione; **i** hippocampal thiobarbituric and reactive substances; MS ($n=6$–8), Control ($n=6$–9); $^*p<0.05$ (t test); results are expressed as the mean ± SEM

controls, [t(12) = 2.595, $p=0.0234$, Fig. 2c]. Regarding catalase activity and GSH concentration in the plasma no statically significant differences were observed [t(12) = 0.577, $p=0.57$, Fig. 2d and t(12) = 0.115, $p=0.91$, Fig. 2e, respectively]. However, mice exposed to MS showed increased TBARS concentration in the plasma when compared to controls [t(12) = 2.932, $p=0.01$, Fig. 2f].

Hippocampal oxidative stress

In the analysis of oxidative stress in the hippocampus, we found a significant reduction in catalase activity in the hippocampus of mice exposed to MS [t(13) = 3.027, $p=0.009$, Fig. 2g]. No differences were found in GSH and TBARs concentration in the hippocampus of MS animals when compared to controls [t(15) = 0.333, $p=0.74$, Fig. 2h and t(14) = 0.663, $p=0.51$, Fig. 2I, respectively].

Discussion

In this study, we used a MS model to investigate the impact of early life stress on anxiety-like behavior and oxidative stress of male BALB/cJ mice. The main findings of the present study are: (1) mice exposed to MS exhibit increased anxiety-like behavior in the open field and light/dark tests;

(2) animals exposed to MS showed increased levels of cholesterol; (3) when compared to control animals, mice exposed to MS showed increased TBARs in plasma and decreased catalase activity in the hippocampus.

Regarding the impact of stress exposure on anxiety-like behavior we observed that early life stress significantly reduced the time in the central zone of the OF. This data goes in line with several other studies that reported similar findings (Lambás-Señas et al. 2009; Romeo et al. 2003; Vazquez et al. 2005). Furthermore, animals exposed to stress spent less time in the light compartment of the LD box, while opting to stay longer in the darker and safer area of the apparatus, which was also shown by previous reports (Jin et al. 2018; Wang et al. 2012a, b). These findings suggest that early life stress increases anxiety-like behavior of adult male mice. Surprisingly, we did not observe differences in the EPM, which is one of the most classical apparatus to measure anxiety behavior in rodents (Handley and Mithani 1984). Nonetheless, while some studies reported that early life stress augments anxiety-like behavior in the EPM test (Grissom et al. 2012; Salzberg et al. 2007), some evidences did not find the same effect and showed similar results when compared to our study (Llorente-Berzal et al. 2012; Loi et al. 2017; Tan et al. 2017). It seems that the EPM provides variable results regarding the impact of stress on anxiety-like behavior, while it appears to be a great apparatus to detect the effect of anxiolytic or anxiogenic drugs (Lister 1987).

The classical tasks used in this study to evaluate anxiety-like behavior in mice are based on the "approach-avoidance" conflict that the animals face when encountering a novel environment, in which they naturally tend to explore the new area, but also evaluate the possibility of being exposed to threats (Aupperle et al. 2011). It is understood that when rodents explore areas of the apparatus that are more exposed and could impose possible threats for longer periods of time the less anxiety-like behavior they present (i.e., center of OF, open arms of EPM and light compartment of the LD), but if the animals stay in protected areas (i.e., close to the walls in the OF, the closed arms in the EPM and the dark compartment in the LD) and avoid the natural instinct of rodents to explore a new environments, the more anxiety-like behavior they present (Arrant et al. 2013; Kraeuter et al. 2019; Seibenhener and Wooten 2015). Nevertheless, Belzung and Le Pape (1994) showed that different anxiety tests (among which, EPM and LD) seem to measure different components of anxiety-like behavior, given that the parameters usually utilized in these tests had low inter-test correlation. Even though all three tests are based on the "approach-avoidance" conflict, each of tasks produce a distinct threat to the animal, which consists of: unprotected and large space in the OF, unsecure and high area in the EPM and brightly illuminated space in the LD. These differences on how each task impose a threat to rodents could possibly explain why we observed differences when comparing MS animals to controls on the OF and the LD, but did not see the same effect on the EPM.

When assessing biochemical parameters, it has been previously shown that alterations in cholesterol levels have been reported in patients exposed to psychosocial stress (Catalina-Romero et al. 2013) and psychiatric disorders (Pereira 2017). For example, schizophrenic patients with a history of early life adversity (ELA) had higher levels of total cholesterol when compared to patients without ELA (Misiak et al. 2015). In depressed patients, a history of childhood physical violence was related to lower levels of total cholesterol. These findings show that alterations in cholesterol levels play a role in the relationship between childhood trauma and the development of certain conditions. In accordance with these findings, we observed that, even though all animals were fed the same diet, those exposed to MS showed significant increase in plasma cholesterol levels. Nevertheless, other studies that employed the MS paradigm did not see significant changes in cholesterol (Ohta et al. 2014; Paternain et al. 2012).

Despite the effect of MS on cholesterol, Paternain et al. (2016) did not observe significant changes in triglyceride or glucose levels, which is similar to our data. Some studies showed that prenatal stress has been associated with altered glucose homeostasis in adults (D'mello and Liu 2006; Uriarte et al. 2013). Furthermore, activation of the HPA axis induces gluconeogenesis (Rao 2015) and triglyceride synthesis (Wang et al. 2012a, b). Gluconeogenesis and triglyceride synthesis are energetically expensive, and having an extended increase in these processes due to a hyperactivity of the stress response system could be deleterious to the organism. Therefore, it is possible that our results show an economical adaptation to the ELS challenges. Nevertheless, further studies addressing this issue are needed to claim this hypothesis.

Lipid peroxidation, along with other oxidative and nitrosative stress biomarkers, have been recently associated with childhood physical neglect (Moraes et al. 2018). Similarly, we found that male mice exposed to ELS showed an increase in plasmatic lipid peroxidation when compared to controls. Interestingly, we did not find a significant difference in the TBARS hippocampal levels. Previous studies have found significant increases in the TBARS levels in different brain tissues of rodents exposed to early life stress (Bernhardt et al. 2017; Neves et al. 2015; Réus et al. 2018), but one study failed to see significant differences (Menezes et al. 2017). To the best of our knowledge, this is the first publication to investigate MS induced changes in TBARS levels in both the brain and plasma, and further research is necessary to elucidate the relationship between ELS and TBARS in different tissues, and its consequences in behavioral responses.

ROS are formed as a by-product of normal oxygen metabolism, mostly from mitochondria respiration. The

antioxidant defense system plays an important role protecting the organism from the damaging effects of ROS (Lü et al. 2010). Antioxidant activity is remarkably reduced in the brain when compared to other tissues (Driver et al. 2000; Mori et al. 2007), which causes it to be more susceptible to oxidative damage. Catalase and GSH are major components of the antioxidant defense system that reduces hydrogen peroxide (H_2O_2) to water through distinct mechanisms (Aoyama and Nakaki 2012; Sani et al. 2006). GSH usually acts on lower concentrations of H_20_2, whereas catalase is more active in higher concentrations of H_20_2 (Baud et al. 2004). In accordance with the findings from Réus et al. (2018), we observed that MS animals had significantly lower levels of catalase in the hippocampus, but no alteration in the plasma was observed. This selective central nervous system (CNS) alteration supports the vulnerability to free radical attacks in the brain (Wang and Michaelis 2010). Furthermore, we did not observe significant central and peripheral alterations in GSH activity in animals exposed to maternal separation, which is consistent with a recent study showing that exposure to maternal deprivation did not change the GSH levels in the hippocampus and prefrontal cortex of adult rats (Neves et al. 2015).

Finally, it is important to clarify that our study has some limitations. The hippocampal analysis was performed without analyzing separately each subregion of the structure which could have impacted the oxidative stress results. The free-hand technique used in this study has this limitation, but future studies addressing each subregion should be performed. In addition, it is well known that other brain regions (such as prefrontal cortex and amygdala) regulates HPA, but in our study we focused only in hippocampus since this brain region has been suggested as a major target of stress mediators and for exploratory purpose regarding metabolic markers. It is important to highlight that stress could disrupt different processes in hippocampus leading to altered response. For example, the study of Revest et al. (2009) experimentally inhibited the neurogenesis of hippocampus suggesting that this manipulation was implicated in anxiety-like behavioral response. Future studies addressing the previously mentioned brain regions could help to shed light on the effects of early-life stress in oxidative markers in the brain. Another limitation refers to the lack of analysis in baseline conditions (before the behavioral tests). In early-life stress studies, it was demonstrated that both baseline and post-test evaluations are significant affected by early stress exposure, narrowing the extent of our conclusions considering that we did not evaluated how maternal separation per se affects oxidative stress. Furthermore, our experiments were performed in the end of the light cycle, in which animals tend to have increased levels of corticosterone. We opted to perform the experiments in this time point because rodents are nocturnal animals and would be more active to interact with the apparatus.

In conclusion, our data indicate that exposure to MS protocol during the first two weeks of development induced anxiety-like behavior in male mice. We also found increased cholesterol levels in the plasma, as well as increased TBARS. This supports the possibility that early exposure to stressful events could impact peripheral metabolism later in life. In addition, mice exposed to MS also showed decreased CAT activity in the hippocampus, suggesting that early-life stress could impact oxidative stress markers in the brain in a long-term basis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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