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Moderate aerobic training counterbalances the deleterious effect of undernutrition on oxidative balance and mitochondrial markers

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The state of Maternal Protein Malnutrition (MPM) is associated with several deleterious effects, including inflammatory processes and dysregulation in oxidative balance, which can promote neurodegeneration. On the other hand, it is known that aerobic exercise can promote systemic health benefits, combating numerous chronic diseases. Therefore, we evaluate the effect of aerobic exercise training (AET) on indicators of mitochondrial bioenergetics, oxidative balance, endoplasmic reticulum stress, and neurotrophic factor in the prefrontal cortex of malnourished juvenile *Wistar* rats. Pregnant *Wistar* rats were fed with a diet containing 17% or 8% casein during pregnancy and lactation. At 30 days of life, male offspring were divided into 4 groups: Low-Protein Control (LS), Low-Protein Trained (LT), Normoprotein Control (NS), and Normoprotein Trained (NT). The trained groups performed an AET for 4 weeks, 5 days a week, 1 h a day per session. At 60 days of life, the animals were sacrificed and the skeletal muscle, and prefrontal cortex (PFC) were removed to evaluate the oxidative metabolism markers and gene expression of *ATF-6*, *GRP78*, *PERK and BDNF*. Our results showed that MPM impairs oxidative metabolism associated with higher oxidative and reticulum stress. However, AET restored the levels of indicators of mitochondrial bioenergetics, in addition to promoting resilience to cellular stress. AET at moderate intensity for 4 weeks in young *Wistar* rats can act as a non-pharmacological intervention in fighting against the deleterious effects of a protein-restricted maternal diet.

Keywords Physical exercise, Maternal low protein diet, Cellular stress, Oxidative stress, Brain

Protein malnutrition is a serious public health problem, which occurs mainly in developing countries among children[1]. Furthermore, when it occurs during early life, it has adverse effects on the anatomical, physiological, and biochemical parameters in the various organs, among them the brain[2]. Studies have associated protein malnutrition with reduced rate of brain development in humans and in animals[3]. The prefrontal cortex (PFC), is an essential brain structure located in the anterior part of the frontal lobe, responsible for forming goals, planning action strategies, selecting cognitive and motor skills for performance, inhibitory control and working memory[4,5]. The PFC form distinct networks making connections with different brain areas forming diverse networks; the maturation of these networks over the development correlates with improvement of functions, therefore alteration in these networks are associated in neuropsychiatric disorders, such as schizophrenia, cognitive impair, depression and negative symptoms[6–8]. Tamási et al.[9] reported that rats subjected to MPM showed reduced PFC

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excitability markers, associating this with the emergence of behavioral and neuropsychiatric disorders later in life, including attention deficits and schizophrenia[9].

Combined to effects on behavior, MPM is responsible for negative changes in energy metabolism, due to an excessive production of Reactive Oxygen Species (ROS)[10]. In situations of cellular stress, electrons are unpaired, a situation related to reduced antioxidant defenses, and which produces damage to the macromolecules, characterizing a state of Oxidative Stress (OS)[11]. These conditions can provide dysfunction in mitochondrial bioenergetics, affecting the amount and availability of Adenosine Triphosphate (ATP) and apoptosis. Previous study in literature using a metabolomics, have linked prenatal undernutrition with decrease in GSH levels and mitochondrial dysfunction in PFC and the authors speculates that the OS might be the pathogenesis of prenatal undernutrition induced schizophrenia[12].

Furthermore, another cellular component affected by the malnutrition state is the endoplasmatic reticulum (ER), responsible for lipid biosynthesis and mainly for protein folding[13]. A defect in protein folding promotes deposition of unfolded proteins in the ER lumen, stimulating the activation of cellular stressors such as the Activating Transcription Factor 6 (ATF6), Endoplasmatic Reticulum Kinase (PERK), Glucose Regulated Protein 78 (GRP78), associated with signaling pathways linked Parkinson's and Alzheimer's diseases. A study in hippocampus demonstrated that aerobic exercise training (AET) improves mitochondrial function and increases enzymatic and non-enzymatic antioxidant capacity, as well as declines ER stress markers[14,15]. Studies in non-brain tissues indicate that AET promotes improvements in mitochondrial functionality. This improvement results in a significant reduction in ER stress, through an increase in the activity of chaperones, which in turn enhances the folding of proteins. This entire biological process culminates in a decrease in overproduction of ROS, with a subsequent increase in antioxidant defenses.

There is evidences suggesting that exercise has a positive effect on ER stress when using control diets. However, the impact of AET on the PFC of malnourished juvenile rats is not well understood. This study aims to assess how moderate physical training affects mitochondrial function, oxidative balance, ER stress markers, and BDNF levels in the PFC of malnourished juvenile Wistar rats.

Material and methods

The experimental animal design used in this study was approved by the Ethics Committee on the Use of Animals from the Federal University of Pernambuco with protocol number 0060/2018. All animal experiments complied with ARRIVE guidelines and have been carried out in accordance with the UK Animal (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU for Animal Experiments, and the National Research Council Guide to the Care and Use of Laboratory Animals.

Animals, diet, and experimental groups

Eight female *Wistar* rats were used from the colony of our Institution. The animals were kept in an experimental laboratory with a temperature of 22° C ± 2, light–dark cycle of 12/12 h. At 70 days old, the rats were placed in a cage to mate in a ratio of 1 male to 2 females, during the mating period. The detection of pregnancy was detected through a vaginal smear confirming the presence of sperm[10]. On the first day of pregnancy, the females were divided into two dietary groups: Normo protein diet (NP; n = 4; 17% casein) or Low protein diet (LP; n = 4; 8% casein), both with the same energetic value as previously described[14], the description of all diet ingredients is presented in Supplementary Table 1. During lactation, the dams received the same experimental diet as in the gestational period, according to experimental group. After weaning (21 days of age), all offspring received a normo protein diet (Casein 17%) to supply all nutritional needs during the growth phase. At 30 days of life, the pups were subdivided into 4 groups: NS: Normoprotein diet sedentary (n = 7; Body weight [BW]: 99.5 ± 3.5 g) ; NT: Normoprotein diet trained (n = 10; BW: 104.2 ± 3.2 g) ; LS: Low- protein diet sedentary (n = 12; BW:70.0 ± 3.2 g) and LT: Low-protein diet trained (n = 11; BW: 65.3 ± 4.5 g). All the rats used for the experiments were randomly selected, respecting only two puppies per dam not to have litter effects.

Aerobic exercise training

At 30 days of age, the AET protocol at moderate intensity was used with an Inbramed treadmill (model KT 10200) adapted for rats, according to the experimental protocol previously published previously[16], which consists of 4 weeks of aerobic training with a frequency of 5 days/week and duration of 60 min/day at 50% of the maximum oxygen volume (VO_2max). Each rat underwent a maximal stress test on the 6th day of each of the 4 weeks of training. The control group remained in the cages, in the same environment as the trained animals.

Euthanasia, tissue collection and protein quantification

At 60 days of age and 48 h after the last AET session, the rats were euthanized by decapitation using guillotine. The skeletal muscle Extensor Digitorum Longus (EDL) and PFC were removed in less than one minute and immediately frozen. The tissue was homogenized in a cold extraction buffer (100 mM Tris base, pH 7.4; 1 mM EDTA; 10 mM sodium orthovanadate; 2 mM phenylmethylsulfonylfluoride (PMSF); 1% Nonidet). After homogenization, samples were centrifuged at 4 °C at 1180 × g for 10 min, and the supernatants were used for protein quantification according to the Bradford method[17].

Activity of citrate synthase

Citrate synthase is the first enzyme in the Krebs cycle and is very important for the catalysis and condensation of acetyl CoA with oxaloacetate for the formation of citrate[18]. Moreover, this enzyme is an indicator of trainability, as described by Le Page et al.[19]. Briefly, a reaction was produced in a mixture of Tris–HCl (pH = 8.2), magnesium chloride (MgCl), ethylenediamine-tetra-acetic acid (EDTA), 0.2–5.5 dithiobis (2-nitrobenzoic acid)

(E = 13.6 µmol/(mL.cm), 3 acetyl CoA, 5 oxaloacetate and 0.3 mg/mL of homogenized tissue. The enzymatic activity was evaluated by measuring the change in the absorbance rate at 412 nm for 3 min at a temperature of 25 °C. Citrate levels are expressed as mM/min/mg protein[20].

Kinetics of pyridine nucleotide oxidation (Nicotinamide adenine dinucleotide (NAD^+)/reduced nicotinamide adenine dinucleotide (NADH)

Changes in oxidation and reduction of pyridine nucleotides in mitochondrial suspension (1 mg/ml) were observed in a spectrofluorometer (OMEGA, Germany) using excitation and emission lengths of 340 and 370 nm, respectively. The extent of oxidation of the pyridine nucleotides was calculated as a function of the fluorescence decay, with temperature control and agitation. Internal calibration was performed by adding known quantities of NAD or NADH[21].

Lipid peroxidation assay

Lipid peroxidation was also evaluated through substances reactive to thiobarbituric (TBARS). The methodology was according to Buege and Aust[22]. Briefly, 300 µg of protein were mixed with 30% (w/v) trichloroacetic acid and 10 mM TRIS buffer (pH 7.4) in equal volumes. After centrifugation, equal amounts of samples and thiobarbituric acid were mixed, followed by boiling at 100 °C for 15 min. The pink pigment formed was evaluated at 535 nm and expressed as mM/ mg protein[22].

Carbonyl assay

The procedures described by Reznick and Packer (1994) were used. 300 mg of protein concentration was added to 30% (w/v) TCA and centrifuged for 14 min at 1.180 g. The pellet was resuspended in 10 mM 2,4-dinitrophenylhydrazine and incubated in a dark room for 1 h with shaking every 15 min. The samples were washed and centrifuged three times in ethyl acetate buffer and the pellet was resuspended in 6 M guanidine hydrochloride and incubated for 30 min at 37 °C. The samples read at 370 nm and expressed as mM/mg protein[23].

Superoxide dismutase activity (SOD)

SOD activity was determined according to Misra and Fridovich (1972). 300 µg of protein was added to 0.05 M of a carbonate buffer with EDTA (pH 10.2). The reaction was initiated with the addition of 150 mM epinephrine at 30 °C. The decrease in absorbance was monitored for 1.5 min at 480 nm and results express in mM/min/mg protein[24].

Catalase activity (CAT)

CAT was performed as described by Aebi (1984). 300 µg of the protein supernatant was used, in addition to 50 mM of the phosphate buffer (pH 7.0) and 0.3 M of hydrogen peroxide. Samples was monitored at 240 nm for 3 min at 20 °C, the result was expressed as mM/min/mg protein[25].

Glutathione S transferase (GST)

The activity of GST was performed as previously described by Habig et al.[26]. 200 mg protein was added to 0.1 M phosphate buffer (pH 6.5) containing 1 mM EDTA. 60 mM reduced glutathione and 30 mM 1-chloro-4,4-dinitrobenzene were added to start the reaction. The assay was conducted at 340 nm, for 1 min, the result expressed as mM/min/mg protein[26].

Reduced glutathione (GSH) and oxidized glutathione (GSSG)

Reduced glutathione was assayed as previously described by Hissin and Hilf[27]. To assess GSH levels, the samples were diluted tenfold in 0.1 M phosphate buffer containing 5 mM-EDTA (pH 8.0). After this, the sample was incubated with 1 mg/ml o-phthaldialdehyde (OPT) at room temperature for 15 min and thereafter fluorescence evaluated at 350 excitations and 420 nm emission. The oxidized glutathione (GSSG) levels were evaluated by incubation of samples with 40 mM Nethylmaleimide for a period of 30 min in RT followed by addition of 100 mM NaOH buffer, the result expressed as µM/mg of protein. The REDOX state was determined by the ratio of GSH/GSSH[27].

Total thiols

The total and protein-bound sulfhydryl group contents were determined as described by Aksenov and Markesbery[28]. The reduction of 5,5-dithiobis (2-nitrobenzoic acid) by thiol groups was measured in homogenates of 200 µg PFC, resulting in the generation of a yellow-stained compound, TNB, whose absorption was measured spectrophotometrically at 412 nm, results expressed in mM/mg protein[28].

mRNA evaluation and gene expression analysis by real time PCR

Total RNA was extracted from prefrontal cortex tissue using Trizol reagent and the guanidine isothiocyanate method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA pellets were washed in 75% ethanol and centrifuged at 7500 × g for 5 min at 4 °C, air-dried, and dissolved in DEPC-treated ultrapure water. RNA quantification was performed in a NanoDrop 2000 spectrophotometer (Thermo Scientific, US), and purity was assessed using the ratio of 260/280 nm absorbance (10). Real-time polymerase chain reaction (RT-PCR) experiments for β2-microglobulin (β2M); ATF6, PERK; GRP78; and BDNF genes were performed using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, USA)[29]. Samples were processed in duplicate, and the cycle threshold (Ct) values of each targeted gene were normalized to the β2M Ct

determined in an identical sample. Relative mRNA expression was determined using the 2ΔΔCt method[24], the sequences and accession numbers are described in Table 1.

Statistical analysis

Descriptive analysis of the data was performed and expressed as mean and Standard Deviation (SD). All data obtained in the present study were subjected to normality analysis of data using the Shapiro Wilk test. Parametric data were analyzed by the ANOVA Two-Way test of variance, followed by Tukey's post-test to observe multiple comparisons. Non-parametric data were analyzed by the Kruska-Wallis's test, followed by Dunn's post-test for multiple comparisons. Finally, we calculated the effect size using the Cohen's d test, which establishes the magnitude of the effects as small (d = 0.2–0.5); medium (d = 0.5–0.8) and large (d = > 0.8), are presented in Supplementary Tables 3,4 And 5. The level of significance in all cases was maintained at 5%. The statistical analyses were performed in GraphPad Prism v.9.0 software (GraphPad Software Inc, La Jolla, CA, USA).

Results

Effect of aerobic exercise training on oxidative metabolism

Initially, we performed an evaluation of citrate synthase (CS) activity in the extensor digitorum longus (EDL) muscle (Fig. 1) to demonstrate aerobic trainability in *Wistar* rats exposed to 4 weeks of AET. When comparing the isolated effect of MPM, no statistically significant differences were observed in the groups ($p = 0.81$). However, when comparing the NT vs NS groups, to visualize the isolated impact of AET, a significant increase of 169.56% in Citrate Synthase levels was observed after 4 weeks of training ($p = 0.02$). Similarly, in the LT and NT groups, no significant differences were observed ($p = 0.99$). When the effects between MPM and AET were compared, however, a 64.30% increase in citrate synthase activity was registered in the LT group compared to the LS group ($p = 0.03$).

Additionally, CS in the PFC (Fig. 2A) was also evaluated. This enzyme is responsible for mediating the formation of citric acid in the Krebs cycle, which is the product of a condensation relationship between acetyl-coenzyme A (Acetyl-CoA) and oxalacetate. Our data demonstrated a significant reduction in CS activity in response to MPM ($p = 0.02$). Furthermore, there were no significant differences between the NT vs NS groups ($p = 0.58$). Similarly, in the LT and NT groups significant differences were not found ($p = 0.40$). However, the LT showed a significant increase of CS ($p = 0.03$), compared to the LS group, this data strongly suggests that AET promotes adaptations in markers of oxidative metabolism in different tissues.

NAD^+/NADH oxidation

Changes in oxidation and reduction of pyridine nucleotides were evaluated. These compounds act as indicators of mitochondrial function in oxidative phosphorylation. For the levels of Nicotinamide Adenine Dinucleotide in its oxidized form (NAD^+), no significant differences were observed among the groups (Fig. 2B). Similarly,

Gene	Forward (5')	Reverse (3')	Accession number
β2M	TGA CCG TGA TCT TTC TGG TG	ACT TGA ATT TGG GGA GTT TTC TG	NM_012512
ATF6	TCG AAG GGA TCA CCT GCT AT	CGA GGA GCT TTT GAT GTG GA	NM_001107196
PERK	TGT GCA GGA AGG AGA ACC TTA	GGC CAA CAT TAA TCA GAT TCC ATA C	NM_001399818
GRP78	CGT CGT ATG TGG CCT TCA CT	ATT CCA AGT GCG TCC GAT GA	NM_0013083
BDNF	ACG GTC ACA GTC CTT GAA AAG	GGA TTG CAC TTG GTC TCG TA	NM_001270630

Table 1. Gene sequence and accession number.

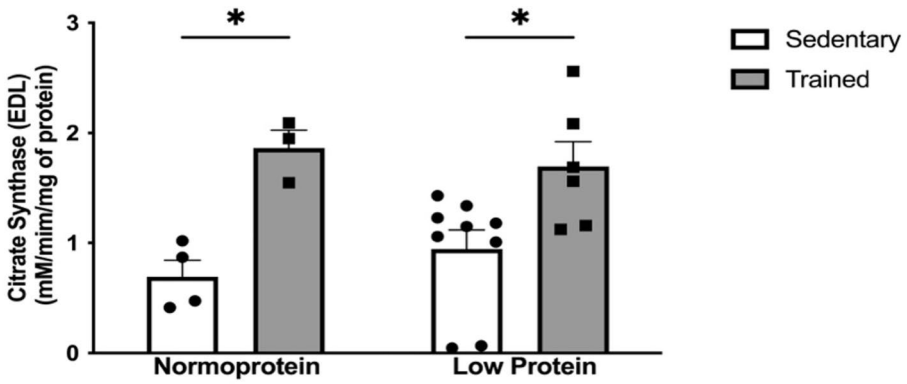

Figure 1. Citrate synthase activity in skeletal muscle of juvenile Wistar rats after four weeks of AET; (NS; n = 5; LS; n = 9; NT; n = 4; LT; n = 6); *p < 0.05.

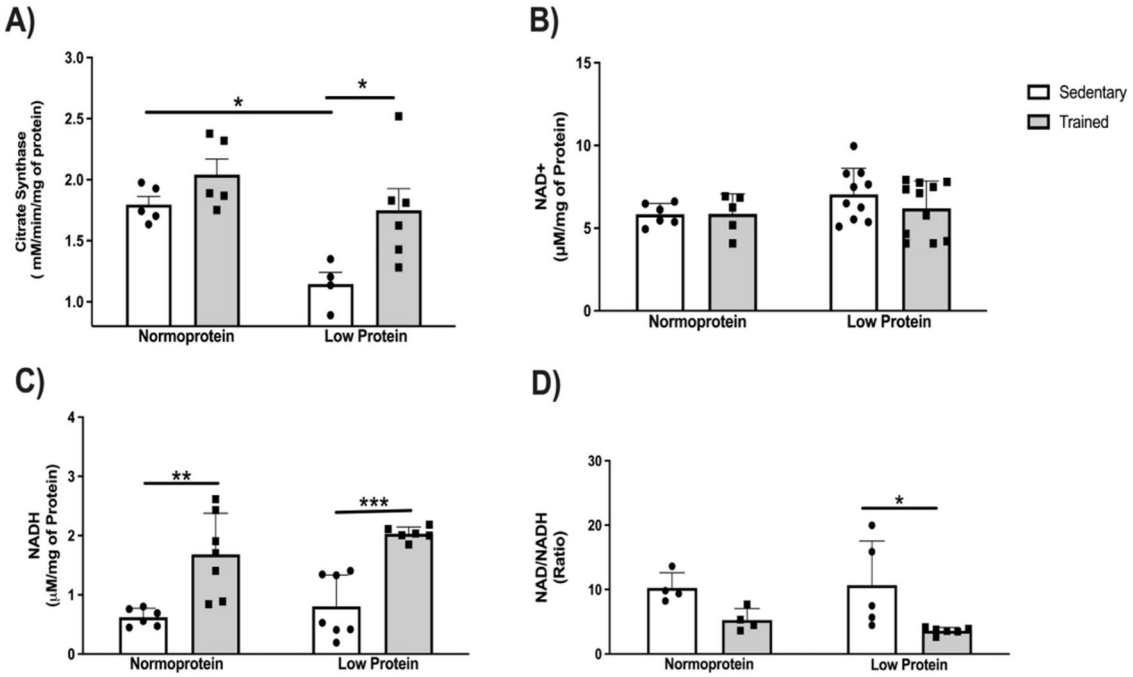

Figure 2. (**A**) Citrate synthase activity (NS; n = 5; LS; n = 5; NT; n = 5; LT; n = 6); and Oxidoreduction kinetics of pyridine nucleotides in the prefrontal cortex of juvenile Wistar rats after four weeks of AET; (**B**) NAD + (NS; n = 4; LS;n = 6;NT;n = 6;LT;n = 8); (**C**) NADH (NS;n = 6;LS;n = 7;NT;n = 7;LT;n = 6); (**D**) NAD + /NADH ratio (NS; n = 4; LS; n = 5; NT; n = 4; LT; n = 6); *p < 0.05; **p < 0.01.

related to the levels of Nicotinamide Adenine Dinucleotide in its reduced form (NADH), no significant differences between LS and NS groups were found (p = 0.09), (Fig. 2C). But a significant increase was found in the NT group (NT:1.684 ± 0.69 uM/mg of protein vs NS: 0.6216 ± 0.15 uM/mg of protein, p = 0.002) compared to the NS after 4 weeks of AET. Moreover, LT showed a high level of NADH (LT:2.031 ± 0.11 uM/mg of protein vs LS: 0.803 ± 0.53 uM/mg of protein, p = 0.0005), compared to the LS group. The oxidoreduction ratio (NAD^+/NADH) showed no significant differences between LS vs NS and NT vs NS groups (p = 0.99; p = 0.28, respectively). In contrast, the LS group obtained a significant increase in this ratio (LS: 10.70 ± 6.84 U/A vs LT: 3.610 ± 0.54 U/A, p = 0.03), when compared to the LT group (Fig. 2D). This data also suggests that AET was able to alter cellular metabolism by modulating the levels of electron acceptors, fundamental for cellular energy homeostasis.

Oxidative stress biomarkers

To elucidate the effects of MPM on OS markers, the levels of Thiobarbituric acid reactive species (TBARS), one of the mediators of the lipid peroxidation process were evaluated. In addition, the levels of carbonyls, in response to increase of protein oxidation was assessed. In the TBARS levels, no significant differences were observed when comparing the LS and NS groups (LS:4.183 ± 1.93 mM/mg of protein vs NS: 3.914 ± 0.95 mM/mg of protein, p = 0.99) (Fig. 3A). Equally, no significant differences were observed in the NT vs NS and LT vs NT groups, respectively (NT: 3.245 ± 1.96 mM/mg of protein vs NS: 3.914 ± 0.95 mM/mg of protein, p = 0.89, LT: 4.005 ± 1.43 mM/mg of protein vs NT: 3.245 ± 1.96 mM/mg of protein, p = 0.82), as well as LT and LS (LT: 4.005 ± 1.43 mM/mg of protein vs LS: 4.183 ± 1.93 mM/mg of protein, p = 0.99). In the carbonyls assay, no significant differences were found between the LS and NS groups (LS: 46.61 ± 14.56 mM/mg of protein vs NS: 43.57 ± 19.38 mM/mg of protein, p = 0.97), as well as between the NT and NS groups (NS: 43.57 ± 19.38 mM/mg of protein vs NT: 48.35 ± 13.04 mM/mg of protein, p = 0.93). However, the LS group showed a significant increase in carbonyls levels (LS: 46.61 ± 14.56 mM/mg of protein vs LT: 27.54 ± 9.69 mM/mg of protein, p = 0.03) when compared with the LT group after AET for 4 weeks. Although there are no significant differences in lipid peroxidation, our findings indicate that AET was able to modulate protein oxidation (Fig. 3B).

Enzymatic antioxidant system

After the evaluation of the oxidative stress biomarkers, we evaluated antioxidant balance: superoxide dismutase (SOD), catalase (CAT) and glutathione S transferase (GST). SOD enzyme responsible for catalyzing the dismutation of superoxide into oxygen (O_2) and hydrogen peroxide (H_2O_2). No significant differences were found between LS and NS groups (LS: 3.495 ± 0.88 mM/min/mg of protein vs NS: 4.755 ± 0.54 mM/min/mg of protein, p = 0.07). The absence of significant differences was also observed between NT and NS groups. (NT: 5.622 ± 1.36 mM/min/mg of protein vs NS: 4.755 ± 0.54 mM/min/mg of protein, p = 0.35). Regarding the LT and NT groups, there were no significant differences (LT: 5.119 ± 0.77 mM/min/mg of protein vs NT: 5.622 ± 1.36 mM/min/mg of protein, p = 0.72). In the LT and LS groups, however, there was a significant increase, 31.74% (LT: 5.119 ± 0.77 mM/min/mg of protein vs LS: 3.495 ± 0.88 mM/min/mg of protein, p = 0.006), (Fig. 3C).

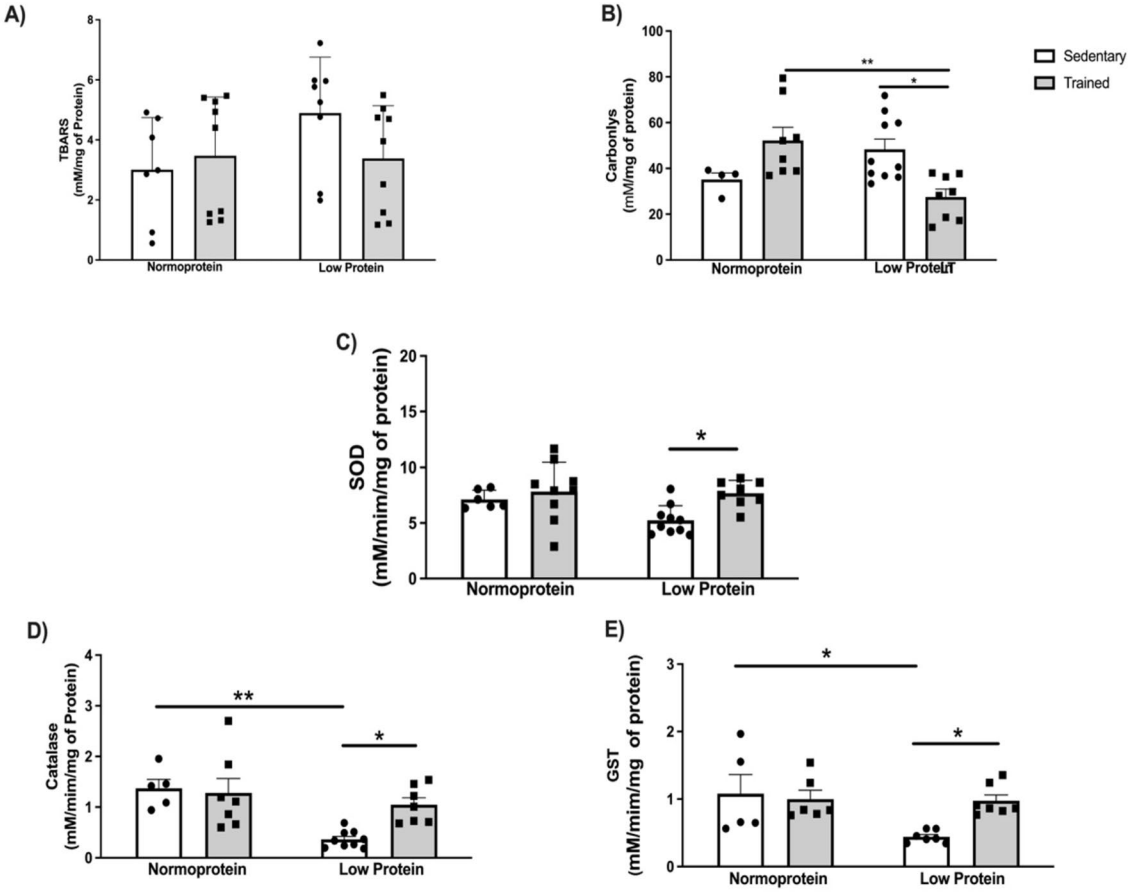

Figure 3. Levels of oxidative stress markers; (**A**) TBARS (NS = 5; LS = 8; NT = 8; LT = 7); (**B**) Carbonyls (NS; n = 5; LS; n = 11; NT; n = 7; LT; n = 8); and Antioxidant enzymatic activity in the prefrontal cortex of juvenile Wistar rats after four weeks of AET; (**C**) SOD (NS; n = 6; LS; n = 10; NT; n = 8; LT; n = 8); (**D**) Catalase (NS; n = 5; LS; n = 9; NT; n = 9; LT; n = 7); (**E**) GST (NS; n = 5; LS; n = 7; NT; n = 6; LT; n = 7); *p < 0.05; **p < 0.01.

Next, the CAT (Fig. 3D) that operates on the decomposition of H_2O_2 was analyzed by adding samples with H_2O_2 as a substrate to assess the isolated effect of MPM by comparing the LS and NS groups. LS group presented a significant decrease in CAT activity (LS: 0.1216 ± 0.05 mM/min/mg of protein vs NS: 0.457 ± 0.13 mM/min/mg of protein, p = 0.003), in relation to the NS group. For the same experience, in the NT vs NS and LT vs NT groups, there were no significant differences (NT: 0.4275 ± 0.25 mM/min/mg of protein vs NS: 0.4574 ± 0.13 mM/min/mg of protein, p = 0.98, LT: 0.3496 ± 0.12 U/mg of protein vs NT: 0.4275 ± 0.25 mM/min/mg of protein, p = 0.77). Comparing the LT and LS groups, we observed that the LT group demonstrated a significant increase in CAT activity later, after 4 weeks of AET (LT: 0.3496 ± 0.12 mM/min /mg of protein vs LS: 0.1216 ± 0.05 mM/min /mg of protein, p = 0.03) in relation to LS group.

In sequence, we also assessed GST activity (Fig. 3E), a key enzyme in the metabolic detoxification process through the reduction of xenobiotic compounds, from the promotion of nucleophilic attacks by reduced glutathione (GSH) on electrophilic atoms of carbon, sulfur, and nitrogen. Furthermore, this process promoted the catalysis and conjugation of GSH. Our results demonstrated a significant reduction in GST activity in the LS group of (LS: 0.441 ± 0.09 mM/min/mg of protein vs NS: 1.078 ± 0.64 mM/min/mg of protein, p = 0.02) compared to the NS group. On the other hand, in the NT and NS groups, no significant differences were observed (NT: 1.000 ± 0.32 mM/min/mg of protein vs. NS:1.078 ± 0.64 mM/min /mg of protein, p = 0.98). No significant differences were observed either in the LT and NT groups (LT: 0.975 ± 0.23 mM/min/mg of protein vs NT: 1.000 ± 0.32 mM/min/mg of protein, p = 0.99). In contrast, the LT and LS groups, the LT group underwent a significant increase in GST activity (LT: 0.975 ± 0.23 mM/min/mg of protein vs LS: 0.441 ± 0.09 mM/min/mg of protein, p = 0.04) after AET protocol with the LS group. In summary, four weeks of AET were able to potentiate the antioxidant enzymatic activity in animals that previously was subjected to MPM insult.

Non-enzymatic antioxidant system

At the end of the evaluation of the enzymatic system, we assessed the non-enzymatic system. The level of GSH is important in various cellular events, such as antioxidant defense, regulation of cell metabolism, gene expression, signal transduction, cell proliferation and apoptosis. In our study, no significant differences were observed between the LS and NS groups (LS: 1007.111 ± 203.90 µM/mg of protein, vs NS:1111.508 ± 427.3 µM/

mg of protein, p = 0.96). Likewise, when comparing the NT and NS groups no significant differences were obtained, either (NT: 832.047 ± 174.221 µM/mg of protein vs NS: 1111.00 ± 427.3 µM/mg of protein, p = 0.62); (Fig. 4A). However, in the LT vs LS and LT vs NT groups, a significant increase was found in the LT for both comparisons (LT: 1836.109 ± 530.2 µM/mg of protein vs LS: 1007.11 ± 203.9 µM/mg of protein, p = 0.001, LT: 1836.109 ± 530.195 µM/mg of protein vs NT: 832.047 ± 174.2 µM/mg of protein, p = 0.001). Then, oxidized glutathione (GSSG) was analyzed. This component acts secondarily in the deactivation of ROS, for the preservation of thiol groups present in proteins and estimation of the REDOX state in biological systems (Fig. 4B). In the LS and NS groups, no significant differences were observed (LS:1.344 ± 0.19 µM/mg of protein vs NS: 0.713 ± 0.28 µM/mg of protein, p = 0.22). Similarly, no significant differences were seen in the NT and NS groups (NT:1.287 ± 0.56 µM/mg of protein vs NS: 0.713 ± 0.28 µM/mg of protein, p = 0.24), as well as in the LT vs NT (LT: 1.491 ± 0.59 µM/mg of protein vs NT: 1.287 ± 0.56 µM/mg of protein, p = 0.86) and LT vs LS groups (LT:1.491 ± 0.59 µM/mg of protein vs LS:1.344 ± 0.19 µM/mg of protein, p = 0.95).

We analyzed the GSH/GSSG ratio (Fig. 4C), known as the reduction and oxidation state (REDOX), which indicates the transfer of electrons with variability in reactivity, being an indicator of cellular health; reduced GSH constitutes up to 98% of cellular GSH under normal conditions. In the LS and NS groups, no significant differences were found (LS: 855.102 ± 218.3 U/A vs NS: 678.781 ± 278.0 U/A, p = 0.93). However, when comparing the NT and NS groups, the NT group registered a significant increase in the ratio between non-enzymatic components (NT: 1615.135 ± 712.2 U/A vs NS: 678.781 ± 278.0 U/A, p = 0.04). Regarding the LT vs NT (LT: 1105.757 ± 572.4 U/A vs NT: 1615.135 ± 712.2 U/A, p = 0.35) and the LT and LS groups (LT: 1105.757 ± 572.4 U/A vs LS: 855.102 ± 218.3 U/A, p = 0.80), there was no significant difference.

The sulfhydryl group is responsible primarily for the antioxidant activity of plasma proteins (Fig. 4D). A comparative analysis of sulfhydryl levels in the LS and NS groups showed no significant difference (LS: 0.095 ± 0.03 mM/mg of protein vs NS: 0.107 ± 0.01 mM/mg of protein, p = 0.74). Similar findings were observed when comparing the NT vs NS and LT vs NT groups, respectively (NT:0.126 ± 0.02 mM/mg of protein vs NS: 0.107 ± 0.01 mM/mg of protein, p = 0.42, LT: 0.129 ± 0.01 mM/mg of protein vs NT: 0.126 ± 0.02 mM/mg of protein, p = 0.98). However, when comparing the LT and LS groups, the LT group obtained a significant increase (LT: 0.129 ± 0.01 mM/mg of protein vs LS:0.095 ± 0.03 mM/mg of protein, p = 0.01) in relation to the LS group. In this context, our results also point out that AET can modulate non-enzymatic antioxidant defense in the PFC of rats submitted to MPM.

ER stress mRNA levels

After the analyses related to oxidative stress, further indications were evaluated of the effect of maternal protein malnutrition and AET on genes linked to ER stress. Initially, we evaluated GRP78, which facilitates the assembly and folding of proteins. In the LS group, a significant increase in gene expression was found compared to the NS group (LS: 8.474 ± 0.39 U/A vs NS:1.000 ± 0.38 U/A, < 0.0001). A comparison of the NT and NS groups

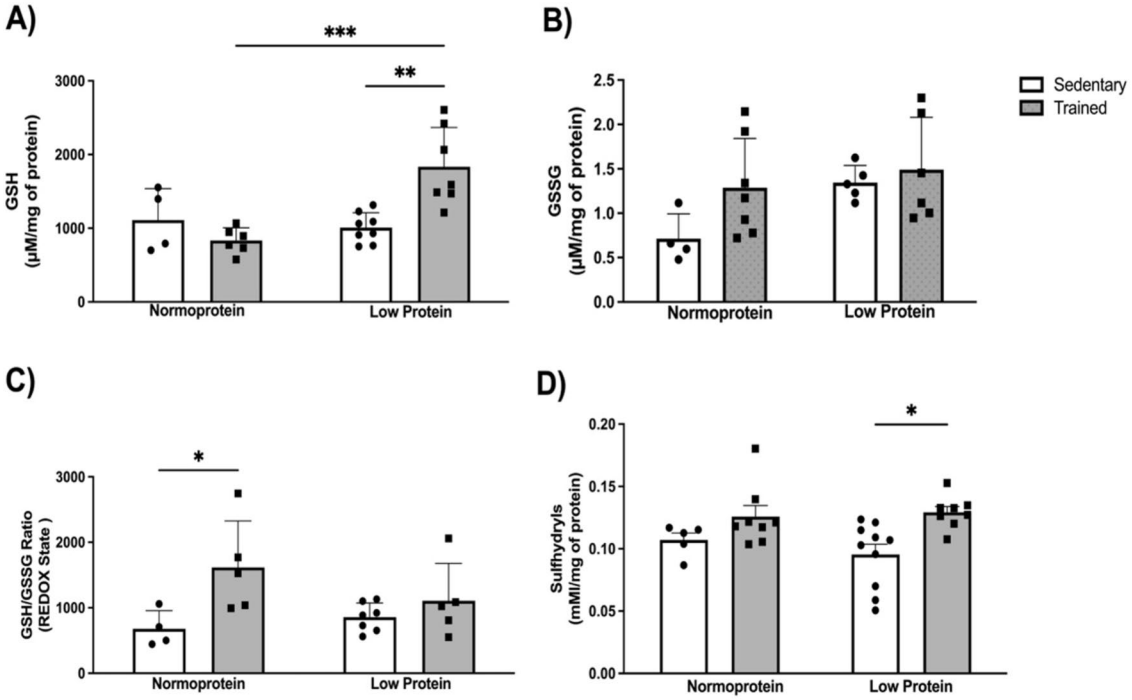

Figure 4. Non-enzymatic antioxidant activity in the prefrontal cortex of juvenile Wistar rats after four weeks of AET; (**A**) GSH (NS; n = 4; LS; n = 8; NT; n = 6; LT; n = 7); (**B**) GSSG (NS; n = 4; LS; n = 6; NT; n = 7; LT; n = 7); (**C**) GSH/GSSG (NS; n = 4; LS; n = 7; NT; n = 5; LT; n = 5); (**D**) Sulfhydryl's (Total Thiols); (NS; n = 5; LS; n = 8; NT; n = 8; LT; n = 8); *p < 0.05; **p < 0.01; ***p < 0.001.

showed no statistically significant changes (NT: 1.543 ± 0.17 U/A vs NS: 1.000 ± 0.38 U/A, p = 0.626). However, when comparing the groups that suffered MPM, the LT group showed a significant reduction in the expression of this marker when compared to the LS group (LT:3.300 ± 0.11 U/A vs LS:8.474 ± 0.39 U/A, p < 0.0001). Finally, a significant decrease in the expression of GRP78 in the NT group was observed when compared to the LT group, demonstrating the effectiveness of AET under different nutritional conditions (LT: 3.300 ± 0.11 U/A vs NT:1.543 ± 0.17 U/A, p = 0.006), (Fig. 5A). The next step was to evaluate PERK, responsible for controlling the homeostasis of the endoplasmic reticulum through the signaling of stress situations, apoptosis, and autophagy. No significant differences were found among any of the experimental groups (LS: 1.485 ± 0.37 U/A vs NS: 1000 ± 0.29 U/A, p = 0.73; NT: 1.032 ± 0.36 U/A vs NS:1.000 ± 0.29 U/A, p = 0.99; LT: 1.710 ± 0.26 U/A vs LS: 1.485 ± 0.37 U/A, p = 0.96, LT: 1.710 ± 0.26 U/A vs NT: 1.032 ± 0.36 U/A, p = 0.49); (Fig. 5B).

Related to the previous genes, the ATF6 is an important molecular signal for ER stress under conditions of poor protein folding and protein degradation. Initially, the LS group showed a significant increase in the expression of this gene compared to the NS group (LS: 76.321 ± 0.509 U/A vs NS: 1.000 ± 0.459 U/A, p < 0.0001). However, between the NT and NS groups there were no significant differences (NT: 1.487 ± 0.482 U/A vs NS:1.000 ± 0.459 U/A, p = 0.99). Finally, a reduction in *ATF6* expression levels was identified in the LT group when compared to the LS group (LT: 30.110 ± 2.564 U/A vs LS: 76.321 ± 0.509 U/A, p < 0.0001); when the LT and NT groups were compared, however, we observed an increase in ATF6 gene expression in the LT group (LT: 30.110 ± 2.564 U/A vs NT: 1.487 ± 0.482 U/A, p < 0.0001), (Fig. 5C). Taken together, our data indicate that AET decreased the gene expression of ER stress markers in the PFC of rats exposed to MPM.

Brain-derived neurotrophic factor mRNA levels

Brain-Derived Neurotrophic Factor (BDNF) is an important regulator of neural morphofunctionality and cognitive function. The BDNF was evaluated, and we observed that MPM was able to increase BDNF expression significantly (LS: 32.480 ± 0.934 U/A vs NS: 1.000 ± 0.838 U/A, p < 0.0001). When the AET response was evaluated in isolation, the NT group obtained a significant increase in BDNF expression compared to the NS group (NT: 15.450 ± 0.614 U/A vs NS: 1.000 ± 0.838 U/A, p < 0.0001). Surprisingly, the LT group showed significant reduced BDNF levels when compared to the LS group (LT: 6.600 ± 2.564 U/A vs LS:32.480 ± 0.934 U/A, p < 0.0001), (Fig. 5D). In the LT group, however, there was a significant decrease in the expression of BDNF, when compared to normal-nourished rats that underwent AET for 4 weeks (LT: 6.600 ± 2.564 U/A vs LS: 32.480 ± 0.934 U/A, p < 0.0001). Briefly, our data indicate that four weeks of AET wasn't potent enough to increase the expression of BDNF in protein malnutrition.

Discussion

Studies have shown that several brain areas including the cortex, cerebellum, and brainstem, when exposed to protein malnutrition, are more vulnerable to cellular stress and dysfunction[30,31]. To test this, we investigated the effects of AET on mitochondrial bioenergetic markers, oxidative balance and gene expression related of ER stress in the PFC of *Wistar* rats subjected to MPM, during pregnancy and postnatal critical periods for growth and development, according to the scientific literature[14,15,32–34]. In summary, our work observed that AET at

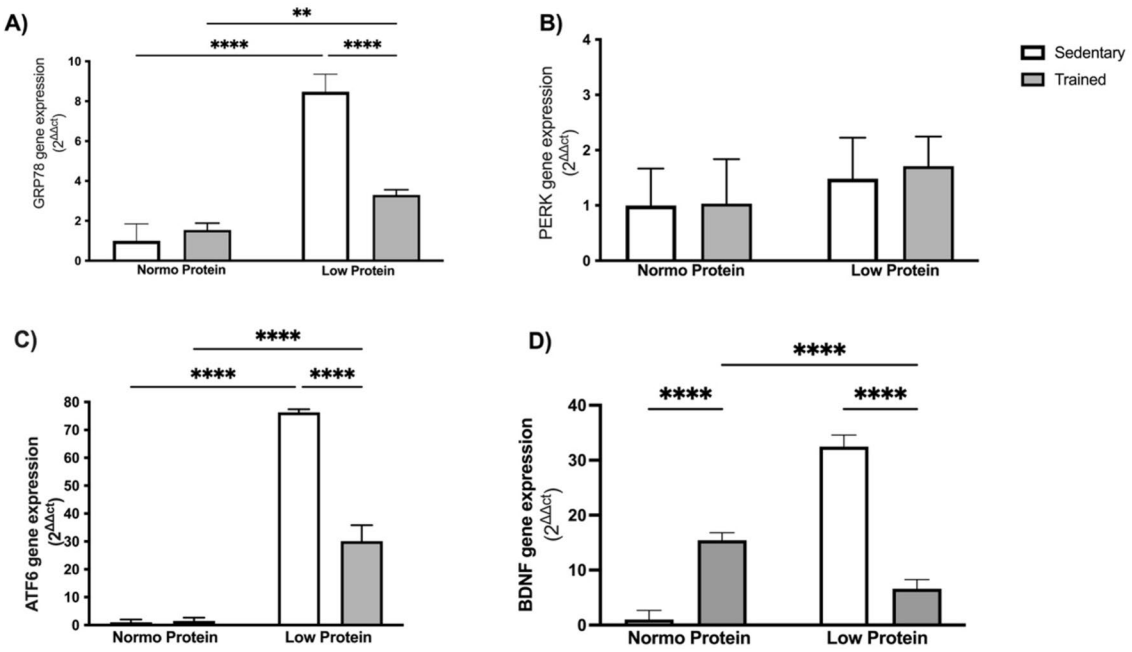

Figure 5. mRNA levels in the prefrontal cortex of juvenile Wistar rats after four weeks of AET; (**A**) GRP78 (NS; n = 5; LS; n = 5; NT; n = 4; LT; n = 5); (**B**) PERK (NS; n = 5; LS; n = 4; NT; n = 5; LT; n = 4); (**C**) ATF6 (NS; n = 5; LS; n = 5; NT; n = 6; LT; n = 5); (**D**) BDNF; (NS; n = 4; LS; n = 5; NT; n = 5; LT; n = 5); *p < 0.05; **p < 0.01.

moderate intensity was able to partially restore the changes caused by maternal protein restriction in the indicators of mitochondrial function, antioxidant defense system and ER stress in the PFC.

Initially, our data demonstrated increased levels of citrate synthase and NADH markers of oxidative metabolism in the LT rats when compared to the LS group. Previous studies have suggested that MPM can promote damage to mitochondrial function, including the reduction of membrane electrical potential and oxygen consumption in central and peripheral tissues, which may cause mitochondrial dysfunction. This condition is associated with several factors, including excessive production of ROS, reduced antioxidant defenses and dysregulation of calcium metabolism[29]. Pedroza et al., using the same model of MPM associated with AET, observed similar results in the activity of citrate synthase and NADH. Additionally, after the intervention, the trained rats showed an increase in oxygen consumption and mitochondrial respiratory control rate, pointing to the effectiveness of this protocol in promoting adaptations in mitochondrial control under unfavorable nutritional conditions, and thus providing protection against the emergence of pathological conditions during adulthood[14]. This suggests that the AET can improve mitochondrial bioenergetics in different tissues.

Next, we analyzed the TBARS and Carbonyl levels indicative of oxidative stress, as well as the enzymatic and non-enzymatic antioxidant defenses. After 4 weeks, the LT group showed a decrease in protein oxidation, an increase in the activity of SOD, CAT, and GST. Carbonyl groups, in the form of aldehydes and ketones, are found in protein side chains, especially through the oxidation of amino acids including arginine, lysine and threonine[35]. A previous study by Pedroza et al.[14], using the same MPM model in cardiac tissue, observed an increase in ROS production associated with higher levels of lipid peroxidation and protein oxidation. Our data did not observe changes in lipid peroxidation levels, demonstrating that each tissue can have different responses to similar stimuli[14]. In addition, excessive oxidation of this biological component in the brain is related to behavioral disorders and neurodegenerative diseases such as Alzheimer's and Parkinson's[36]. Therefore, to mitigate ROS effect, the action of different antioxidant defenses is essential.

Primarily, SOD catalyzes the reaction of the superoxide anion into hydrogen peroxide, which is then detoxified into oxygen and water by catalase. On the other hand, GST integratively acts by catalyzing the conjugation of GSH to xenobiotic substrates to detoxify the intracellular environment. Corroborating our findings, Moradi-Kor observed high activity of SOD and glutathione peroxidase in the PFC after 15 days of voluntary physical exercise in female adolescent Wistar rats in an anxiety and depression model[37]. Furthermore, systematic review and meta-analysis studies conducted by De Souza confirm that aerobic exercise can increase the antioxidant capacity in different brain regions and promote morphofunctionality benefits[38]. Besides these observations, it's important mention that the exercise is not a "magical" tool and always will induce improve in antioxidant defense or decrease oxidative stress biomarkers. A systematic review published in 2013 already showed that exercise can induce positive, negative or non-effect in brain[39], corroborating with this review, recent published paper demonstrate that in peripheral tissue associated to SNC (i.e. sciatic nerve), exercise doesn't improve antioxidant defense, nor decrease oxidative stress marker[40].

The non-enzymatic system also plays a fundamental role in combating oxidative damage, acting in symbiosis with enzymatic antioxidants[41]. GSH plays a key role in removing reactive species. Moreover, these components act together with their enzymes, known as the glutathione system, responsible for the detoxification of ROS and nitrogen species (RNS) and electrophiles produced by xenobiotics[42]. In prefrontal cortex, we did not observe statistical difference between control and malnourished group in GSH and thiols levels. However, when we apply AET, the levels of GSH and total thiols increased significantly. These results differ from Neves et al.[43] who did not observe increase in GSH levels after 8 weeks of AET in the PFC and hippocampus of Wistar rats submitted to MPM. The variability in the experimental design associated with the age that the training and the experiments were applied and the structure of physical training related to intensity, duration, and frequency may be related to the discrepancy of these results[44]. In our case we observed a positive effect of the exercise in the group that suffer a nutritional insult, suggesting that for individual with nutritional imbalance, exercise can restore the homeostasis. From our data, compared to previous data in literature, we can speculates that the exercise effects will dependent of the pre-insult (i.e. normonourished or protein restriction), and the structure of the training protocol.

To understand possible mechanisms involved in MPM and AET effect, we evaluated the ER stress markers and BDNF, important regulators of neurodevelopment and cognitive function[45]. Our data showed that AET was able to reverse the effects of protein malnutrition by significantly decreasing the expression of GRP78, and ATF6, important components in the ER stress cell signaling pathway. Our work is the first study to assess the effects of AET under conditions of protein deficit in ER stress in PFC. Nevertheless, research with different tissues such as blood, heart and skeletal muscle and experimental models, including studies in humans, has demonstrated that the decrease in the expression of these factors by AET promotes protection against cardiometabolic and neurogenerative diseases, although the mechanisms for promoting these benefits have not been fully elucidated[46].

Surprisingly, BDNF levels were lower after AET in a study by LT. Wang and Xu[47], suggested that exposure to protein malnutrition is able to modulates BDNF levels significantly, leading to worse memory and decreased learning performance in rats[47]. Our data does agree with other data presented in the literature, where different modalities of physical exercise have been reported as responsible for increasing BDNF levels, resulting in high levels of neuronal plasticity and improvement in cognitive function[48–50]. In states of nutritional deprivation, the body has the ability to prioritize or direct the nutrient flow to vital organs that are fundamental for our survival, specially the brain[51]. The increased levels of BDNF expression observed in LS, was an additional surprise for our analysis, and further studies may be need to understand which mechanism are related, since previous data in literature evaluating BDNF gene expression on hippocampus from rats at 30 and 90 days of age, that was submitted to maternal protein restriction, demonstrated a decrease in the BDNF levels[52]. Besides our data goes in opposite direction compared to previous studies, we can speculates that this increase in BDNF observed in LS may be related to the modulatory response associated with the necessity for growth, development and control of cerebral homeostasis associated with several others neurochemical factors or also due to the positive feedback activated

by the lower level of BDNF protein, however more studies are need to confirm our hypothesis. Although, its relevant consider that previous data in literatures suggested that hypocaloric diets can significantly increase BDNF levels and acts in the prevention of sarcopenia in humans[53] and previous data from our laboratory evaluating in hippocampus applying the same experimental design showed also an increase in BDNF mRNA levels[15].

As there are few studies of the relation between AET and BDNF under MPM conditions, future studies using different brain regions, including orbitofrontal, dorsolateral, ventromedial, hypothalamus, or even brainstem, should be performed. We believe a better understanding of the possible causal relationship between these parameters has clinical relevance, especially in countries with higher malnutrition levels. In addition, comparing the impacts of MPM associated with different physical exercise protocols in other brain areas would allow a substantial elucidation of the mechanisms responsible for these alterations in the nervous system and give possible clues for therapeutical interventions.

The present study presents new knowledge about the positive impacts of AET at moderate intensity on markers of mitochondrial function, enzymatic and non-enzymatic antioxidant balance, as well as the expression of genes linked to ER stress in MPM conditions in the PFC. This is the first study in the scientific literature that aims to observe such impacts. In this study, some limitations were found, including the need to investigate a greater number of genes linked to ER stress and neurotrophic factors linked to neuroplasticity, as well as the quantification of their protein levels. Additionally, we recommend the observation of cellular and molecular results associated with cognitive and behavioral data, which was not the objective of the present study but would help in the broad understanding of the impacts of AET on the functionality of the PFC. Furthermore, their results point to new frontiers of knowledge to be produced, in relation to (1) through which mechanisms effectively does AET at moderate intensity promote such benefits, (2) what are the repercussions on the functionality of the PFC, through future studies that aim to analyze tests linked to planning, management and execution of tasks corroborating findings in humans.

In conclusion, AET using a speed pattern at moderate intensity (6.6 m/min) for 4 weeks in young adult Wistar rats was able to combat the deleterious effects of a protein-restricted maternal diet about the parameters of mitochondrial bioenergetics, protein oxidation, antioxidant balance and markers of ER stress in the PFC.

Data availability

All genes used in the present study have their respective accession numbers β2-microglobulin (β2M) [Accession number: NM_012512]), ATF6 [Accession number: NM_001107196]), PERK [Accession number: NM_001399818]), GRP78 [Accession number :NM_0013083]) and BDNF [Accession number: NM_001270630]), this information was taken from the National Center for Biotechnology Information website. https://www.ncbi.nlm.nih.gov. The data supporting this study's findings are available from the corresponding author upon reasonable request.

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Author contributions

MSSF: Conceptualization; Data discussion; Formal analysis; Investigation; Statistical analysis; Writing original draft. AASP, DGMS: Methodology; Formal analysis; Investigation; SCAS and ARP: Methodology; Formal analysis; Investigation; Formal analysis; Investigation; MPF: Data discussion; Statistical analysis; Writing original draft, Writing—review & editing; FHY; LPA and CJL: Conceptualization; Funding acquisition; Grant administration; Supervision; Writing—review & editing.

Competing interests
The authors declare no competing interests.

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