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Sex-dependent effect on mitochondrial and oxidative stress parameters in the hypothalamus induced by prepubertal stress and access to high fat diet

Ana Paula Toniazzo[a,*], Danusa Mar Arcego[a], Camilla Lazzaretti[b], Carina Mota[a], Carlos Eduardo Schnorr[a,c], Letícia Ferreira Pettenuzzo[a], Rachel Krolow[a], Jose Claudio Fonseca Moreira[a], Carla Dalmaz[a,b]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Porto Alegre, Rio Grande do Sul, Brazil
[b] Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Departament of Civil and Environmental, Universidad de la Costa, Calle 58 #55-66, Barranquilla, Atlántico, Colombia

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ABSTRACT

Objective: Some factors related to lifestyle, including stress and high-fat diet (HFD) consumption, are associated with higher prevalence of obesity. These factors can lead to an imbalance between ROS production and antioxidant defenses and to mitochondrial dysfunctions, which, in turn, could cause metabolic impairments, favoring the development of obesity. However, little is known about the interplay between these factors, particularly at early ages, and whether long-term sex-specific changes may occur. Here, we evaluated whether social isolation during the prepubertal period only, associated or not with chronic HFD, can exert long-term effects on oxidative status parameters and on mitochondrial function in the whole hypothalamus, in a sex-specific manner.
Methods: Wistar male and female rats were divided into two groups (receiving standard chow or standard chow + HFD), that were subdivided into exposed or not to social isolation during the prepubertal period. Oxidative status parameters, and mitochondrial function were evaluated in the hypothalamus in the adult age.
Results: Regarding antioxidant enzymes activities, HFD decreased GPx activity in the hypothalamus, while increasing SOD activity in females. Females also presented increased total thiols; however, non-protein thiols were lower. Main effects of stress and HFD were observed in TBARS levels in males, with both factors decreasing this parameter. Additionally, HFD increased complex IV activity, and decreased mitochondrial mass in females. Complex I-III activity was higher in males compared to females.
Conclusion: Stress during the prepubertal period and chronic consumption of HFD had persistent sex-specific effects on oxidative status, as well as on its consequences for the cell and for mitochondrial function. HFD had more detrimental effects on females, inducing oxidative imbalance, which resulted in damage to the mitochondria. This HFD-induced imbalance may be related to the development of obesity.

1. Introduction

The prevalence of obesity increased in the last four decades, and remains a challenge for public health worldwide (Arroyo-Johnson and Mincey, 2016). This increase has been attributed to changes in lifestyle (Ballal et al., 2010), in which sedentary habits (Stein and Colditz, 2004), the consumption of high-fat diets (HFD) (Buettner et al., 2007), and exposure to stress (Adam and Epel, 2007; Huneault et al., 2011) are highly associated.

Stress exposure and high-fat diet (HFD) during sensitive periods of development, such as the prepubertal period, could be associate with the rising rates of obesity observed in children and adolescents. An early exposure to these two factors can induce sex–specific effects on hormonal signaling related to energy balance (Toniazzo et al., 2018) and also program metabolism in juvenile animals (Krolow et al., 2013). In this context, the prepubertal period (immediately prior to the onset of puberty) is critical for sexual maturation (McCormick and Mathews, 2007), and to the development of neuronal circuits that control energy homeostasis and stress responses. In this context, the prepubertal period (immediately prior to the onset of puberty) is critical for sexual maturation, and to the development of neuronal circuits that control energy homeostasis and stress responses (McCormick and Mathews, 2007).

One animal model of stress exposure during the prepubertal period

* Corresponding author. Depto Bioquímica, UFRGS Ramiro Barcelos, 2600 Lab.37, 90035-003, Porto Alegre, RS, Brazil.
E-mail address: aninha.toniazzo84@gmail.com (A.P. Toniazzo).

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is social isolation: In rodents, social interactions are rewarding and crucial for the social and emotional development of these animals (Douglas et al., 2003, 2004; Panksepp and Lahvis, 2007). Therefore, exposure to social isolation in the prepubertal period may have persistent effects on emotion, behavior, and metabolism (Pervanidou and Chrousos, 2012; Arcego et al., 2017), and it models stress to which children may be exposed during the beginning of their school years.

Additionally, stress exposure, both during the development or in the adulthood, can induce changes in eating behavior (Ely et al., 1997). According to the intensity and duration of stress exposure, it may cause both, increases and decreases in food intake (Ely et al., 1997; Silveira et al., 2000; Pecoraro et al., 2004; Groesz et al., 2012). In response to stress, rats demonstrates increased motivation for the ingestion of "comfort foods" (sucrose and fat) (Dallman et al., 2003, 2005), and stressed animals many times choose diets rich in fat, which is associated with obesity, especially when it is chronically consumed (Peckham and Entenman, 1962; Teegarden and Bale, 2008). The excess of energy supply by HFD may contribute to the increase of reactive oxygen species (ROS) production (Frohnert and Bernlohr, 2013) that, in turn, may lead to mitochondrial dysfunction in muscle (Bonnard et al., 2008) and in brain (Freeman et al., 2013). In agreement, the mediators of the stress response also exert several effects on mitochondrial biogenesis, metabolism, ROS generation, and apoptosis (Manoli et al., 2007). In adults, acute stress is associated with increases in mitochondrial biogenesis, and the enzymatic activity of some respiratory chain complexes. Conversely, chronic stress can lead to abnormal mitochondrial biogenesis, respiratory chain dysfunction, decreased ATP production, increased ROS generation, lipid peroxidation, mitochondrial and nuclear DNA damage, and increased cell apoptosis and/or necrosis (Manoli et al., 2007). Additionally, there are evidences that mitochondrial function (Guevara et al., 2009, 2011), brain mitochondrial respiration, oxidative stress (Gaignard et al., 2015), and mitochondrial biogenesis are sex-dimorphic (Sharma et al., 2014).

The main function of mitochondria is to produce energy for cells in form of adenosine triphosphate (ATP). However, aside from ATP production, the mitochondria participates in the ROS production and elimination (Bournat and Brown, 2010). The imbalance between ROS production and antioxidant defenses (oxidative stress) may culminate in cellular damage (Halliwell, 1997), and, as mentioned above, it may impair mitochondrial function (Frohnert and Bernlohr, 2013). The brain is highly affected by mitochondrial dysfunction due to its high metabolic demand for energy, provided mainly from mitochondrial oxidative metabolism, and due to its vulnerability to oxidative stress (Sohal et al., 1990). However, specific brain regions appear to be particularly susceptible to injury, and in models of obesity, the hypothalamus is highly susceptible to changes (Velloso and Schwartz, 2011; Valdearcos et al., 2017), probably due to its relation with appetite, body weight gain, and glucose homeostasis (Gyengesi et al., 2012). In this sense, excessive consumption of high-fat diets causes damage to neurons in the hypothalamus, resulting in energy metabolism imbalance, a characteristic found in obesity (Velloso and Schwartz, 2011; Cavadas et al., 2016). HFD diets may alter mitochondrial dynamics, thus interfering in energetic homeostasis (Carraro et al., 2018), and the mitochondrial dysfunction is, among other mechanisms, involved in hypothalamic damage in obesity (Dietrich et al., 2013).

Considering (a) the ability of early environment, including stress exposure and HFD access, two highly prevalent events in the present human societies, in programming hypothalamic functions, and considering (b) that these factors may affect oxidative status and mitochondrial functions, the aim of this work was to investigate the effects of exposure to stress in the prepubertal period and chronic access to HFD, on the long-term oxidative status and oxidative damage to cells. We also aimed to evaluate respiratory complexes activities and mitochondrial mass in adult male and female rats.

2. Materials and methods

2.1. Subjects

All proceedings were performed in strict accordance to the recommendations of the Brazilian Society for Neurosciences (SBNeC), Brazilian Law on the use of animals (Federal Law 11.794/2008) and were approved by the Institutional Ethical Committee (CEUA-UFRGS 27714). All efforts were made to minimize animal suffering, as well as to reduce the number of animals used. Wistar rats from our own breeding colony were housed in Plexiglas cages (65 × 25 × 15 cm) with the floor covered with sawdust and maintained on a standard 12 h dark/light cycle (lights on between 7:00 h and 19:00 h), temperature of 22 ± 2 °C. At postnatal day (PND) 21, males and females were weaned and separated according to sex. Half of the animals were housed in standard cages in groups of 3–5 animals (control); the other animals were submitted to stress by social isolation (isolated in a smaller home cage, 27 × 17 × 12 cm) (Douglas et al., 2004; Arcego et al., 2014). Only one male and one female per litter were used in each group. Different diets were offered to the animals: (a) standard lab chow; (b) both standard chow and HFD. These last animals were free to choose between standard chow and HFD. Therefore, four groups of each sex were obtained: (1) controls + standard chow (17 males and 18 females); (2) controls + standard chow and high-fat diet (16 males and 15 females); (3) isolated + standard chow (16 males and 17 females) and (4) isolated + standard chow and HFD (13 males and 15 females). During 40 days, beginning on PND 21, both HFD and standard chow were offered *ad libitum*, according to the groups. Isolation was maintained from PND 21 to 28 and during this period the animals were not handled, except for the cleaning of the cages. On PND 28, isolated animals were returned to regular home cages in groups of three to five. A timeline of the experimental design is shown in Fig. 1. At PND 60 the animals were killed by decapitation, brains were immediately dissected on ice to remove the hypothalamus and biochemical analyses were performed.

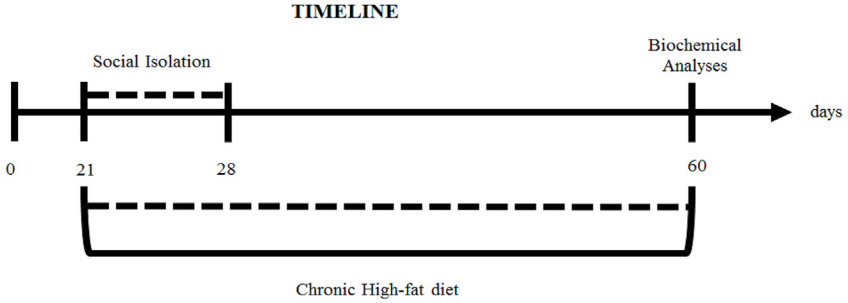

Fig. 1. Timeline of the experimental design. At postnatal day (PND) 21, animals were separated according to sex, and housed in groups (control) or isolated for 7 days. They received standard lab chow, or both standard chow and high fat diet from PND 21 to PND 60.

Table 1
Nutritional composition/100 g of the food used in the studies performed. HFD: high fat diet.

Diet	Energy (Kcal)	Total Protein (g)	Total carbohydrate (g)	Total Fat (g)
Standard chow[a]	301.2	22	44.3 (from starch)	4.0 (0.6 from saturated and 3.4 from unsaturated fat)
HFD (Arcego et al., 2014)	588	28	25 (12.5 from starch and	42 (16 from saturated and 26 from unsaturated fat)

[a]Nuvilab®
[b](Arcego et al., 2014) Isolation during the prepubertal period associated with chronic access to palatable diets: Effects on plasma lipid profile and liver oxidative stress. Neurochem Res. 2013 Sep; 38(9):1791–800.

2.2. Diets

The nutritional compositions of standard lab chow and high-fat diet are displayed in Table 1. The HFD was enriched with fat (42%) from lard and soy oil. In addition, the diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch (Arcego et al., 2014). This ratio soy oil/lard has larger amounts of saturated and monounsaturated fatty acids, to reproduce the consumption of fat in western diets that have higher percentages of these types of fat, such as "fast foods".

In a previous study (Toniazzo et al., 2018), we demonstrated that during stress week, the isolated animals and the animals with access to HFD consumed more calories, however they did not present higher body weight gain during this period. After isolation, from PND 28 to 60, males consumed more calories than females and consequently present higher body weight gain than females. At PND 60, animals with access to HFD had more gonadal fat and HFD increased retroperitoneal fat especially in males.

2.3. Biochemical analysis

2.3.1. Preparation of the samples for biochemical measurements

At PND 60 to 62, animals were killed by decapitation at approximately 13:00 h, after 6 h of fasting. Trunk blood was collected with heparin, and plasma separated by centrifugation and frozen at −80 °C (for evaluation of glucose and total cholesterol). The whole hypothalamus was quickly dissected out and stored at −80 °C until analysis.

2.3.2. Plasma glucose and cholesterol levels

Glucose (N = 4–5/group) and total cholesterol (N = 4–5/group) were measured using commercial kits from Wiener Laboratorios (Rosario, Argentina). The analysis of total cholesterol and glycemia were performed in duplicate.

2.3.3. Assessment of oxidative stress parameters

Whole hypothalamus were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate were centrifuged at 1000 × *g* for 10 min at 4 °C and the supernatants were used. The oxidative stress parameters were performed in duplicate.

2.3.4. Superoxide dismutase activity (SOD)

Superoxide dismutase activity (N = 6–7/group) was determined using the RANSOD kit (Randox Labs., USA), based on the procedure described by Delmas-Beauvieux et al. (1995). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed by spectrophotometric analysis at 492 nm at 37 °C. The inhibition of the chromogen production is proportional to the activity of SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

2.3.5. Glutathione peroxidase activity (GPx)

Glutathione peroxidase activity (N = 5–8/group) was determined using a RANSEL kit (Randox Labs., USA), based on the method described by (Paglia and Valentine, 1967). In this essay, glutathione peroxidase (GPx) catalyses the oxidation of glutathione (GSH) by Cumene Hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

2.3.6. Catalase activity (CAT)

Catalase activity (N = 5–8/group) assessment is based upon establishing the rate of H_2O_2 degradation at 240 nm at 25 °C by spectrophotometric analysis (Aebi, 1984). CAT activity was calculated in micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

2.3.7. Evaluation of free radical production by the chemical oxidation of dichlorodihydrofluorescein (DCFH)

Samples (N = 6–8/group) were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100 µM) at 37 °C for 30 min. DCFH-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen/nitrogen species. The formation of the fluorescent derivative dichlorofluorescein (DCF) was monitored by excitation and emission wavelengths of 488 and 525 nm, respectively, using a spectrum photometer. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmol of DCF formed per mg of protein (Sriram et al., 1997).

2.3.8. Determination of total thiol content and non-protein thiols

Total thiol (N = 5–10/group) content was evaluated as an estimation of the oxidative damage to proteins, as described by (Ellman, 1959). Total thiol content was determined by the absorbance after 60 min at 412 nm. The non-protein thiols a sample aliquot was reacted with trichloroacetic acid (10% v/v), centrifuged (10,000 g, 10 min), and the supernatants were used to measure the level of SH. Results are expressed as nmol SH/mg protein.

2.3.9. Determination of thiobarbituric acid-reactive species (TBARS)

Samples (N = 5–8/group) were deproteinized with 10% trichloroacetic acid (1:2) and centrifuged at 10,000 g for 10 min. The supernatant (100µL) was transferred in duplicate to a 96 well microplate and mixed with 0.67% TBA (100µL). The mixture was heated in a microplate heater at 100 °C for 20 min. The mixture was cooled and the absorbance was measured at 532 nm with a SpectraMax i3x Spectrophotometer (Molecular Devices, San Jose, CA, USA). TBARS levels are represented as nmol TBARS/mg protein. TBARS levels in the sample were quantified using a standard curve. The TBARS data were represented separately for males and females.

2.3.10. Respiratory chain activity determination

The entire hypothalamus were freshly homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base), pH 7.4, for determination of respiratory chain complex activities. The homogenates were centrifuged at 1000 g for 10 min at 4 °C and the supernatants were immediately maintained at −80 °C until analyses.

The activities of the electron transport chain (ETC) complexes I–III, II and IV were determined according to standard methods previously described in the literature (Schapira et al., 1990; Weis et al., 2012) and performed in duplicate. The activity of complex I–III (complex I + CoQ + III) (N = 4–6/group) was assessed by measuring the increase in absorbance due to cytochrome *c* reduction at 550 nm, according to the method described by Schapira et al. (1990). Complex I–III activity was calculated as the rotenone sensitive NADH: cytochrome *c* reductase activity. The activity of complex II (succinate: DCIP oxyredutase) (N = 4–6/group) was determined according to Fischer et al. (1985), by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm, in a medium containing sodium succinate, sodium azide, and rotenone and DCIP. Cytochrome *c* oxidase (COX, complex IV) activity (N = 4–6/group) was determined according to Rustin et al. (1994), following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm. The activities of the respiratory chain complexes were calculated and expressed as nmol per min per mg of protein.

2.3.11. Mitochondrial mass and membrane potential measurements

Mitotracker was used for mitochondrial function analysis in cell suspensions of whole hypothalamus obtained by mechanical dissociation with PBS containing collagenase to favor digestion to a density of about 200,000 cells/mL. Dissociated cells were then decanted for 10 min. To assess mitochondrial potential (ΔΨ) (N-7-10/group) and mass (N-7-10/group), MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG) dyes were employed (Pendergrass et al., 2004; Khanal et al., 2011). MTG is a green-fluorescent fluorophore that accumulates in mitochondria independent of mitochondrial membrane potential and has been used to measure mitochondrial mass (Rodriguez-Enriquez et al., 2009). MTR is a lipophilic cationic fluorescent dye that is concentrated inside mitochondria because of the negative mitochondrial membrane potential and has been used to measure mitochondrial membrane potential (Pendergrass et al., 2004). MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock concentration. Dissociated cells were stained with 100 nM MTR and 100 nM MTG for 45 min at 37 °C in a water bath in a dark room according to method described by (Pendergrass et al., 2004) with some modification.

2.3.12. Flow cytometry analysis

Samples stained with MTR and MTG dyes were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). MitoTracker dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples without stain) were included for setting up the machine voltages. Controls stained with a single dye were used to set compensation. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: red (FL-3; 670 nm long pass) and green (FL-1; 530 nm/30). Fluorescence emissions were collected using logarithmic amplification. In brief, data from 10.000 events were acquired and mean relative fluorescence intensity was determined after exclusion of debris events from the data set. All flow cytometric acquisitions and analyses were performed using CELLQuest Pro data acquisition (BD Biosciences) and FlowJo analysis software. The mean fluorescence intensity (MFI) of each channel was evaluated. The data were plotted as dot plot with mass in y axis and potential on x axis.

2.3.13. Protein assay

The protein concentration was determined in the samples using the method described by Lowry et al. (1951) with bovine serum albumin as the standard.

2.4. Statistical analysis

Data are expressed as mean ± S.E.M of the mean and analyzed using three-way ANOVA, with *isolation stress, diet* and *sex* as factors. All analyses were performed using SPSS software and a $P \leq 0.05$ was considered significant.

3. Results

3.1. Plasma cholesterol levels and glycemia

Results from plasma cholesterol measurements were analyzed using a three-way ANOVA, and are shown in Supplementary Table S1). A main effect of *diet* was observed, since total cholesterol increased in all animals receiving HFD [$F(1,31) = 6.05$, $P = 0.020$]. Results also showed that stress increased total cholesterol in males (interaction between *stress x sex* [$F(1,31) = 6.84$, $P = 0.014$]) but decreased in females. In addition, glycemia increased in stressed males with access to *HFD* [interaction between *stress* x *diet* x *sex*: $F(1,26) = 12.5$, $P = 0.002$]. It is important to note that glycemia increased only with the interaction of *stress* x *diet* factors, and while when considering these factors *per se* no increase was observed.

3.2. Antioxidant enzyme activities and free radical production

Oxidative stress parameters were analyzed to investigate whether there was an oxidative imbalance in the entire hypothalamus of adult male and female rats subjected to isolation stress during the pre-pubertal period, when associated to chronic HFD. When SOD activity was evaluated at PND 60 (Fig. 2A), a main effect of *sex* was observed [three-way ANOVA, $F(1, 45) = 40.22$, $P < 0.001$], since female rats showed higher hypothalamic SOD activity than males. Results also showed an effect of *HFD* [$F(1,43) = 7.83$, $P = 0.008$] decreasing GPx activity (Fig. 2B) in males and females, while both CAT activity (Fig. 2C) and free radical production, as evaluated by the DCFH test, were not altered ($P > 0.05$) (Fig. 2D).

3.3. Total thiols, non-protein thiols and thiobarbituric acid reactive substances

A main effect of *sex* was observed on total thiol and non-protein thiol contents: total thiol content was higher in females [$F(1, 54) = 4.38$, $P = 0.041$] and non-protein thiols were higher in males [$F(1, 41) = 5.08$, $P = 0.030$] (see Fig. 3C and D).

With regard to thiobarbituric acid reactive substances (TBARS) levels, the exposure to stress decreased TBARS levels in males [$F(1,27) = 8.77$, $P = 0.006$] (see Fig. 3A). In addition, access to the high-fat diet also decreased TBARS levels in males [$F(1,27) = 13.29$, $P = 0.001$]. No effects were detected on TBARS levels in females ($P > 0.05$) (see Fig. 3B).

3.4. Respiratory chain enzyme activities

Enzymatic analysis of mitochondrial electron transport chain (ETC) activities in the entire hypothalamus was performed (Fig. 4). A main effect of *sex* was observed on Complex I-III activity [three-way ANOVA, $F(1,33) = 89.89$, $P < 0.05$], since this activity was lower in females compared to males (Fig. 4A). Besides, an interaction between *diet* and *sex* [$F(1, 34) = 4.60$, $P = 0.039$] showed that females receiving HFD present increased Complex IV activity; in contrast, males receiving HFD presented decreased Complex IV activity (Fig. 4C). Complex II activity was not significantly altered ($P > 0.05$) (Fig. 4B).

3.5. Mitochondrial mass and membrane potential

Analyses of hypothalamic cells labeled with MTG and MTR are shown in Fig. 5. A three-way ANOVA showed an interaction between *diet* and *sex* [$F(1, 55) = 5.36$, $P = 0.024$], suggesting that females receiving HFD had lower mitochondrial mass (Fig. 5A). No effect was

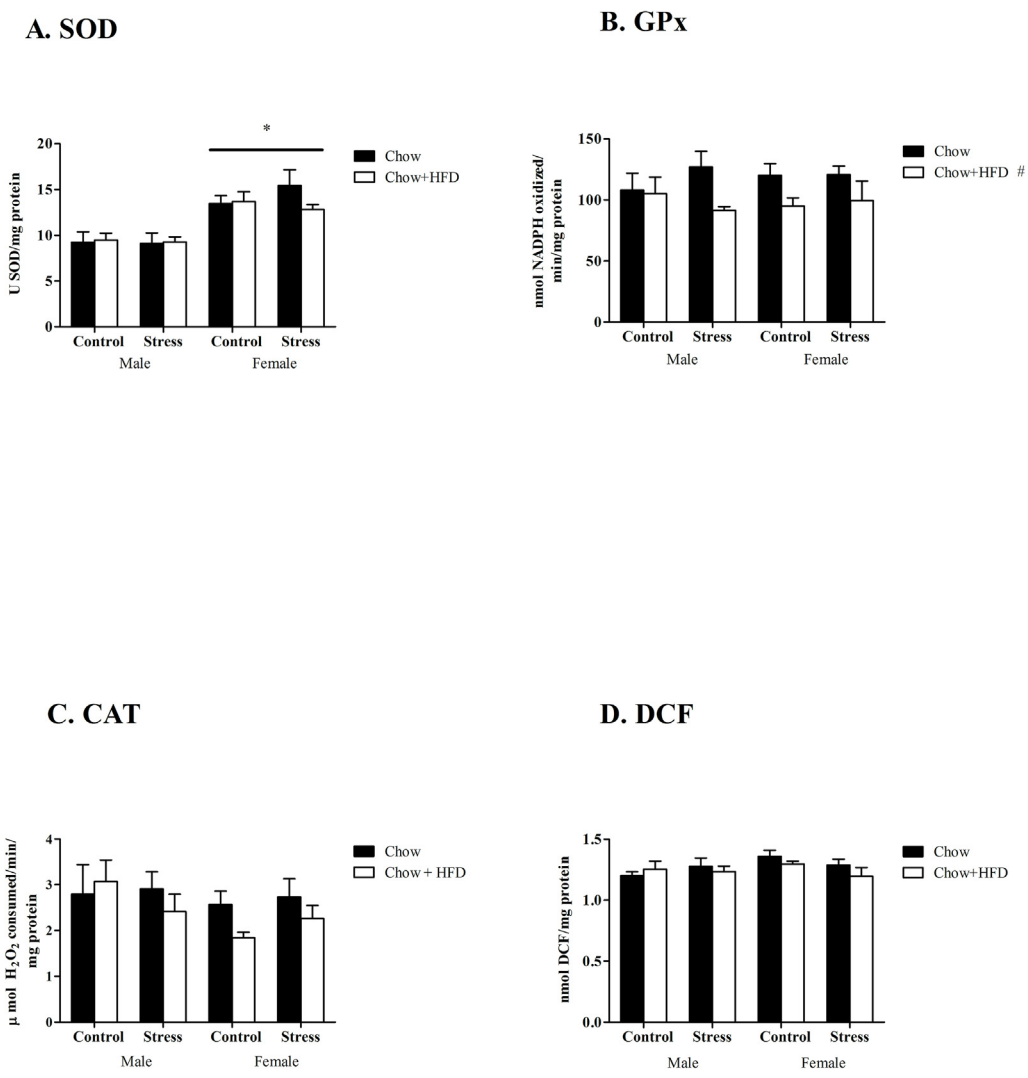

Fig. 2. Effect of isolation stress during the prepubertal period, with or without chronic access to HFD on antioxidant enzyme activities, and free radicals (DCFH test) production in hypothalamus of adult male and female rats. Data are expressed as mean ± S.E.M., N = 5–8/group. **(A) SOD (expressed as U SOD/mg protein).** Three-way ANOVA showed an effect of sex ($P < 0.001$), since females displayed higher SOD activity than males. **(B) GPx (expressed as nmol NADPH oxidized/min/mg protein).** Three-way ANOVA showed that HFD decreased GPx activity ($P = 0.008$). **(C) CAT (expressed as micromoles of H_2O_2 consumed/min/mg protein).** No effect was observed ($P > 0.05$). **(D) DCFH production (expressed as nmol of DCF formed/mg** protein). No effect was observed ($P > 0.05$). # Effect of diet; * Effect of sex.

detected on membrane potential ($P > 0.05$) (Fig. 5B).

4. Discussion

The findings of the current study demonstrate sex differences in the activities of antioxidant enzymes and mitochondrial bioenergetics after both pre-pubertal stress and HFD exposure. Additionally, some effects of high-fat diet on mitochondrial function and oxidative defenses were sex-specific, as well as an effect of exposure to social stress isolation during the prepubertal period on lipid peroxidation.

Our results showed that SOD activity was higher in females compared to males. SOD is an enzyme that dismutates the superoxide radical, producing hydrogen peroxide (H_2O_2). H_2O_2 is a substrate for peroxidases (CAT and GPx), which degrade H_2O_2 into water (H_2O) (Halliwell, 2006). This increase in SOD activity without a proportional increase in peroxidase activity, such as observed in females, may indicate an overload of peroxide challenge (Pinho et al., 2006). However, this hypothetical accumulation of H_2O_2 was not reflected in changes in DCF test, which measures exposure to reactive species. It is interesting that females also showed lower Complex I-III activity when compared to males. Since this complex is a site of electron leakage, for the complexes I and III are the main mediators of ROS production (Leloup et al., 2011), its lower activity could suggest a lower flow of electrons through the respiratory chain, and lower production of superoxide, thereby explaining why there were no alterations in free radicals production in females. Interestingly, evidences indicates that estrogen increases the expression of mitochondrial electron transport chain proteins, including cytochrome c and complex IV subunits, and also increases complex IV enzyme activity (Duckles et al., 2006). Here, we did not found sex differences in the activities of these components of the electron transport chain in rat hypothalamus.

Few studies have investigated the sex-specific effects of HFD on oxidative status, especially in hypothalamus. This structure is important for the control of metabolism and food intake through the integration of several signaling pathways (Drougard et al., 2015). In recent years, it has been proposed that the reactive oxygen species (ROS) modulates energy balance by acting in different neurons in hypothalamus (Drougard et al., 2015). In this study, access to HFD decreased GPx activity in both, males and females. In neurons, the antioxidant enzyme GPx provides the main pathway of H_2O_2 catabolism (Dringen et al., 1999). Therefore, the decline in GPx activity can contribute to an increased intracellular accumulation of H_2O_2, enhancing cell oxidative damage (Walczewska et al., 2010). It is noteworthy that, in females receiving HFD, the association between increased SOD activity and decreased GPx activity may result in H_2O_2 accumulation. It is known that low concentrations the H_2O_2 modulate cellular signaling processes (Rice, 2011), while high concentrations can induce oxidative stress (Armogida et al., 2012). Moreover, H_2O_2 can react directly with thiol residues in redox-sensitive proteins, and it may alter the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG), thus altering the redox status of the cell (Schafer and Buettner, 2001). In the present study, however, no reductions in thiol groups were observed in HFD

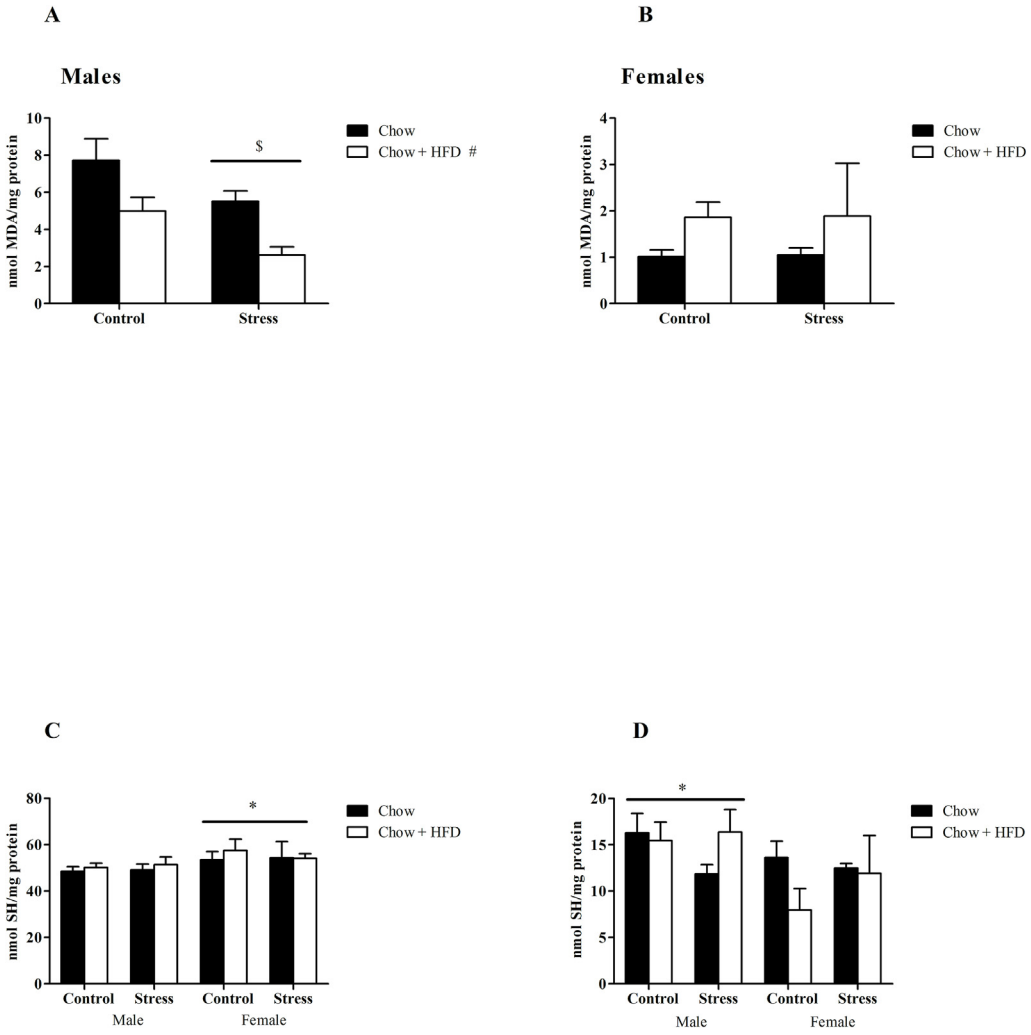

Fig. 3. Effect of isolation stress during the prepubertal period, with or without chronic access to HFD on redox parameters in hypothalamus of adult male and female rats. Data are expressed as mean ± S.E.M., N = 5–10/group. **(A) TBARS (nmol MDA/mg protein) levels in males.** There were main effects of *stress* (P = 0.006; exposure to stress decreased TBARS levels in males), and *diet* (P = 0.001; access to HFD decreased TBARS in males). **(B) TBARS (nmol MDA/mg protein) levels in females.** No effect was observed (P > 0.05). **(C) Total thiols (nmol SH/mg protein).** With regard to total thiols and non-protein thiols, a main effect of *sex* was noted, with total thiols being higher in females (P = 0.041), and **(D) Non-protein thiols (nmol SH/mg protein)** higher in males (P = 0.030). $ Effect of stress; #Effect of diet; *Effect of sex.

animals. It should be considered that some studies have shown that a long-term HFD intake increases oxidative stress and causes mitochondrial dysfunctions in distinct tissues, such as muscle and brain (Yokota et al., 2009; Ballal et al., 2010). However, to our knowledge, this is the first study evaluating the effects of chronic HFD on oxidative status in the hypothalamus considering sex-specific differences. In this context of higher SOD activity observed in females with HFD-induced decrease in GPx, our findings suggest that the female hypothalamus is more susceptible to an oxidative imbalance and consequently to a possible cell damage.

As pointed out previously, the nervous tissue is particularly vulnerable to oxidative stress (Halliwell, 2006). In this sense, oxidative stress can lead to oxidation of biomolecules such as lipids, proteins and DNA, resulting in damage to cellular organelles, particularly mitochondria (Miao and St Clair, 2009). The oxidation of protein residues, such as sulfhydryl groups, cause conformational changes, protein unfolding and degradation (Lyras et al., 1997). Interestingly, females had higher hypotalamic total thiol contents than males, suggesting lower oxidation of protein sulfhydryl residues and, therefore, higher protection from protein damage compared to males. On the other hand, females had decreased non-protein thiol content than males. It is known that reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells, and it is an important non-enzimatic antioxidant for the detoxification of electrophilic components and peroxides (Anderson, 1998). Therefore, GSH deficiency may increase cellular oxidative damage (Townsend et al., 2003). In contrast to this finding, some studies have shown the protective effects of estrogen on oxidative stress in nervous tissues, emphasizing that females are more protected against an oxidative imbalance than males (Razmara et al., 2007; Vina et al., 2011). One limitation of our study is that the estrous cycle was not evaluated. However, we must consider that variations of the circulating hormones could lead to higher variability of the parameters evaluated, and, despite these possible variations, statistical significant differences were observed between sexes and in females subjected to HFD.

The TBARS assay is widely used for ex vivo and in vitro measurements (Tsai and Huang, 2015; Everson et al., 2018; Pasquali et al., 2018) of the effects of different treatments on lipid peroxidation. Although its specificity has been questioned (Draper and Hadley, 1990; Devasagayam et al., 2003), it has been accepted as an empirical window on the process of lipid peroxidation. Although the comparison between different tissues can be affected by different amounts of polyunsaturated lipids, we believe it is possible to compare the same tissue when the animals are submitted to different treatments. In the present study, interestingly, isolation stress decreased hypothalamic lipid peroxidation in males. In contrast to our results, rats exposed to acute or chronic stress have been reported to present increased lipid peroxidation in distinct brain structure (Sosnovskii and Kozlov, 1992; Liu et al., 1996; Solin and Liashev Iu, 2013; Herbet et al., 2017). It must be considered, however, that this evaluation was made in the adult age, a long time after exposure to stress. It is possible that in these animals the hypothalamus underwent an adaptation, turning this structure more resilient to oxidative damage.

High-fat diets can compromise mitochondrial membranes leading to perturbations in membrane fluidity (Tsalouhidou et al., 2006) and

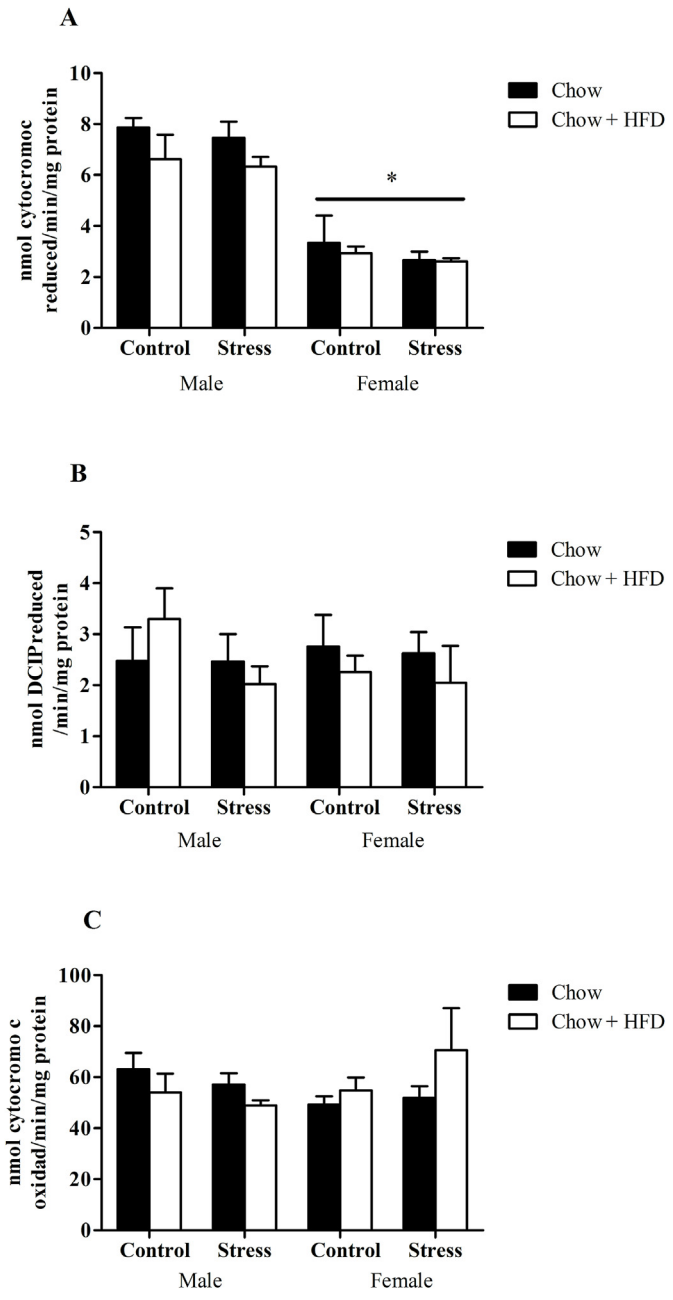

Fig. 4. Effect of isolation stress during the prepubertal period, with or without chronic access to HFD on respiratory chain activity. Data are expressed as mean ± S.E.M., N = 4–6/group. **(A) Complex I–III activity (nmol cytocrome *c* reduced/min/mg protein).** There was a main effect of *sex* ($P < 0.001$) on Complex I-III activity that was smaller in females. **(B) Complex II activity (nmol DCIP reduced/min/mg protein).** No effect was observed ($P > 0.05$). **(C) Complex IV activity (nmol cytocromo c oxidized/min/mg protein).** HFD increased Complex IV activity in females and decreased this activity in males (three–way ANOVA interaction of *diet* x *sex,* $P = 0.039$).

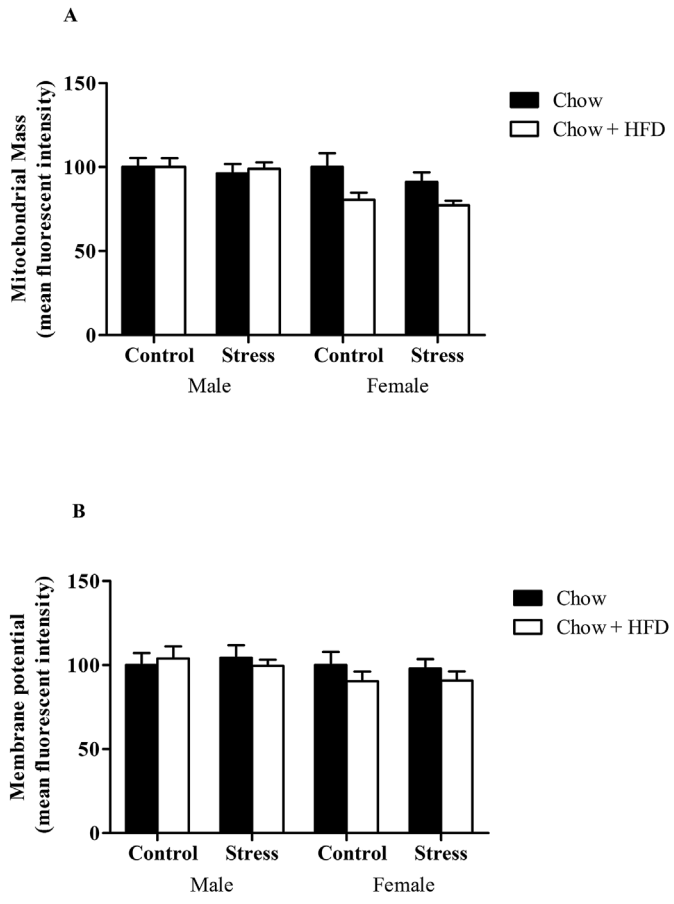

Fig. 5. Effect of isolation stress during the prepubertal period, with or without chronic access to HFD on cells labeled with Mitotracker Green (mitochondrial mass) or Mitotracker Red (mitochondrial potential) in the hypothalamus of adult male and female rats. Data are expressed as mean ± S.E.M., N = 6–10/group. **(A) Mitochondrial mass.** Three-way ANOVA showed and interaction between *diet* x *sex* ($P = 0.024$), since the access to HFD decreased mitochondrial mass in females. **(B) Mitochondrial potential.** No effect was observed ($P > 0.05$).

production of mitochondrial ROS (Yu et al., 2014). Previous experiments in mice have shown that HFD decreases expression of genes involved in oxidative phosphorylation (OXPHOS), genes encoding proteins in complexes I, II, III, and IV of the electron transport chain as well as transcription factors and cofactors in skeletal muscle (Sparks et al., 2005). The consumption of HFD increased Complex IV activity only in females, while this activity was reduced in males. It is possible that the Complex IV is more active in females as a compensatory mechanism, in response to the excess of energy supplied through HFD. This dissociation between the effects of HFD in males and females has been observed previously: males receiving HFD presented a lower T3/T4 ratio and a higher caloric efficiency compared to controls, while females did not show these effects (Toniazzo et al., 2018).

Mitochondrial dysfunction, including reduction of mitochondrial mass, has been reported in peripheral tissue of obese individuals or in animal models after high fat diet (Bournat and Brown, 2010). We observed that HFD decreased mitochondrial mass in the hypothalamus from females. In agreement, sex-differences in mitochondrial biogenesis have been previously reported (Sharma et al., 2014). It is possible that the oxidative stress induced by HFD consumption in females is responsible for this altered mitochondrial dynamics, according to what was discussed above for the increased Complex IV activity. Moreover, mitochondrial dysfunction could be linked to defects in fatty acid oxidation, and it has been proposed that hypothalamic fatty acid oxidation/accumulation may be involved in the regulation of feeding and glucose homeostasis (Duca and Yue, 2014).

In summary, the present findings show that males and females respond differently to the effects of stress in the prepubertal period and to HFD consumption. Females were more susceptible to an oxidative imbalance and a possible cell damage. The chronic consumption of HFD induced changes in mitochondria mainly in females, such as lower mitochondrial mass and increased Complex IV activity. Our data reinforce the importance of investigating the effects of environmental factors on oxidative status and on mitochondrial function considering

sex-specific effects. Additionally, these data may collaborate with the investigation of the mechanisms involved in the development of obesity and contribute to determine prevention measures and new therapies in order to attend sex -different particularities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.01.008.

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