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Isolation during the prepubertal period associated with chronic access to palatable diets: Effects on plasma lipid profile and liver oxidative stress

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HIGHLIGHTS

- Isolation stress during early life leads to oxidative imbalance in the liver.
- These effects were accentuated with a high-fat diet.
- The high-fat diet increased abdominal fat and plasma glucose and leptin levels.
- The high-fat diet increased plasma cholinesterase activity.

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ABSTRACT

Pre-puberty is a critical period for the final maturation of the neural circuits that control energy homeostasis, as external stimuli such as exposure to diets and stress may influence the adaptive responses with long-term repercussions. Our aim is to investigate the effects of isolation stress during early life and of chronic access to palatable diets, rich in sugar or fat, on the metabolic profile (glycemia, plasma lipids, leptin and cholinesterase activity) and oxidative stress parameters in the livers of adult male rats. We observed changes mainly in animals that received the high-fat diet (increased body weight and abdominal fat in adults, as well as increased plasma glucose, and cholinesterase activity), and most of these effects were further increased by exposure to stress. High-fat diet also affected the rats' lipid profile (increased cholesterol, LDL-cholesterol and triglycerides); these effects were more marked in stressed animals. Additionally, exposure to stress led to an oxidative imbalance in the liver, by increasing production of reactive species, as well as the activity of antioxidant enzymes (superoxide dismutase and catalase); these effects were accentuated with the high-fat diet (which also caused a severe reduction in glutathione peroxidase activity). Taken together, these results show that the pre-pubertal period constitutes a critical window for stressful interventions during development, leading to alterations in metabolic parameters and increased oxidative stress during adulthood that may be more pronounced in animals that receive a high-fat diet.

1. Introduction

The consumption of diets rich in sugar and fat, along with a sedentary lifestyle, is associated with increased obesity prevalence [1]. Levels of obesity have been increasing in children and adolescents, creating concern, as exposure to diets rich in calories during this period of development could modify the maturation of neuronal circuits and lead to dysfunction or diseases during adulthood [2]. In addition, it has been suggested that environmental factors, such as exposure to stress, are strongly implicated in this higher prevalence of obesity [3].

A stressor is defined as a challenge to the organism that can potentially disrupt homeostasis and, therefore, requires a physiological response. During development, when the plastic capacity is maximal, these adjustments become more important. The childhood and adolescence are critical periods for the maturation of the neural circuits that control energy homeostasis and stress responses [4]. Early life events, such as childhood stress, may have long term effects on behavior and metabolism [5,6]. In these periods, one of the most potent stressors, in both humans and animals, is social isolation [7–9], which can lead to behavioral, anatomical and neurochemical changes that may remain during adulthood [8,10].

Exposure to stress induces a variety of responses, including activation of the sympatho-adrenomedullar system, release of catecholamines, and activation of the hypothalamic–pituitary–adrenal (HPA) axis, culminating in the release of glucocorticoids (GCs) [11]. The

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metabolic effects of the GCs include increased plasma glucose due to gluconeogenesis and glycogen degradation, as well as inhibition of glucose uptake in some tissues, mobilization of amino acids from extrahepatic tissues, stimulation of lipolysis in adipose tissue and increased metabolic rate [12,13]. Animal studies show that stress may both increase and decrease food ingestion depending on the duration and intensity of the stress [14–18]. In human and animal studies, many factors have been implied in these effects related to stress and feeding behavior, including autonomic nervous system activation [12], effects of hormones related to the stress response, such as CRH and glucocorticoids [1,19], leptin [20,21], and/or stress activation of neural systems involved in the cognitive, rewarding, and emotional aspects of ingestive behavior [12]. Stress exposure may increase food intake and insulin levels, facilitating the development of obesity and the metabolic syndrome [22,23]. Conversely, the regulation of the HPA axis will depend on the type of palatable food consumed: Studies using rodents have shown that diets high in calories and sugar reduce this axis response to stress [24], while diets rich in fat enhance stress-induced levels of glucocorticoids [25,26].

Additionally, studies in animals and humans, as well as in tissue cultures, have reported that stress exposure and elevated GCs levels increase the generation of reactive oxygen species (ROS) [27–30]. When there is an imbalance between antioxidant defenses and oxidative species, oxidative stress occurs [31], leading to damage to cell structures like proteins, lipids, membranes and DNA, which have been observed both in humans [32,33] and in rodents [34,35]. Moreover, some studies using animal models have presented evidence that the presence of ROS and excessive intake of fatty foods can lead to breaks in cellular DNA [36–40]. In this context, the consequences of stress exposure in animals with *ad libitum* access to palatable diets require a better understanding.

Since the pre-pubertal period is critical for development, being important to the stress response and for the emergence of eating disorders, the aim of our study is to verify whether stress by social isolation during the pre-puberty period in animals with chronic access to palatable diets until adulthood may alter oxidative stress parameters in the liver, and metabolic profiles such as plasma lipids, plasma glucose and leptin. Serum cholinesterase activity was also measured, since relationships between the activity of this enzyme and hyperlipidemia, diabetes, and obesity have been reported [41].

2. Material and methods

2.1. Experimental subjects

All animal proceedings were performed in strict accordance with the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Law on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee. All efforts were made to minimize animal suffering, as well as to reduce the number of animals used.

Animals were housed in home cages made of Plexiglas (65 × 25 × 15 cm) with the floor covered with sawdust, and were maintained on a standard 12 h dark/light cycle (lights on between 7:00 h and 19:00 h), temperature of 22 ± 2 °C. On postnatal day (PND) 21, sixty-three Wistar rats were weaned. Only male pups were used from each litter, and these pups were divided into six groups, in such a way that only one animal per litter was used in each group. Male pups were weighed at PND 21 and distributed into 3 groups, according to the diet that they received: (1) receiving standard lab chow (44.3% carbohydrate, 22% protein and 4% fat); (2) receiving both chow and a diet with a high content of simple carbohydrate [42] and (3) receiving chow and a high-fat diet (25% carbohydrate, 28% protein and 42% fat). Therefore, animals from these last two groups could choose the diet they consumed from the two diets available. Half of the animals on each diet were housed in groups of 4; the other half were stressed by isolation (one animal in a smaller home cage, 27 × 17 × 12 cm) [43], in such a way that six groups were obtained; controls receiving chow (CC), controls receiving chow and high-carbohydrate diet (HCC), controls receiving chow and high-fat diet (HFC), isolated animals receiving chow (IC), isolated animals receiving chow and high-carbohydrate diet (HCI), and isolated animals receiving chow and high-fat diet (HFI). The isolation stress occurred between postnatal days 21 and 28. On PND 28, isolated animals were returned to regular home cages (65 × 25 × 15 cm) in groups of four. During 40 days, beginning on PND 21, amounts of palatable diets and standard lab chow were offered *ad libitum*. At postnatal day 60, the animals were killed by decapitation and biochemical evaluations were performed.

2.2. Diets

Studies have shown that diets rich in simple carbohydrates or in fat have distinct effects on HPA axis response to stress [24–26]. Therefore, we used a standard chow, a diet rich in sugar and a fat-enriched diet. The nutritional compositions of each diet used are displayed in Tables 1 and 2. The high-carbohydrate diet (HCD) used in this study was enriched in simple carbohydrates, and made with condensed milk, sucrose, vitamins and a salt mix, powder standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet is similar to that of a standard lab chow, however most carbohydrates in the palatable diet were sucrose [42]; in contrast, the standard lab chow contained carbohydrates obtained mainly from starch.

The high-fat diet (HFD) used in the study was enriched with fat (42%) from lard and soy oil. In addition, this diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch [adapted from 44]. This ratio soy oil/lard has a larger amount of saturated and monounsaturated fatty acids, to reproduce the consumption of fat in the western diets, that have higher percentage of these types of fat, such "fast foods". However, we added 1.6% (w:w) of soy oil to provide a minimal amount of n3 fatty acids for an adequate ratio of n6:n3 fats [45].

2.3. Food consumption

Previously weighed quantities of standard lab chow and palatable diets were offered and the remaining amounts of pellets were measured each day to evaluate consumption. The food consumption was measured per cage and the amount of food consumed was divided by the number of animals per cage to determine mean consumption per animal. To verify the amount of kilocalories consumed per animal, we multiplied the amount of food ingested by the caloric content per gram of chow or diets. The standard lab chow has a caloric content of 3.01 kcal/g, whereas the high-carbohydrate diet has a caloric content

Table 1
Nutritional composition/100 g of the food used in the studies performed. HCD: high carbohydrate diet; 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 (0.62 from saturated and 3.4 from unsaturated fat)
HCD[b]	346.3	27	40 (13.3 from starch and 26.7 from sucrose and lactose)	8.7 (3.04 from saturated and 5.6 from unsaturated fat)
HFD[c]	588	28	25 (12.5 from starch and 12.5 from sucrose)	42 (16 from saturated and 26 from unsaturated fat)

[a] Nuvilab®.
[b] Souza CG, et al. Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like behavior. Life Sci, 2007, 81:198-203.
[c] Adapted from Ziegler DR, et al. A ketogenic diet increases protein phosphorylation in brain slices of rats. J Nutr 2002, 132:483-487.

Table 2
Composition of vitamin and mineral salt mixture of mg/100 g in diet.

Diet	Vitamin mixture	Mineral salt mixture
Standard chow	Vitamin A, 13,000[a]; vitamin D3, 2000[a]; vitamin E, 34[a]; vitamin K3, 0.3; vitamin B1, 0.5; vitamin B2, 0.6; vitamin B6, 0.7; vitamin B12, 2.2; niacin (nicotinic acid), 6; pantothenic acid (calcium D-pantothenate), 2; folic acid, 0.1; biotin [D-(+)-biotin], 0.005; choline, 190.	Sodium, 270; iron, 5; manganese, 6; zinc, 6; copper, 1; iodine, 0.2; selenium, 0.005; cobalt, 0.15; fluorine, 8.
HCD and HFD	Vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-α-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacine, 4; pantothenic acid, 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; pyridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin, 0.04; vitamin B12, 0.003.	NaCl, 557; KI,3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4$–$7H_2O$, 108; $MnSO_4$–H_2O, 16; $ZnSO_4$–$7H_2O$, 2.2; $CuSO_4$–$5H_2O$, 1.9; $CoCl$–$6H_2O$, 0.09.

[a] UI/KG.

of 3.46 kcal/g and the high-fat diet has a caloric content of 5.8 kcal/g (being 15% and 93%, respectively, more caloric than standard chow).

2.4. Abdominal fat dissection and preparation of the samples for biochemical measurements

Forty days after receiving these diets, the animals were killed by decapitation (following 6 h of fasting). The two major portions of abdominal fat (gonadal and retroperitoneal adipose tissue depots) and adrenal glands were carefully dissected and weighed. Trunk blood was collected into tubes with EDTA for glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL), leptin, and cholinesterase activity determination. Plasma was separated and frozen until the day of analysis. The liver was perfused with cold saline, dissected out and stored at −80 °C until analysis, when it was homogenized in 50 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged at 1000 ×*g* for 10 min at 4 °C and the supernatant was assayed for oxidative stress parameters using the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and the evaluation of antioxidant enzyme activity.

2.5. Biochemical analysis

Leptin was measured by ELISA (Abnova Corporation, Jhongli City, Taoyuan County, Taiwan). Plasma glucose, total cholesterol, HDL-cholesterol and triglycerides were measured with commercial kits; glucose, total cholesterol, and triglycerides were measured using kits from Wiener Laboratorios (Rosario, Argentina), and HDL-cholesterol was measured using a kit from Labtest Diagnóstica S.A. (Minas Gerais, Brazil). LDL-cholesterol was evaluated using the Friedewald formula [46].

2.5.1. Cholinesterase activity evaluation

Plasma cholinesterase (ChE) activity was determined by the method of Ellman et al., [47] with some modifications. Hydrolysis rate was measured at an acetylthiocholine (AcSCh) concentration of 0.8 mM in 1 mL assay solutions with 100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). Fifty microliters of diluted rat serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2 min (intervals of 30 s) at 25 °C. All samples were run in duplicate. Specific enzyme activity was expressed as mmol acetylthiocholine hydrolyzed per hour per milligram of protein.

2.5.2. Superoxide dismutase activity

Superoxide dismutase activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux, et al. [48]. 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 spectrophotometrically at 492 nm at 37 °C. The inhibition of the production of the chromogen 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.

2.5.3. Glutathione peroxidase activity

Glutathione peroxidase activity was determined according to Wendel [49], with modifications. The reaction was carried out at 37 °C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione, and 0.4 U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

2.5.4. Catalase activity

Catalase activity assessment is based upon the spectrophotometric establishment of the rate of H_2O_2 degradation at 240 nm at 25 °C [50]. 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}.

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

The samples were incubated with 2′-7′-dichlorodihydrofluorescein diacetate (100 μM) at 37 °C for 30 min. DCFH is released by cellular esterases and oxidized 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 formation of oxidized reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmol of DCF formed per mg of protein [51].

2.5.6. Determination of total thiol content

This assay is based on the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups, which become oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm [52].

2.5.7. Protein assay

The protein concentration was determined in the samples using the method described by Lowry et al., [53], with bovine serum albumin as the standard.

2.6. Statistical analysis

Data are expressed as mean ± SE of the mean, and analyzed using two-way ANOVA, with isolation stress and diet as factors. For body weight and caloric intake, Repeated Measures ANOVA was used (the within subjects factor was time; the between subjects factors were stress and diet). With regard to Repeated Measures ANOVA, the Greenhouse–Greisser correction was applied when necessary, considering the violation of the sphericity assumption, as shown by the Mauchly test. ANOVA tests were followed by the Tukey multiple range test, when indicated. All analyses were performed using SPSS software and $P \leq 0.05$ was considered significant.

3. Results

3.1. Body weight and caloric consumption

Body weight gain and caloric consumption were analyzed during the period of stress and until 60 days of life (Fig. 1 for body weight and Figs. 2 and 3 for caloric consumption). During the first week (period of isolation), animals receiving the high-fat diet gained less weight than the other groups [two-way ANOVA, $F(2,44) = 12.145$, $P < 0.001$, followed by Tukey post-hoc]. There was also an interaction between stress and diet [$F(2,44) = 3.407$, $P < 0.05$], since stressed animals did not present a reduced weight gain when receiving the high-fat diet (Fig. 1A). With regard to the caloric consumption during this first week, the animals with access to a high-carbohydrate diet had a higher caloric consumption, compared to the other groups [two-way ANOVA, $F(2,22) = 52.92$, $P < 0.001$, followed by Tukey post-hoc], as displayed in Fig. 2A. There was a marginally-significant interaction between isolation stress and diet on caloric consumption during this period [$F(2,22) = 2.87$, $P = 0.07$], since isolated animals receiving the high-fat diet had an increased caloric consumption. The caloric consumption evaluated considered both palatable diets and standard chow consumed. When evaluating just the consumption of high-carbohydrate or high-fat diets, we observed an interaction between isolation stress and diet [repeated measures ANOVA, $F(1,16) = 10.87$, $P = 0.005$]; while both groups (isolated or controls) receiving the high-carbohydrate diet had a marked increase in consumption, only stressed animals receiving the high-fat diet showed an increased consumption with time; diet consumption also increased as the days passed by [$F(6,96) = 8.47$, $P < 0.001$] (Fig. 2B). Additionally, there was an interaction between diet and time [$F(6,96) = 4.37$, $P = 0.001$], since animals with access to the high-carbohydrate diet consumed more food than those receiving the high-fat diet and increased this consumption as time passed. Accordingly, there was a main effect of diet [$F(1,16) = 212.45$, $P < 0.001$], with the high-carbohydrate diet being more consumed. When considering body weight until 60 days of age, animals gained weight [repeated measures ANOVA, $F(5,220) = 3142.48$, $P < 0.001$] and there were interactions between body weight along time and with stress [$F(5,220) = 2.59$, $P < 0.05$] and between time and diet [$F(10,220) = 5.49$, $P < 0.001$]. There was also a main effect of diet [$F(2,44) = 4.97$, $P = 0.01$], since animals receiving the high-carbohydrate diet presented a higher weight gain than animals receiving regular chow (Tukey post-hoc, $P < 0.05$) (Fig. 1B). Accordingly, as observed in Fig. 3A, caloric consumption was higher in animals with access to the high-carbohydrate diet [$F(2,7) = 148.56$, $P < 0.001$, followed by Tukey post-hoc]. In addition, there was an effect of time [$F(4,28) = 30.31$, $P < 0.001$], since caloric consumption increased over time. The higher caloric consumption was due to increased ingestion of the diet and not the chow (Fig. 3B) [$F(1.5,20) = 20.57$, $P < 0.001$ for time; $F(1.5,20) = 5.81$, $P < 0.05$ for the interaction between time and diet; $F(1,5) = 461.67$, $P < 0.001$ for diet].

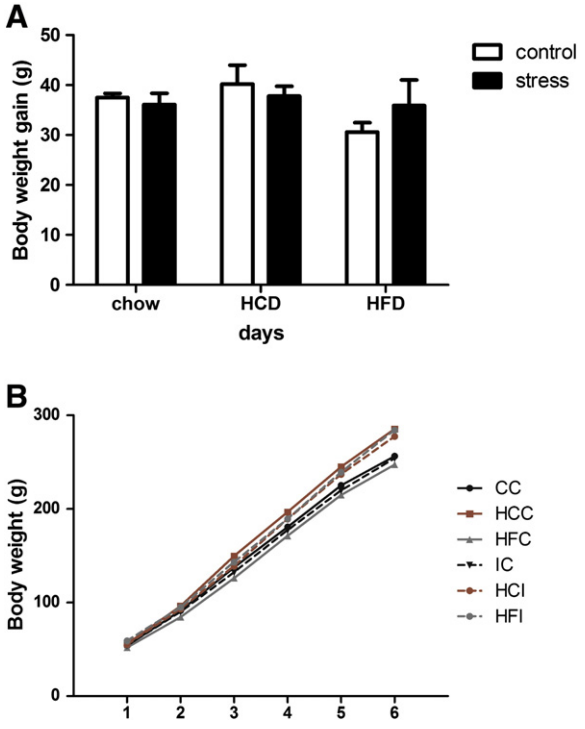

Fig. 1. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on body weight gain. A. Body weight gain during the period of stress isolation. Two-way ANOVA showed a significant effect of diet ($P < 0.001$) and an interaction between stress and diet ($P < 0.05$). B. Body weight until 60 days of age. Repeated measures ANOVA showed an effect of diet ($P < 0.01$) and interactions between weight over time and stress ($P < 0.05$) and time and diet ($P < 0.001$). Data are expressed as mean ± SEM, N = 10–15/group.

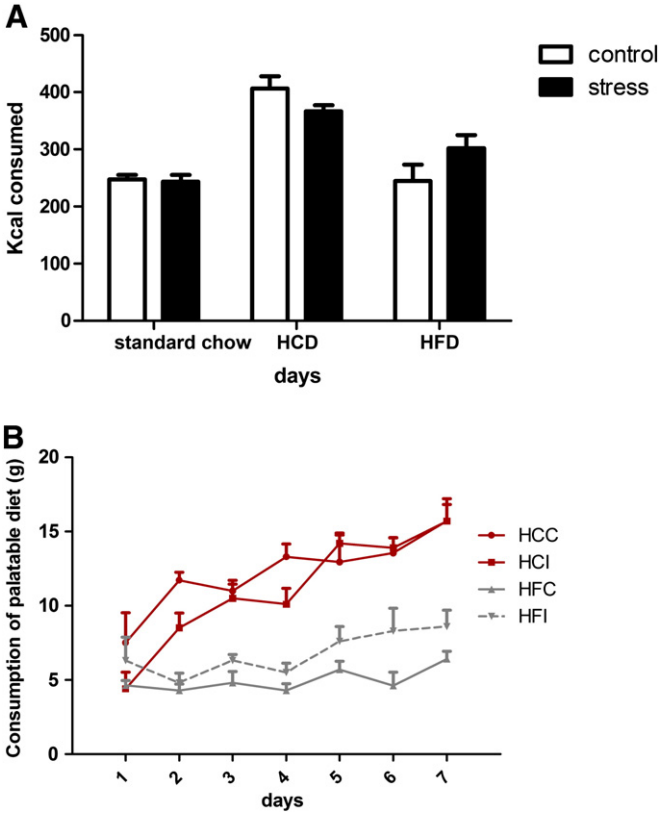

Fig. 2. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on consumption of palatable diets during the period of isolation stress. A. Caloric consumption. Two-way ANOVA showed an effect of diet ($P < 0.001$). HCD had a higher caloric consumption compared to other groups. B. Consumption of HCD and HFD. Repeated measures ANOVA showed an interaction between isolation stress and diet ($P = 0.005$) and diet and time ($P < 0.001$), effect of diet ($P < 0.001$) and time ($P < 0.001$). Data are expressed as mean ± SEM, N = 4–10/group.

3.2. Abdominal fat and adrenal gland weight

Fat deposition and adrenal gland weight were analyzed in adults and are shown in Table 3. Significant differences were observed both on retroperitoneal fat [Two-way ANOVA; $F(2,45) = 23.88$, $P < 0.001$] and gonadal fat [$F(2,45) = 32.35$, $P < 0.001$]. The groups that received

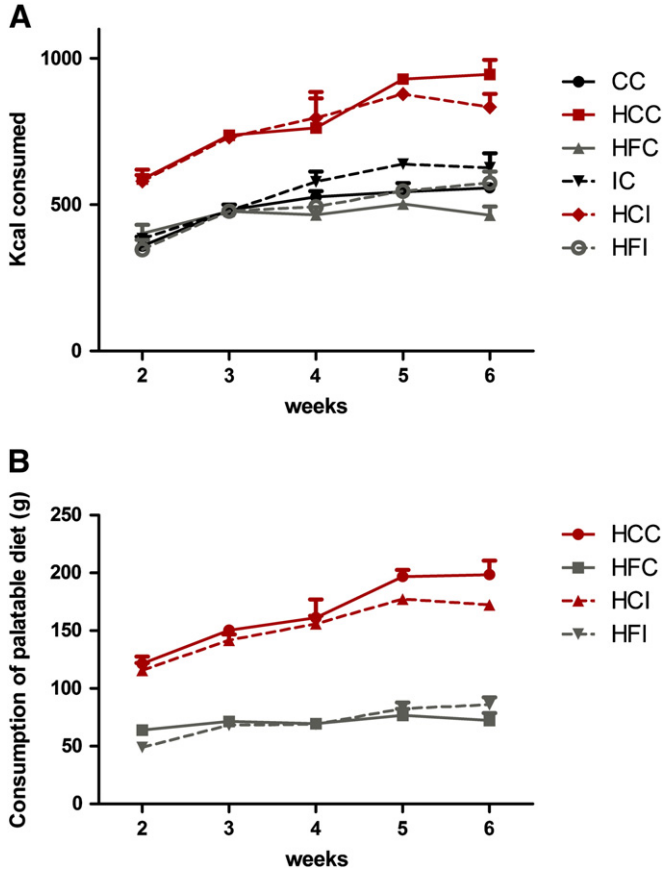

Fig. 3. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on consumption of palatable diets until 60 days of age. A. Caloric consumption. Repeated measures ANOVA showed effect of diet ($P < 0.001$) [higher in animals with HCD] and time ($P < 0.001$). B. Consumption of palatable diets. Repeated measures ANOVA showed an effect of time ($P < 0.001$), diet ($P < 0.001$) and an interaction between time and diet ($P < 0.05$). Data are expressed as mean ± SEM, N = 2–4/group.

palatable diets had increased abdominal fat, when compared to the group receiving regular chow, and the group receiving the high-fat diet had the highest fat accumulation (Tukey post-hoc, $P < 0.05$). Furthermore, a significant interaction between stress and diet was found in gonadal fat [$F(2,45) = 3.44$, $P < 0.05$] and this interaction was almost significant in the retroperitoneal fat [$F(2,45) = 2.79$, $P = 0.07$], where stress decreased the fat weight in the HCD and increased it in the HFD. With regard to adrenal gland weight, there was a marginally significant effect of exposure to stress during the prepubertal period [$F(1,45) = 3.83$, $P = 0.057$], where stress increased adrenal gland weight. If the adrenal gland weight is expressed as the adrenal/body weight ratio, the result remains the same.

3.3. Plasma lipid levels and serum cholinesterase activity

Results from plasma lipid measurements, as well as serum cholinesterase activity, were analyzed using a two-way ANOVA and are shown in Table 4. The levels of total cholesterol showed a marginally significant effect of diet [$F(2,27) = 3.11$, $P = 0.06$]. No differences were found in HDL-cholesterol levels ($P > 0.05$). However, a significant interaction between stress and diet was verified on LDL-cholesterol levels [$F(2,27) = 3.54$, $P < 0.05$], as well as a main effect of stress [$F(1,27) = 4.08$, $P = 0.05$], where stress during the prepubertal period was found to reduce LDL-cholesterol during adulthood, but not in those animals receiving the high-fat diet. Additionally, there was an almost significant interaction between stress and diet on triacylglycerol levels [$F(2,21) = 3.02$, $P = 0.07$]. Analyses of cholinesterase activity showed a significant difference between diet groups [$F(2,22) = 19.07$, $P < 0.001$, followed by Tukey post-hoc], since the animals receiving the HFD presented higher plasma enzyme activity.

3.4. Plasma glucose and leptin levels

As displayed in Table 5, rats with access to a HFD had higher glucose plasma levels than the other groups [two-way ANOVA, $F(2,27) = 3.77$, $P < 0.05$, followed by Tukey post-hoc]. Exposure to stress caused a reduction in leptin levels [$F(1,21) = 8.07$, $P < 0.01$], whereas both palatable diets increased these levels [$F(2,21) = 12.15$, $P < 0.001$].

3.5. Antioxidant enzyme activities, total thiol content and free radical production in the liver

Oxidative stress parameters were analyzed to verify whether there was an oxidative imbalance in the liver after exposure to isolation stress during the prepubertal period, when palatable diets were chronically offered (Fig. 4). When evaluating SOD activity, an interaction between stress and diet was shown [two-way ANOVA, $F(2,21) = 3.48$, $P < 0.05$]: isolation stress led to increased SOD activity in the group receiving the HFD. Exposure to stress increased CAT activity [$F(1,21) = 34.03$, $P < 0.001$] and there was an interaction between stress and diet [$F(2,21) = 9.92$, $P < 0.001$], since the increased activity induced by stress exposure was higher in the group receiving standard chow. For GPx activity, there was an expressive effect of diet, with both palatable diets decreasing this activity; however, a really marked decrease was observed in animals receiving HFD [$F(2,21) = 361.51$, $P < 0.001$, followed by Tukey post-hoc]. In relation to total thiol content, there was an effect of diet [$F(2,21) = 4.83$, $P = 0.01$; no significant difference was observed in post-hoc test]. Additionally, free radical production, as evaluated by the DCFH test, showed an effect of diet [$F(2.21) = 7.46$, $P < 0.005$] and an interaction between stress and diet [$F(2,21) = 5.44$, $P = 0.01$], since stress increased free radical production in the liver, particularly in the animals receiving palatable diets.

Table 3
Effect of isolation stress during the prepubertal period and chronic access to palatable diets on weights of retroperitoneal and gonadal fat and adrenal glands in adult rats.

	Control			Stress		
	Chow	HCD	HFD	Chow	HCD	HFD
Retroperitoneal fat	4.16 ± 0.43	7.70 ± 0.75[a]	7.91 ± 0.58[b]	4.21 ± 0.53	5.66 ± 0.65[a]	8.73 ± 0.99[b]
Gonadal fat	3.31 ± 0.24	5.87 ± 0.54[a]	6.45 ± 0.55[b]	3.16 ± 0.35	4.12 ± 0.47[a]	7.04 ± 0.97[b]
Adrenal glands	0.061 ± 0.005	0.056 ± 0.005	0.061 ± 0.005	0.062 ± 0.003	0.071 ± 0.004	0.068 ± 0.007

Fat tissue and adrenal gland weight were evaluated and expressed in mg. Data are expressed as mean ± SEM, N = 10–12/group. There was an effect of diet on retroperitoneal fat (two-way ANOVA, $P < 0.001$) and gonadal fat ($P < 0.001$); interaction between stress and diet on retroperitoneal fat ($P < 0.05$) and gonadal fat ($P = 0.07$); effect of stress ($P = 0.057$) on adrenal glands.
[a] Significantly different from chow and HFD.
[b] Significantly different from chow and HCD (Tukey post-hoc, $P < 0.05$).

Table 4
Effect of isolation stress during the prepubertal period and chronic access to palatable diets on total plasma cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride levels, and plasma cholinesterase (ChE) activity in adult rats.

	Control			Stress		
	Chow	HCD	HFD	Chow	HCD	HFD
Total cholesterol	54.23 ± 5.45	46.43 ± 4.45	52.89 ± 3.38	43.18 ± 4.62	47.67 ± 3.05	61.65 ± 5.11
HDL-Cholesterol	24.10 ± 3.00	28.63 ± 4.56	26.07 ± 1.13	27.67 ± 5.94	29.28 ± 4.98	33.57 ± 3.23
LDL-cholesterol	47.49 ± 6.82	39.74 ± 4.60	39.73 ± 4.75	26.26 ± 5.15	29.12 ± 2.87	45.60 ± 5.30
Triglycerides	86.84 ± 17.61	109.71 ± 18.38	64.56 ± 10.11	53.77 ± 11.14	53.68 ± 1.50	87.61 ± 15.27
ChE activity	0.412 ± 0.02	0.417 ± 0.01	0.592 ± 0.04[a]	0.380 ± 0.02	0.462 ± 0.04	0.530 ± 0.02[a]

Plasma lipids are expressed as mg/dL of plasma (mean ± SEM; N = 5–7/group). Cholinesterase activity is expressed as μmol AcSCh/h. mg protein (N = 4–5/group). Two-way ANOVA showed an interaction between stress and diet ($P < 0.05$), and an effect of stress ($P = 0.05$) on LDL-cholesterol; and an effect of diet ($P < 0.001$) on cholinesterase activity.
[a] Significantly different from chow and HCD (Tukey post-hoc, $P < 0.001$).

4. Discussion

In the present study, we evaluated the effect of chronic access to distinct palatable diets (when the animals could choose between these diets and standard chow), associated with stress by isolation early on in life, in adult male rats. Animals receiving HCD showed a higher weight gain compared to the control and higher calorie consumption when compared to other groups. However, the group receiving HFD presented the highest accumulation of abdominal fat, and this was further increased when associated with stress. The association of stress with HFD increased LDL-cholesterol levels. In the groups receiving the other diets, however, stress appeared to be beneficial, reducing LDL-cholesterol. The high-fat diet also increased plasma glucose levels and cholinesterase activity. In addition, the two palatable diets increased leptin levels, while stress caused a decrease. In the liver, stress induced an oxidative imbalance, and the group that received the high-fat diet was the most affected.

The animals with access to a high-fat diet demonstrated a lower weight gain during the first week, and the caloric consumption of these animals was similar to that of controls, suggesting a lower caloric efficiency in these animals. However, stressed animals that received this diet increased their consumption, and presented a weight gain that was similar to that of the controls. On the other hand, animals that had access to a high-carbohydrate diet had a higher caloric intake during the first week, mainly due to the increased consumption of the palatable diet, even though the weight gain of these animals did not present an equivalent increase. Stress increased consumption of both palatable diets, supporting the hypothesis that palatable food may be used as compensation during periods of stress ("comfort foods") [54].

After one week of isolation stress, the animals were returned to living in groups, but were still given access to palatable diets. Considering body weight until 60 days of age, the group that received the HCD had a higher weight gain, reflecting their increased consumption. The same did not occur in animals that received the HFD. Furthermore, of the stressed groups, those receiving palatable diets presented increased body weight. Access to both diets (HCD and HFD) led to increased abdominal fat depots (both gonadal and retroperitoneal fat), probably reflecting the higher consumption in the HCD. In the case of the HFD, the increased deposition of fat suggests a lower lean body mass, as there was no increase in weight when compared to the controls. Therefore, the HCD appears to be more palatable, inducing higher consumption and leading to a higher weight gain in these animals. In addition, both diets showed increased abdominal fat, which is an important risk factor for metabolic syndrome [55,56]. Moreover, stress exposure further increased abdominal fat in the HFD group, while the opposite was observed in animals receiving the HCD. This may be explained by the fact that high-fat diets enhance stress-induced levels of glucocorticoids [25,26], and thus may increase fat accumulation, while diets that are high in sugar reduce the HPA axis response to stress [24].

The adrenal glands were only slightly increased in stressed animals. However, since this measurement is performed in adulthood, and isolation stress was applied in childhood, this result suggests a long-lasting effect of isolation on the HPA axis function. In rats, during the prepubertal period, this axis functions in a different way compared with during adulthood, and habituation to stressors is less efficient [57]. This result adds further support to the notion that, during early life, the brain is very susceptible to genetic influences and environmental experiences [58–61], and that early adverse experiences may lead to abnormal behavior associated with alterations of the HPA axis [8,62].

In relation to plasma lipid levels, it is known that there is a relationship between cardiovascular disease and high intake of saturated fat [63,64], as well as increased plasma levels of total cholesterol, triglycerides and LDL-cholesterol, and decreased HDL levels [65–67]. Accordingly, in our study, the high-fat diet animals presented higher levels of LDL-cholesterol, and a tendency towards increased triglycerides and total cholesterol; however these findings were observed only in the animals that had been stressed during the prepubertal period. On the other hand, HDL-cholesterol levels were not significantly different. Stress by social isolation is an aversive event that increases the activity of the HPA axis [4,43]. It is possible that exposure to stress during the prepubertal period permanently programs the organism's metabolism; this effect could be associated with the alterations observed in the lipid profile in the HFD group. Studies show that glucocorticoids can increase circulating fatty acids through an increase in dietary fat intake [68]; these findings support our observations, since HFD consumption increased during isolation stress. This effect, however, did not persist until adulthood, showing that programming of the ingestion of this type of diet was not modified in the long term, while HCD continued to be more consumed for some weeks after stress. Glucocorticoids are also known to increase lipogenesis and VLDL secretion from liver [68], which

Table 5
Effect of isolation stress during the prepubertal period and chronic access to palatable diets on plasma glucose and leptin levels in adult rats.

	Control			Stress		
	Chow	HCD	HFD	Chow	HCD	HFD
Glucose	132.3 ± 9.4	128.8 ± 2.7	144.1 ± 10.5*	120.7 ± 5.8	131.7 ± 4.5	147.9 ± 2.8*
Leptin	4.98 ± 0.44	9.32 ± 1.35**	10.92 ± 0.89**	4.83 ± 0.22	6.46 ± 1.15**	7.57 ± 1.12**

Data are expressed as mean ± SEM, N = 5–7/group for plasma glucose; N = 4–5/group for leptin. Plasma glucose is expressed as mg/dL; leptin is expressed as ng/mL. Two-way ANOVA showed an effect of diet ($P < 0.05$) on plasma glucose levels; an effect of stress ($P < 0.01$) and diet ($P < 0.001$) on leptin levels.
* Significantly different from the other groups (Tukey post-hoc, $P < 0.05$).
** Significantly different from chow (Tukey post-hoc, $P < 0.001$).

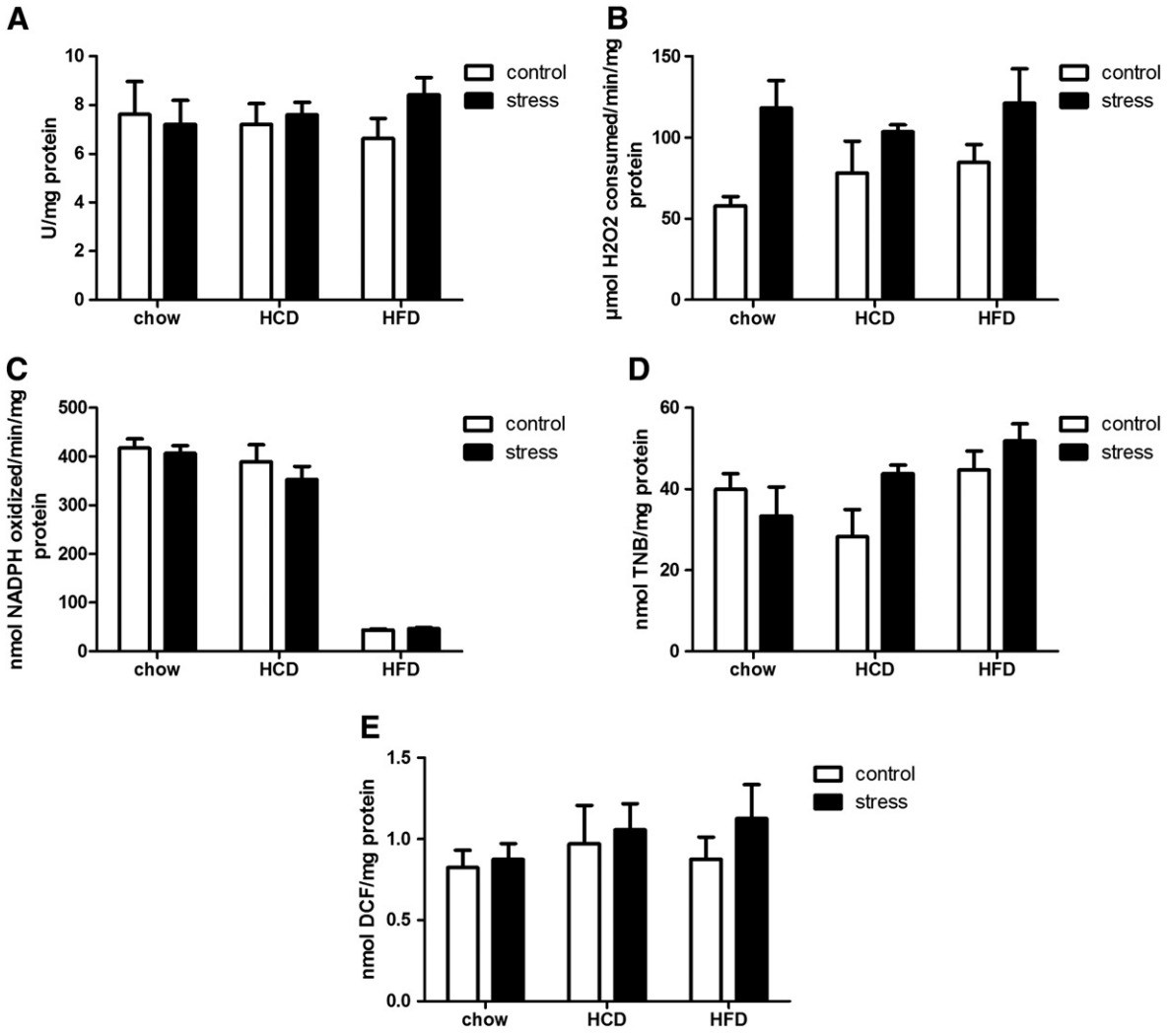

Fig. 4. Effect of isolation stress during the prepubertal period with chronic access to palatable diets on antioxidant enzyme activities, total thiol and free radicals (DCFH test) production in liver of adult rats. Data are expressed as mean ±SEM. N = 5–7/group. A. SOD (expressed as U/mg protein), B. CAT (expressed as micromoles of H_2O_2 consumed/min/mg protein), C. GPx (expressed as nmol NADPH oxidized/min/mg protein), D. total Thiol (expressed as nmol TNB/mg protein) and E. DCFH (expressed as nmol of DCF formed/mg protein). Two-way ANOVA showed an interaction between stress and diet on SOD ($P < 0.05$), and CAT ($P < 0.01$) activities, and on DCFH ($P = 0.01$); effect of stress on CAT activity ($P < 0.01$); effect of diet on GPx activity ($P < 0.001$), on total thiols ($P = 0.01$) and on DCFH ($P < 0.005$).

could help to explain the tendency to increase plasma triglycerides in the stressed group receiving HFD, and the synergic effect between stress and HFD, since it has been suggested that HFD enhances stress-induced levels of these hormones [25,26]. Depending on the intensity and duration of stress exposure, both lipolytic and antilipolytic effects may be observed in adipocytes, although the mechanisms by which these metabolic effects occur are still unclear [69–72]. Additionally, stressed animals had a decrease in LDL-cholesterol levels (except the group receiving HFD), when compared to the control group receiving regular chow, and stress had a tendency to increase cholesterol levels, which was more pronounced in animals that received the HFD. Both the adrenal and non-adrenal (ovarian and testicular) syntheses of steroid hormones employ LDL-cholesterol mainly from the circulation [73,74]. Thus, it is possible that the decrease in LDL-cholesterol levels that occurs as a result of stress is the consequence of the redirection of these lipids to the synthesis of steroid hormones; in other words, stress-induced steroidogenesis (which would be in agreement with the slightly increased adrenals in these animals). In summary, the effects of the consumption of a high-fat diet on serum lipid profile depend on the previous history of the animal, and exposure to stress during the prepubertal period endangers these animals, when this type of diet is associated.

One possible modulator of stress-induced eating is leptin [75,76]. This hormone is secreted from adipose tissue and influences energy homeostasis, immune and neuroendocrine function [77–79]. Production of leptin correlates positively with adipose tissue mass [80], and circulating leptin levels are involved both in the signaling of energy stores and in food intake. We found that the two palatable diets increased leptin levels, which can be correlated with the increase in abdominal fat found in these animals. Increased leptin levels may act in the hypothalamus to decrease appetite [78]. Interestingly, consumption in animals receiving the high-carbohydrate diet remained high. This may have occurred due to a lower sensitivity to leptin in the hypothalamus, where high plasma leptin concentrations do not induce reduction in food intake, suggesting resistance to the effects of endogenous leptin [81,82]. Furthermore, stress exposure decreased leptin levels in those animals given palatable diets (non-standard). Previous studies in the literature showed elevated leptin levels in human patients under glucocorticoid therapy [83,84], which could result from an inhibitory role on the action of leptin [85,86], which is suggested to contribute to "leptin resistance". Our finding that stress exposure decreased leptin levels does not agree

with the above reports, and this may be due to the fact that we used sub-acute stress during the developmental phase.

Animals that received the high-fat diet presented increased levels of blood glucose compared to other groups. As shown in the literature, the high-fat diet contributes to impaired glucose tolerance and insensitivity to the blood-glucose lowering effect of insulin [87]. This has been related to an impaired insulin binding and/or glucose transporters, due to changes in the fatty acid composition of the membrane induced by dietary fat modification [88]. Moreover, plasma cholinesterase activity was also higher in animals fed on the high-fat diet. Although the exact physiological function of plasma cholinesterase is unclear, reports from the literature suggest a relationship between the increased activity of this enzyme and hyperlipidemia, diabetes, and obesity [41], which are all risk factors for coronary artery disease [89] and heart disease [90]. Thus, increased levels of blood glucose and activity of plasma cholinesterase in animals receiving HFD are possibly risk factors for metabolic syndrome and cardiovascular disease in these animals.

In liver, exposure to isolation stress increased SOD activity in the group receiving HFD; stress also increased CAT activity in all the diet groups. GPx activity decreased with palatable diets, and most markedly in the HFD group. The production of free radicals was increased by stress, especially in the groups fed with the palatable diets, and total thiol content had an effect of diet, but no difference was observed in the post-hoc test. Thus, these results showed that stress during the pre-pubertal period induced a long-lasting increase in the production of free radicals in the liver, concomitantly with an increased activity of antioxidant enzymes. These increased antioxidant defenses are probably the result of the response to free radical formation, in an effort to protect cells against oxidative damage. This increase seemed to be higher in the HFD group. Moreover, this diet also induced a drastic reduction in GPx activity. It is known that high-fat diet can aggravate oxidative stress [91,92], causing the formation of toxic intermediates that can diminish the activity of antioxidant enzymes [93,94]. It should be pointed out that this vulnerability of the high-fat group to oxidative imbalance is not due to decreased ingestion of antioxidant vitamins, since these animals consumed higher amounts of the diet, and proportional higher amounts of vitamins included in this diet. One possible explanation for the decreased GPx activity may be the rapid consumption and exhaustion of these enzyme molecules, due to reactions with the free radicals generated. Other studies have also shown a decrease in the activity of antioxidant enzymes in the liver in association with high-fat diets [92,95–97]. In our study, however, only GPx activity was significantly reduced by the HFD, and further studies are necessary to understand the mechanism for this marked reduction. This decrease in GPx activity, however, may lead to an oxidative imbalance, leaving the liver vulnerable to an overproduction of hydrogen peroxide produced by the superoxide dismutase enzyme, the activity of which is increased in these animals (HFD associated with stress). This is especially important, considering that data suggest that oxidative stress can lead to various forms of chronic liver injury [98].

5. Conclusion

In conclusion, chronic access to palatable diets, especially a HFD, from the pre-pubertal period until adulthood, leads to changes in parameters related to risk factors for cardiovascular diseases and metabolic syndrome (increased body weight, abdominal fat, and leptin levels, as well as increased glycemia and cholinesterase activity); this condition is made worse by exposure to stress during the pre-pubertal period. Additionally, exposure to stress led to an oxidative imbalance in the liver and this was even more marked in the animals fed the HFD. These data emphasize the importance of considering the previous history of individuals when investigating the effects of diets on metabolic parameters. Also, it is relevant to understand how early life events may program development and metabolism later in life, enabling future preventive measures in childhood, including identifying and treating children at risk in order to decrease their vulnerability to stress-related disorders in adulthood.

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