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ORIGINAL ARTICLE

Maternal low-protein diet in female rat heart: possible protective effect of estradiol

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Several studies have shown that maternal low-protein (LP) diet induces detrimental effects in cardiovascular system and oxidative stress in male animals. Additional studies suggested that female has lower incidence of cardiovascular disease. However until present data, the possible effects of estradiol on the undernutrition during gestational and lactation periods are not discussed. The present study was conducted to evaluate the effects of a maternal LP diet during gestational and lactation period on oxidative balance in the female rat hearts ventricles at two ages. Dams were fed with normal protein (NP) or a LP diet during the gestational and lactation period, and their female offspring were divided into age groups (22 or 122 days, corresponding to a low or high estrogen level) composing four experimental groups. Evaluating the nutritional effect showed an increase in oxidative stress biomarkers and decrease in enzymatic defense in LP-22D compared with NP-22D. In contrast, no changes were observed in malondialdehyde and carbonyls, but an increase in glutathione-*S*-transferase (GST) activity in the LP-122D compared with NP-122D. The global oxy-score in the LP-22D group indicated a predominance of oxidative damage when compared with NP-22D, while in LP-122D group the global oxy-score was restored to NP-122D levels. Evaluating the estradiol effect, our data show a significant decrease in oxidative stress with increase in CAT and GST activity, associated with increase in intracellular thiols. Our data suggest that in situation with low levels of estradiol, hypoproteic diet during gestation and lactation period has detrimental effects on heart, however when estradiol levels raise, the detrimental effects induced are mitigated.

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Key words: estradiol, heart, maternal low-protein diet, oxidative balance

Introduction

A growing number of investigators have been studying possible factors that increase the risk of diseases during the developmental period. These studies have showed that a poor quality of nutrition during the pre- and postnatal periods plays a key factor in the development of several types of diseases.[1–6] Thus, the widespread Developmental Origins of Health and Disease (DOHaD) approach remains a major investigation axis of these studies. According to Barker's hypothesis, depending on the maternal insult, the highly plastic fetal organism is conditioned by an adaptive response to modify the metabolic profile.[7] This adjustment process following early environmental cues is known as 'developmental plasticity' and may result in different phenotypes and permanent changes in adulthood.[8,9]

The well-known Helsinki Birth (ongoing research)[10] and Dutch Famine[11] cohorts represent the precursor findings to the studies of DOHaD that give support to the theory linking maternal exposure to a restricted-nutrient environment and higher predisposition to non-communicable diseases (NCDs), such as diabetes and cardiovascular diseases (CVDs).[12]

CVD represents the major cause of death in the world (46.2% of NCDs deaths in 2012) and the fight against heart diseases has been declared one of the priorities of the World Health Organization. The nutritional deficiency represents one of the strongest inducers of the CVDs and many studies have shown that a maternal low-protein (LP) diet during the gestational and/or lactation periods causes several disorders, including increase in arterial blood pressure,[13,14] in left ventricular pressure,[15] impaired diastolic function,[16] and overexpression of genes associated to pathologic hypertrophy.[17] Oxidative stress is thought to be the underlying process, and has been directly related to onset and development of hypertension.

According to Brawley *et al.*, male rat offspring of dams fed LP diet during pregnancy developed vascular dysfunction and higher systolic blood pressure than the control group.[18] These findings are supported by the studies of Sato *et al.* that reported a similar association between LP diet in the developmental period and elevated blood pressure in male rats. This work suggests that the elevation in blood pressure was due to disruption in the balance of oxidative status in the aorta.[19] The oxidative stress induced by LP not only contributes to an

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increase in peripheral resistance but also affects the heart function. Recent data published by our laboratory, show that maternal LP diet in rat heart impaired the phosphorylative mitochondrial capacity, increased the hydrogen peroxide (H_2O_2) production and lipid peroxidation, decreased the antioxidant activity of some enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione-*S*-transferase (GST)] and decreased the reduced glutathione (GSH), an important intracellular thiol.[20]

Although equally harmful for both genders, the oxidative stress induced by maternal LP diet and its contribution to the development of CVDs later in life seem to be less severe on females.[21,22] Evidence that estrogen plays a crucial role in cardioprotection[23–26] is in line with the antioxidant function attributed to sex hormones. The cardioprotective effect of estrogen was demonstrated by Hernández *et al*. in a study with 150-day old ovariectomized (OVX) rats, which had an increase in blood pressure compared with a sham-group and that was reversed with estrogen replacement.[27] A recent study by Rodríguez-Rodríguez *et al*. with undernourished male and female rats reported no changes in blood pressure in rats at 21 days of life. At this age, male rats had an increased carbonyl and decreased GSH and total thiol levels in the plasma of male rats. These findings were not present in 21-day females.[28] At 180 days of life, male rats became hypertensive and showed significant increase in oxidative stress biomarkers, while females remained normotensive and with an unchanged level of oxidative stress biomarkers.

Even though the literature has demonstrated a possible link between nutrient restriction, heart disease and female hormones, the oxidative status after a nutritional insult in female rat heart is not well understood. The present study was conducted to test the hypothesis that level of oxidative stress in the heart ventricles of female offspring induced by maternal protein restriction during the gestational and lactation periods, depends on the offspring estrogens level.

Methods

Animals and diet

Eighteen female *Wistar* rats were mated (two females/one male) at the age of 70 days. On the first day of pregnancy, determined through the finding of spermatozoa in a vaginal smear, the females were divided into two dietary groups: Normoprotein (NP) diet (17% casein) or LP diet (8% casein), both with the same energetic value as previously described.[4] One day (24 h) after birth, the litters were standardized to nine pups per dam (with a 1:1 male to female ratio, plus one female when possible). The dams were divided in accordance to the experimental groups: immediate effect: NP diet, offspring at 22 days of age (NP-22), and LP diet, offspring at 22 days of age (LP-22); late effect: NP, 122 days of age (NP-122), and LP, 122 days of age (LP-122), composing four experimental groups (NP-22D, NP-122D, LP-22D and LP-122D). Litters from four dams were randomly chosen to constitute each experimental group composing $n = 5$ in each experimental groups. These time points were chosen because in female offspring at 22 days of age, the levels of estradiol are lower than at 122 days of age.[29,30] The mothers were maintained on their respective diets until weaning (21 days). After weaning females were maintained at four females per cage until they reach the experimental age. The late effect groups (122 days) started to receive laboratory chow (Labina; Purina Agribands) until tissue collection at 122 days of age. This study was approved by the Animal Care Committee from the Biological Sciences Center of the Federal University of Pernambuco (UFPE, protocol: 23076.017806/2014-62), in accordance with the recommendations of the National Institutes of Health – *Guide for the Care and Use of Laboratory Animals.*

Determination of estradiol

Blood samples were collected from offspring at 22 and 122 days of age. The blood samples were collected using specific tube collector for serum analysis. After the collection, samples were centrifuged at 10,000 rpm for 10 min at room temperature (RT) and stored at −20°C for the analysis. Estradiol concentration was measured by an enzymatic immunoassay kit (estradiol ELISA; Interkit, Brazil), 25 µl of sample were used according to manufacturer's instructions, and results were expressed as picograms per milliliter. The kit detection limits are in the range of 0–1000 pg/ml.

Necropsy procedures and tissue preparation

The rats were killed at 22 and 122 days of age. In the day of the experiment all rats had the body weight measured using a digital balance (Marte, model S-100 with a sensitivity of 0.001 g) and immediately the rats were killed by decapitation. The hearts were removed in <30 s and placed in a cold extraction buffer [50 mM Tris, pH 7.4; 1 mM ethylenediaminetetraacetic acid (EDTA); 2 mM phenylmethane sulfonyl fluoride (PMSF), 10 mM sodium orthovanadate], after removing the atriums, the ventricles were homogenized in an Ultra-Turraz homogenizer (model T10 BS32; IKA, Germany). After homogenization, samples were centrifuged at 4°C, 4000 rpm for 10 min (Sigma centrifuges, model 1-14K, Germany) and the supernatant used for all assays, including protein content determination by the Bradford protein assay.[31] Each experimental condition was performed with 300 µg of total protein.

Lipid peroxidation evaluation

The lipid peroxidation was evaluated using the malondialdehyde (MDA) levels in accordance with Buege and Aust.[32] Briefly, equal amounts of 30% trichloroacetic acid (w/v) and 3 mM of Tris-HCl buffer were added to the sample. After centrifugation at 5820 rpm for 10 min, the supernatant was mixed with thiobarbituric acid and boiled for 15 min. The pink

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pigment yielded was measured by densitometry at 535 ηm using a spectrophotometer.

Carbonyl content

The protein oxidation was performed as previously described.[33] The samples were centrifuged with 30% trichoroacetic acid (w/v) for 15 min at 4000 rpm. The pellet was re-suspended in 10 mM 2.4 dinitrophenylhydrazine and incubated in a dark room for 1 h with agitation every 15 min. Another centrifugation followed by addition of 6 M Guanidine hydrochloride and 30 min incubation at RT was performed and the protein oxidation was measured by densitometry at 370 ηm in a spectrophotometer.

SOD activity

The SOD is the primary antioxidant enzyme capable of removing superoxide radical by catalyzing its dismutation to oxygen and water, and it is essential for maintaining normal cell development and function.[34] The SOD activity was measured by inhibition of the epinephrine auto-oxidation method. Briefly, in a reaction mix containing 50 mM sodium carbonate buffer (pH 10.2), 5 mM EDTA and samples was added 15 mM of epinephrine and the enzymatic kinetics was analyzed for 2 min at 480 ηm as previously described by Misra and Fridovich.[34]

CAT activity

CAT is an antioxidant enzyme that it is widely distributed within the cell and is capable to catalyze the breakdown of H_2O_2 into water and oxygen, requiring iron as a cofactor attached to the active site. CAT activity was measured as previously described by Aebi,[35] where the enzymatic kinetics of the H_2O_2 decomposition was analyzed for 3 min at 240 ηm. The assay contained 50 mM phosphate buffer (pH 7.0), 300 mM H_2O_2 and sample.

GST activity

GST is an antioxidant enzyme involved in the detoxification of a wide range of toxic agents including peroxide and alkylating agents present in the tissues. The activity of GST was measured by the method described by Habig.[36] The principle of the assay is based on the absorbance of the conjugation of 1-chloro, 1 mM 2,4-dinitrobenzene (CDNB) with 1 mM reduced GSH and sample. Absorbance was measured at 340 nm at 30°C. One unit of enzyme conjugates 10.0 nmol of CDNB with GSH per minute.

Reduced GSH levels

The GSH levels were determined according to Hissin and Hilf[37] by adding the sample to 100 mM phosphate buffer (pH 8.0) and 5 mM EDTA followed by a 15 min incubation with *o*-phthalaldehyde (1 mg/ml) and sample at RT. The samples were measured at 350 and 420 ηm, excitation and emission, respectively, and compared with a GSH standard curve.

Total thiol groups

The measurement of total thiol groups consisted of a cold extraction buffer (50 mM Tris base, pH 7.4; 1 mM EDTA; 2 mM PMSF, 10 mM sodium orthovanadate) added to the samples, followed by incubation with 10 mM 5,5'-dithiobis (2-nitrobenzoic acid) at RT under a dark cover for 30 min. The samples were measured at 412 ηm as described by Ellman.[38]

Calculation of a global score of oxidative status (oxy-score)

The biomarkers of oxidative status described above were used to calculate a global score (oxy-score) for each experimental group as previously described.[28,39] A positive oxy-score indicates prevalence of antioxidant capacity and a negative oxy-score indicates predominance of oxidative damage as follows:

$$\text{Oxy-score} = (\text{ANTIOX} - \text{OXY})$$

where OXY is the standardized values of MDA and protein carbonyl contents; as biomarker of oxidative damage. ANTIOX the sum of standardized antioxidant biomarkers (all enzymatic and non-enzymatic defenses).

Statistical analysis

The data were tested to normality through Kolmogorov–Smirnov analysis, followed by the two-way analysis of variance to assess differences between groups. Comparisons were performed using GraphPad Prism software (GraphPad Software Inc., v.5, La Jolla, CA, USA), and the results were expressed as the mean ± standard error of the mean (S.E.M.). All values were considered significant at $P < 0.05$.

Results

Initially, we measured body weight in female offspring to confirm whether maternal LP diet at gestation and lactation induces differences in body weight at the two target ages. Our data demonstrated that offsprings from hypoproteic group exhibit lower body weight compared with offsprings that received normoproteic diet (NP-22D: 32.5 ± 1.8 *v.* LP-22D: 25.0 ± 0.5 g, $P < 0.05$; NP-122D: 225.3 ± 2.8 *v.* LP-122D: 181.0 ± 1.0 g, respectively, $P < 0.001$; Table 1). In sequence, we measured estradiol levels in female offspring to confirm the difference in hormones levels at the two experimental ages. We observed that females at 22 days of age have significantly lower levels of estradiol compared with females at 122 days of age (NP-22D: 136.9 ± 24 *v.* NP-122D: 282.0 ± 19.0 pg/ml, respectively, $P < 0.01$; Table 2). Evaluating whether the maternal LP diet could interfere on estradiol levels, we next evaluate the estradiol levels on females at 122 days that received hypoproteic diet during development as can be observed in Table 2 (NP-122D = 282.0 ± 19.0 *v.* LP-122D:

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Table 1. *Body weight from female's offsprings at 22 and 122 days of age that received normoprotein diet (NP group;* n = *5 per experimental group) or a low-protein diet (LP group;* n = *5, per experimental group) during the gestation and lactation period*

Groups	Body weight (g)
NP-22 days	32.5 ± 1.8
LP-22 days	25.0 ± 0.5*
NP-122 days	225.3 ± 2.8
LP-122 days	181.0 ± 1.0***

Data expressed as mean ± S.E.M., $^{*}P < 0.05$; $^{***}P < 0.001$ (two-way analysis of variance test).
$^{*}P < 0.05$, difference between NP *v.* LP at 22 days of age; $^{***}P < 0.001$, difference between NP *v.* LP at 122 days of age.

Table 2. *Levels of estradiol in 22 and 122 days of age*

Groups	Estradiol concentration (pg/ml)
NP-22 days	136.9 ± 6.1
NP-122 days	282.0 ± 19.0**
LP-122 days	308.0 ± 20.0

The female's offspring received normoprotein diet (NP group; $n = 5$ per experimental group) or a low-protein diet (LP group; $n = 5$ per experimental group) during the gestation and lactation period. Data expressed as mean ± S.E.M., $P < 0.01$ (two-way analysis of variance test).
$^{**}P < 0.01$, difference between 22 *v.* 122 days of age.

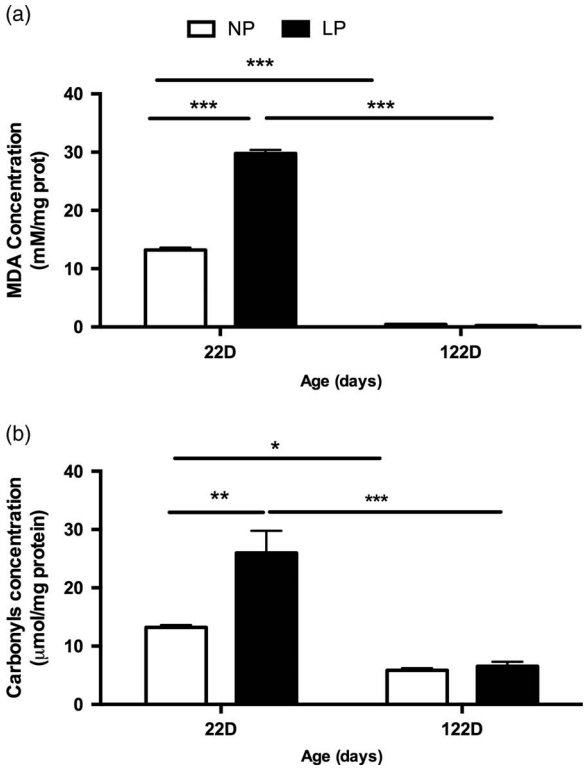

Fig. 1. Oxidative stress biomarkers in 22 and 122 days of age. (*a*) Malondialdehyde (MDA) concentration in 22 and 122 days of age and (*b*) carbonyl content in 22 and 122 days of age. The female's offspring received normoprotein diet (NP group; $n = 5$, per experimental group) or a low-protein diet (LP group; $n = 5$, per experimental group) during the gestation and lactation period. Data expressed as mean ± S.E.M., $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (two-way analysis of variance test).

308.0 ± 20.0 pg/ml), revealing that LP diet did not induce detrimental effect on estradiol levels at least in 122 days of age.

We next investigated the effects of the maternal diet on the heart ventricles of female offspring at 22 and 122 days. Compared with the NP-22 group, analysis of heart ventricles in the LP-22 group, showed a significant increase (125%) in MDA concentration (NP-22D = 13.2 ± 0.4 *v.* LP-22D =29.7 ± 0.6 mmol/mg protein; Fig. 1a). Analyzing the effect of maternal LP diet on animals with 122 days our data showed no significant difference when compared normoproteic against hypoproteic groups. When we analyze the age effect, possible estradiol effect, we observed a significant decrease in the MDA concentration in both groups at 122 days (NP-22D = 13.2 ± 0.4 *v.* NP-122D = 0.44 ± 0.05 mmol/mg protein, $P < 0.001$; LP-22D: 29.7 ± 0.6 *v.* LP-122D: 0.25 ± 0.02 mmol/mg protein, $P < 0.001$; Fig. 1a). Related to carbonyls concentration, hypoproteic diet in animal at 22 days showed a significant increase (96%) in the carbonyl concentration (NP-22D = 13.2 ± 0.4 *v.* LP-22D = 25.9 ± 3.8 µmol/mg protein, $P < 0.01$; Fig. 1b), but no alteration in carbonyl concentrations was observed at 122 days (NP-122D = 5.8 ± 0.37 *v.* LP-122D = 6.6 ± 0.74 µmol/mg protein; Fig. 1b). Analyzing again the age effect, we observed that at 122 days carbonyls concentration dropped in normo- and hypoproteic groups (NP-22D = 13.2 ± 0.4 *v.* NP-122D = 5.8 ± 0.37 µmol/mg protein, $P < 0.05$; LP-22D: 25.9 ± 3.8 *v.* LP-122D: 6.6 ± 0.74 µmol/mg protein, $P < 0.001$; Fig. 1b).

Examining the diet effect on antioxidant enzymes, LP-22 compared with NP-22 revealed that SOD increased by 50% (NP-22D = 17.6 ± 1.6 *v.* LP-22D = 26.4 ± 1.2 U/mg protein, $P < 0.001$; Fig. 2a), but no significant difference was observed at 122 days (NP-122D = 4.8 ± 0.46 *v.* LP-122 = 6.5 ± 0.24 U/mg protein; Fig. 2a). When was examined the age effect, possible estradiol effect, we detected a significant decrease in normo- and hypoproteic groups at 122 days (NP-22D = 17.6 ± 1.6 *v.* NP-122D = 4.8 ± 0.46 U/mg protein, $P < 0.001$; LP-22D: 26.4 ± 1.2 *v.* LP-122D: 6.5 ± 0.24 U/mg protein, $P < 0.001$; Fig. 2a).

Investigating the effect of maternal LP diet on CAT activity was revealed a significantly decreased around 70% in the LP-22D group (NP-22D = 1.2 ± 0.2 *v.* LP-22D = 0.40 ± 0.02 U/mg protein, $P < 0.05$; Fig. 2b), with no differences in CAT activity in the LP group at 122 days

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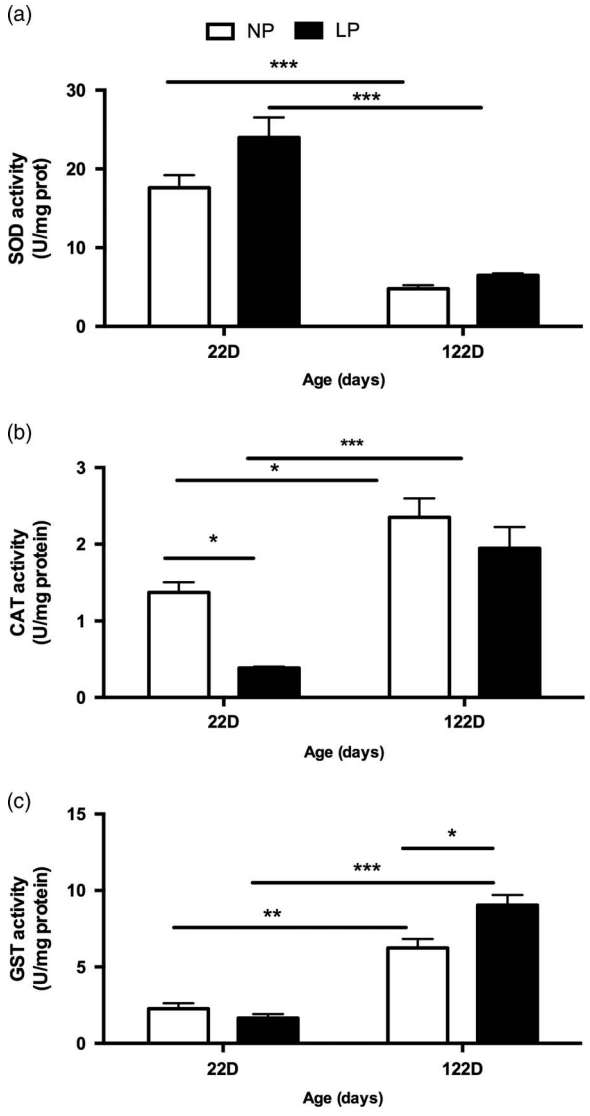

Fig. 2. Enzymatic antioxidant defense in 22 and 122 days of age. (*a*) Superoxide dismutase (SOD) activity, (*b*) catalase (CAT) activity, (*c*) glutathione-*S*-transferase (GST) activity in female's heart ventricles at 22 and 122 days of life. Female offspring received a normoprotein diet (NP group; $n = 5$, per experimental group) or a low-protein diet (LP group; $n = 5$, per experimental group) during the gestation and lactation period. Data expressed as mean ± S.E.M., $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (two-way analysis of variance test).

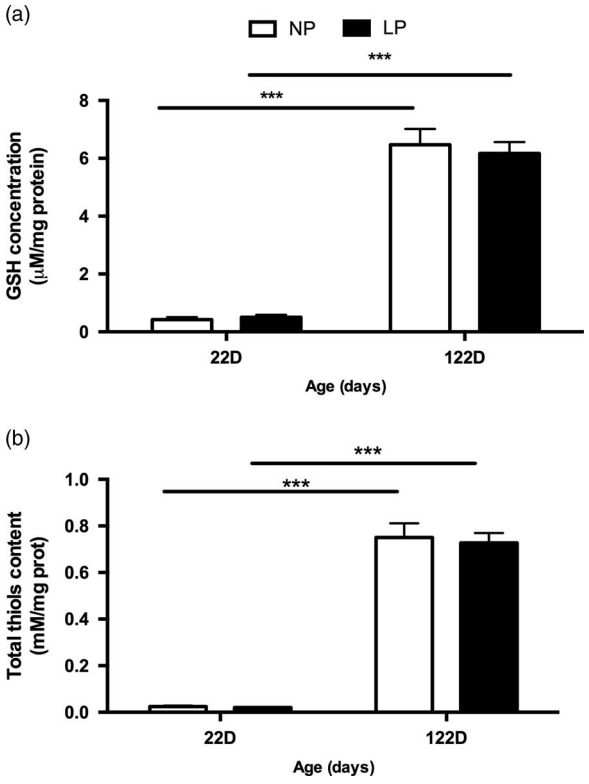

Fig. 3. Non-enzymatic antioxidant defense in 22 and 122 days of age. (*a*) Glutathione (GSH) levels and (*b*) Total thiol groups in female's heart ventricle at 22 and 122 days of life. Female offspring received a normoprotein diet (NP group; $n = 5$, per experimental group) or a low-protein diet (LP group; $n = 5$, per experimental group) during the gestation and lactation period. Data expressed as mean ± S.E.M., $^{***}P < 0.001$ (two-way analysis of variance test).

(NP-122D = 2.35 ± 0.24 *v.* LP-122D = 1.94 ± 0.27 U/mg protein, $P < 0.05$; Fig. 2b). Exploring the possible effect of estradiol, was detected a significant increase in CAT activity in both groups at 122 days, suggesting that the rise in estradiol levels can improve the CAT activity (NP-22D = 1.2 ± 0.2 *v.* NP-122D = 2.35 ± 0.24 U/mg protein, $P < 0.05$; LP-22D: 0.4 ± 0.2 *v.* LP-122D: 1.94 ± 0.27 U/mg protein, $P < 0.001$; Fig. 2b).

Exploring the effect of hypoproteic diet on GST activity, no difference was observed in the GST activity at 22 days of age (NP-22D = 2.27 ± 0.4 *v.* LP-22D = 1.55 ± 0.26 U/mg protein; Fig. 2c). However, when we examined the possible effect of the rise in estradiol levels, we observed that GST was significantly increased (40%) in LP-122 group in comparison with the NP-122 group (NP-122D = 6.24 ± 0.59 *v.* LP-122D = 8.76 ± 0.79 U/mg protein, $P < 0.05$; Fig. 2c), additionally was reveled that at 122 days GST activity was significant elevated in heart ventricle from normo- and hypoproteic groups (NP-22D = 2.27 ± 0.4 *v.* NP-122D = 6.24 ± 0.59 U/mg protein, $P < 0.01$; LP-22D: 1.55 ± 0.26 *v.* LP-122D: 8.76 ± 0.79 U/mg protein, $P < 0.001$; Fig. 2c).

Similarly to GST activity at 22 days, we did not observed any nutritional effect in the GSH level in both age (NP-22D = 0.43 ± 0.08 *v.* LP-22D = 0.50 ± 0.09 μmol/mg protein; NP-122D = 6.47 ± 0.54 *v.* LP-122D = 6.17 ± 0.39 μmol/mg protein; Fig. 3a), however analyzing the age effect we observed a significant increase in GSH levels when comparing with groups at 22 days (NP-22D = 0.43 ± 0.08 *v.* NP-122D = 6.47 ± 0.54 μmol/mg protein, $P < 0.001$; LP-22D = 0.50 ± 0.09 *v.* LP-122D = 6.17 ± 0.39 μmol/mg protein, $P < 0.001$; Fig. 3a). Same effect was observed in total thiol content, no nutritional effect in

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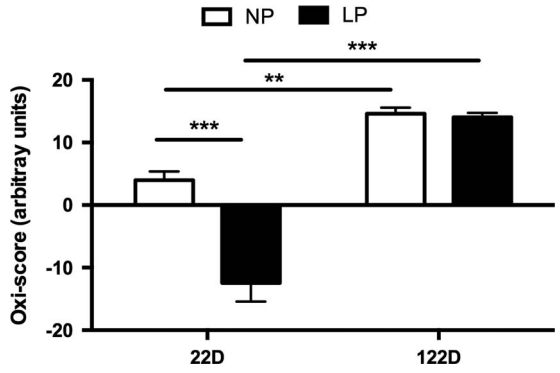

Fig. 4. Global oxidative status (oxy-score) in female's heart ventricle at 22 and 122 days. Female offspring received a normoprotein diet (NP group; $n = 5$, per experimental group) or a low-protein diet (LP group; $n = 5$, per experimental group) during the gestation and lactation period. Data expressed as mean ± S.E.M., $^{**}P < 0.01$; $^{***}P < 0.001$ (two-way analysis of variance test).

both ages (NP-22D = 0.024 ± 0.004 *v.* LP-22D = 0.022 ± 0.001 mmol/mg protein; NP-122D = 0.76 ± 0.06 *v.* LP-122D = 0.72 ± 0.04 mmol/mg protein; Fig. 3b) and increase at 122 days (NP-22D = 0.024 ± 0.004 *v.* NP-122D = 0.76 ± 0.06 mmol/mg protein, $P < 0.001$; LP-22D = 0.022 ± 0.001 *v.* LP-122D = 0.72 ± 0.04 mmol/mg protein, $P < 0.001$; Fig. 3b).

Assessment of global oxy-score, showed a negative effect of the maternal LP diet in the group LP at 22 days when compared with the normoproteic group at 22 days (NP-22D = 3.97 ± 1.42 *v.* LP-22D = − 12.46 ± 2.95; $P < 0.001$; Fig. 4). In the analysis, no significant differences in global oxy-score were found between NP-122D and LP-122D group (NP-122D = 14.63 ± 0.97 *v.* LP-122D = 14.05 ± 0.70; Fig. 4), however the analysis of the oxy-score at 122 days reveals a positive effect of the age, suggesting a decline in the risk of the oxidative damage in female at 122 days of age (NP-22D = 3.97 ± 1.42 *v.* NP-122D = 14.63 ± 0.97, $P < 0.01$; LP-22D = − 12.46 ± 2.95 *v.* LP-122D = 14.05 ± 0.70, $P < 0.001$; Fig. 4).

Discussion

In order to study the possible protective effect of estrogens after a nutritional insult during development (i.e. gestation and lactation), we used the maternal protein restriction strategy during the pre- and postnatal period as experimental model, and evaluated several indicative parameters of oxidative stress in the heart of female rat offspring. We have recently demonstrated that maternal LP diet induces a marked mitochondrial dysfunction and oxidative stress in male offspring at 100 days of age, mainly due to a decrease in antioxidant defense.[20] In the present study, our main finding was that maternal LP diet during the critical period of development differently affects the heart leading to a substantial increase of global score for oxidative damage at an early age (i.e. lower concentration of estrogen), and that this effect is mitigated by an increased circulating level of estrogen at a later age, in addition it's important mention that besides the detrimental effect on heart ventricles, maternal LP diet did not interfere in the estradiol levels at adult age. Our results suggest that even with an impaired oxidative balance while young, adult female offspring exhibits a resistance to oxidative stress due to the enhanced levels of female hormone compared with male adult animals.

Researchers with focus in the developmental process of CVDs not surprisingly have also attributed the onset of these pathologies to an oxidative stress situation.[39–42] Our data showed that the female offspring of mother fed with LP, at 22 days of age (LP-22 group) had a greater lipid peroxidation, shown by the MDA levels. In contrast, lipid damage was attenuated at 122 days of life, suggesting a sexual hormone-mediated adaptation. In agreement with our findings, the study conducted by Hernández *et al.* showed that the increased MDA levels observed in OVX rats at 150 days of age compared with sham-operated animals, was reversed by estrogen replacement in OVX rats.[27] Additional studies have investigated the absence of estrogens on lipid membrane peroxidation. Barp *et al.* found that lipid peroxidation was increased in the heart of 9-week old OVX rats.[43] Hamed *et al.* showed that plasma MDA levels augmented in OVX adult rats.[44] Similarly, Muñoz-Castaneda *et al.* demonstrated an increase in cardiac lipid peroxidation in OVX rats at 90 days of age that was reversed following estrogen replacement.[45] Studies conducted to understand the lower incidence of CVDs in females, demonstrate that endogenous hormones in females confer lower production of reactive oxygen species (ROS), the main oxidant agent responsible to oxidative stress induction.[46] All these data contribute with our hypothesis that elevation of estradiol, observed at 122 days, confers a protection against oxidative stress in female rat hearts.

In addition to lipid peroxidation, carbonyl content was substantially increased in LP-22 group, while no difference was observed in the LP-122 group in comparison with the respective control groups. Additionally was observed that at 122 days carbonyl content was declined in normoproteic and hypoproteic groups. A recently published work reports no differences in the plasma carbonyl content in females at 180 days of age.[28] However, this study, different from our results, found no difference in females at 21 days of age. The discrepancy between our data and Rodriguez's data may be due to the source of material analyzed (i.e. heart *v.* plasma) and/or experimental model (i.e. gestational and lactation period (42 days) *v.* 10 days during gestation). Taken together, our data led us to suggest that developmental protein restriction alters the oxidative status, increasing the formation of oxidant compounds during pre-pubertal age, which is suppressed by the increasing levels of endogenous hormones after sexual maturation.

Oxidative stress status is associated with an exacerbated ROS production, and/or with a deficient antioxidant defense capacity that result in damage to important cell constituents.[47] Following the evaluation of oxidative biomarkers, we further analyzed the antioxidant defense components represented by

both enzymatic and non-enzymatic systems. The first enzyme that decreases reactive species is SOD, which converts superoxide anion ($O_2^{\bullet}$) to hydrogen peroxide (H_2O_2) and that can be further converted to water and oxygen by CAT. In our study, SOD activity increased in both, LP-22 as well as in LP-122, suggesting an enhancement in the superoxide anion ($O_2^{\bullet}$) scavenging system. In addition, we verified a significant reduction in CAT activity in the LP-22 group, but no difference in LP-122 days. Together, our data suggest that the increase in sexual hormone at 122 days may contribute to the increase in enzymatic defense in female rat hearts. Studies from our laboratory demonstrated that a maternal LP diet led to a decrease in SOD and CAT activities in heart and brainstem of male rats at 100 days of age.[4,20] De Bem *et al.*, who showed that plasma SOD was reduced in male rats born to mothers fed LP diet during gestation, corroborate with our findings.[48] Moreover, it has been reported that the SOD and CAT activities are decreased in OVX rats at adult ages,[43,44] and that replacement of estrogen induced an upregulation of these antioxidant enzymes.[45] Altogether, we can infer that protein restriction induce an enzymatic dysfunction, while estrogen has a protecting effect and reverts the injury induced by undernutrition.

Concerning of the non-enzymatic defense system, the LP diet did not alter the GSH or total thiol groups levels in both age groups analyzed in this study, but the age effect reveals an impressive increase in GSH and total thiols in females with 122 days. Rodríguez-Rodríguez *et al.*[28] also reported no difference in the GSH levels young and adult females groups studied. In conclusion, our results suggest that the LP diet seems to modulate preferentially the enzymatic antioxidant system rather than the non-enzymatic system. The fact that the nutritional insult on non-enzymatic defense remained unchanged at both age groups provided an overview of the oxidative status, reinforcing the hypothesis that LP diet modulates enzymatic system. Analysis of the oxy-score exposed a predominance of oxidative damage in LP-22 group and prevalence of antioxidant capacity in the LP-122 group. The study of Rodríguez-Rodríguez *et al.*[28] did not find difference in the oxy-score in young or adult female rats, and this may be associated with the unchanged antioxidant and biomarkers parameters. In addition, Rodriguez-Rodriguez suggested that the no difference observed in females that received maternal LP diet could be attributed to the effect of female's hormones, as there are evidences that estradiol exhibit the capacity to scavenging radical species playing a protective role against oxidative stress.[28]

To our knowledge, the present study is the first to demonstrate that maternal LP diet during gestation and lactation negatively affects the global oxidative score in the heart of female offspring at early age, with restoration to control levels at a later age when estrogen levels increase. Several studies have been conducted to understand how protein restriction during development can induce and/or increase the risk for the manifestation of non-communicable chronic diseases in adulthood. However, the majority of theses studies are conducted in males. It's important to remind that mitochondria are the major site responsible for the production of pro-oxidative agents, and also that the mitochondrial genome is inherited from mother. From the results of our study, we can suggest that female offsprings from dams that undergo nutritional insult, on reaching a reproductive age can decrease the detrimental effect of an excessive production of ROS. This will diminish possible damages in either mitochondrial or nuclear DNA, ensuring the genetic information transfer, with the minimum possible injury in the genome, allowing the species maintenance and conservation. However, further studies are necessary to clarify the mechanisms involved in the possible protective effect induced by estrogens and others sexual hormones, evaluating epigenetics effects of estrogens on mitochondrial function and genome after nutritional insults.

Therefore, the present study suggest that maternal protein restriction induce oxidative damage in female rat heart, however on reaching at reproductive age the increased levels of estrogen slow down oxidative stress injury, suggesting a possible mechanism of protection in female at a reproductive age.

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Conflicts of Interest

None.

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