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Effects of perinatal protein restriction on the oxidative balance in the hypothalamus of 60-day-old rats

Efeitos da restrição proteica perinatal sobre o balanço oxidativo no hipotálamo de ratos de 60 dias de idade

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ABSTRACT

Objective
Evaluate the effects of maternal low-protein diet on the oxidative stress in the hypothalamus of 60-day-old rats.

Methods
Male *Wistar* rats were divided into two experimental groups according to the mother's diet during pregnancy and lactation; control group (NP:17% casein n=6) and a malnourished group (LP:8% casein n=6). At 60 days of life, the rats were sacrificed for the collection of the hypothalamus for further biochemical analysis.

Results
Our results showed an increase in oxidative stress in malnourished group, observed through an increase in carbonyl content (p=0.0357), a reduction in the activity of the glutathione-S-transferase enzyme (p=0.0257), and a reduction in the non-enzymatic antioxidant capacity evidenced by the decrease in the ratio reduced glutathione/oxidized glutathione (p=0.0406) and total thiol levels (p=0.0166).

Conclusion
A low-protein diet during pregnancy and lactation is closely associated with increased oxidative stress and reduced antioxidant capacity in the hypothalamus of sixty-day-old rats.

Keywords: Hypothalamus. Oxidative stress. Protein-energy malnutrition.

RESUMO

Objetivo
Avaliar os efeitos da restrição proteica materna sobre o estresse oxidativo no hipotálamo de ratos de 60 dias de idade.

Métodos
Ratos Wistar machos foram divididos em dois grupos experimentais de acordo com a dieta da mãe durante a gestação e lactação: grupo controle (NP: 17% caseína n=6) e grupo desnutrido (LP: 8% caseína n=6). Aos 60 dias de vida, os ratos foram sacrificados para coleta do hipotálamo para posterior análise bioquímica.

Resultados
Os resultados demonstraram aumento do estresse oxidativo no grupo desnutrido, observado através do aumento do conteúdo de cabonilas (p=0,0357) e redução da atividade da enzima glutationa-S-transferase (p=0,0257) e da capacidade antioxidante não enzimática, evidenciada pela queda da razão glutationa reduzida/glutationa oxidada (p=0,0406) e dos níveis de tióis totais (p=0,0166).

Conclusão
Uma dieta com baixo teor de proteínas durante a gestação e lactação está intimamente associada ao aumento do estresse oxidativo e à redução da capacidade antioxidante no hipotálamo de ratos de 60 dias de vida.

***Palavras-chave**: Hipotálamo. Estresse oxidativo. Desnutrição proteico-calórica.*

INTRODUCTION

According to the Origins of Health and Disease Development theory, have been proposed that adverse conditions such as malnutrition, during critical periods of the development, can predispose the offspring to chronic diseases in adulthood [1-6]. In this sense, experimental evidence demonstrates that maternal protein restriction during pregnancy is related to different outcomes in adulthood, resulting in mitochondrial dysfunction and oxidative stress in the heart, brainstem and kidney [7-9]. From a clinical point of view, the Dutch Famine of 1944 is perhaps the main parallel to the study of malnutrition in humans. In a cohort of 2,414 people aged 50 to 58 years, born in Amsterdam during the famine period, were observed in men greater glucose intolerance, microalbuminuria, atherogenic lipid profile, coronary artery disease, and, for women was observed, a greater risk of breast cancer [10].

According to data already published in literature, increased oxidative stress is among the main molecular outcomes associated with low-protein diets, which ultimately may be associated with functional changes in different tissues [7-9]. In this sense, oxidative stress is understood as a chronic imbalance between the production of pro-oxidant agents and the ability to remove them by the antioxidant systems, with such agents coming mainly from the mitochondria [11]. The central nervous system, is easily affected by the deleterious effects of oxidative stress, due to its high lipid content, high energy demand and low antioxidant capacity [12]. In a protein restriction model, Santana et al. (2019) demonstrated that the brainstem of *Wistar* rats exposed to two consecutive generations of low-protein diet during the gestation and lactation periods showed an overproduction of reactive species added to an impairment of mitochondrial bioenergetics [13]. In addition, increased lipid and protein oxidation along with impaired mitochondrial function has also been demonstrated in the brainstem of *Wistar* rats submitted to a low-protein diet during pregnancy and lactation [14,15].

It's well known that the hypothalamus regulates energy balance through neurons that respond to hormonal and nutritional variation, therefore, can be affected by protein restriction during critical periods of development [16]. Specifically in the hypothalamus, few data are available in the literature to demonstrate the effects of a maternal low-protein diet on oxidative balance. Due to this lack of literature associating the impact of a low-protein diet during gestation and lactation period on oxidative balance, we hypothesize that maternal protein restriction during pregnancy and lactation is associated with an impairment in the hypothalamic oxidative balance of the offspring. To test this hypothesis, after undergoing protein restriction, the evaluation of biomarkers of oxidative stress and enzymatic and non-enzymatic antioxidant defenses in the hypothalamus of 60-day-old rats was performed.

METHODS

Animals and diet

The experimental procedures followed what is recommended by the guidelines of the Institutional Ethics Committee for Animal Research (Approval Protocol nº 0060/2018), meeting the "Principles of Care for Laboratory Animals" described by the National Institutes of Health, Bethesda, MD, USA. Female *Wistar* rats (n=8), between 80 and 90 days old, were paired in a 2 to 1 male ratio and were evaluated daily for pregnancy detection. When pregnant, they were randomly divided into two groups, based on the amount of casein protein in the diet offered: control group (NP, 17% casein); and malnourished group (LP, 8% casein) (Table 1). The diet was carried out at the *Laboratório de Técnica Dietética do Centro Acadêmico de Vitória-Universidade Federal de Pernambuco*, and both the normal diet and the low-protein diet had the same energy value, being offered during pregnancy and lactation, as already described [8,13,17,18]. One day after birth, litter sizes were standardized by eight pups per dams to avoid litter size interference in milk production. To avoid "litter effect", only 2-3 males from each litter were chosen to continue in the study, which were fed with commercial chow (Labina; Purina Agriband, Brazil). At 60 days after birth, the rats were sacrificed to collect the hypothalamus for further biochemical analysis.

Table 1 – Composition of the diets (g/100 g diet).

Ingredients	The amount	
	8% protein	17% protein
Casein (85%)	9.41	20
Dextrin cornstarch	13.2	13
Cellulose	5	5
Sucrose	10	10
Cornstarch	50.34	39.74
Soybean oil	7	7
Choline	0.25	0.25
Methionine	0.3	0.3
Vitamin mix*	1	1
Mineral mix†	3.5	3.5
Energy density (kj/g)	16.26	16.26

Note: *Vitamin mix contained in mg/kg of diet: retinol 12; cholecalciferol 0.125; thiamine 40; riboflavin 30; pantothenic acid 140; pyridoxine 20; inositol 300; cyanocobalamin 0.1; menadione 80; nicotinic acid 200; choline 2720; folic acid 10; p-aminobenzoic acid 100; and biotin 0.6.
†Mineral mix contained in mg/kg of diet: CaHPO4 17 200; KCl 4000; NaCl 4000; MgO 420; MgSO4 2000; Fe2O2 120; FeSO4.7H2O 200 [17,18].

Tissue homogenization

The hypothalamus was homogenized in a buffer containing 50 mM-TRIS, 1 mM-EDTA [pH 7.4], 1 mM-sodium orthovanadate, 1.1 mM PMSF, and 0.1% NP-40. The samples were homogenized (Tecnal, Sao Paulo, Brazil), the homogenates were centrifuged at 1180 x g for ten minutes at four degrees Celsius, and stored at -80°C. Protein concentration was determined by the Bradford method [19].

Evaluation of malondialdehyde production

A total of 150 μg of hypothalamic protein was used to evaluate malondialdehyde (MDA), a product generated after the reaction with thiobarbituric acid (TBA) according Draper et al. [20]. The reaction was initiated by adding 30% trichloroacetic acid (TCA) and Tris-HCl (3 mM) to the samples, after were subjected to centrifugation (10 min at 2500 x g). In the final step, the supernatant was transferred to another tube and mixed with 0.8% of TBA (v/v), and boiled for 30 min. After let the samples cool down at room temperature and read at 535 nm using a spectrophotometer. The results was expressed in mmol/mg of protein [20].

Evaluation of protein oxidation

Used as a marker for oxidative damage to the protein, the carbonyl content was measured according to Reznick and Packer [21]. 30% TCA was mixed with the sample and centrifuged at 1180 x g for 15 min. Then, the pellet was suspended in 10 mM 2,4-dinitrophenylhydrazine and incubated for one hour in the dark, shaking every fifteen minutes. Finally, the samples were centrifuged and washed three times with ethyl/acetate, with the resulting pellet suspended in 6M guanidine hydrochloride and incubated for five minutes at 37°C. The final solution was read at 370 nm and the results expressed in mol/mg of protein [21].

Measurement of superoxide dismutase (EC 1.15.1.1) activity

The total measurement of Superoxide Dismutase (SOD) enzyme activity was measured using the methodology previous described by Misra and Fridovich [22]. 150 μg of protein from hypothalamic supernatants was incubated with 880 ml of sodium carbonate (0.05%, pH 10.2, 0.1 mM EDTA) under a temperature of 30°C and then the reaction was started with 30 mM epinephrine (0.05% acetic acid). The kinetics of epinephrine autooxidation inhibition was evaluated for 90 seconds at 480 nm and the measure of its activity expressed in U/mg of protein.

Measurement of catalase (EC 1.11.1.6) activity

To assess measurement of Catalase (CAT) activity, we follow Aebi [23] protocol. Briefly, the reaction mixture containing 50 mM of phosphate buffer at pH 7.0, 300 mM H_2O_2 and 150 μg of hypothalamic homogenate. The rate constant was obtained at 240 nm during 4 min at 30°C. The activity of this enzyme was demonstrated as U/mg of protein [23].

Measurement of glutathione-S-transferase (EC 2.5.1.18) activity

For the evaluation of GST activity, we applied the protocol from Habig [24]. Initially, 150 μg of samples were placed in 0.1 M phosphate buffer, pH 6.5, with 1 mM EDTA (30°C), and the

reaction was started with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM-GSH. Finally, the 2,4-dinitrophenyl-S-glutathione product was monitored at 340 nm [24].

Measurement of REDOX state (GSH and GSSG)

The Reduced Glutathione (GSH) content was evaluated in a medium with 0.1 M phosphate buffer together with 5 mM EDTA (pH 8.0), and 100 μg supernatant protein. After that, at room temperature, 1 mg/ml of o-phthaldialdehyde was added to the mixture and incubated for fifteen minutes; then fluorescence was measured at 350 excitation and 420 nm emission wavelengths. To measure the amount of oxidized glutathione (GSSG), 200 μg of protein was incubated with N-ethylmaleimide (0.04 M) for thirty minutes at RT and then 0.1 M of NaOH buffer was added to make 0.2 ml. Similarly, for GSH, protein aliquots were added with o-phthaldialdehyde, and fluorescence was measured in the same way as oxidized glutathione. Results for the two glutathiones were compared with standard curves and the REDOX state defined as the ratio of GSH/GSSG [25,26].

Total thiol content

According to the methodology previously described [27], using 5,5-dithio-bis (2-nitrobenzoic acid (DTNB). Briefly, part of the hypothalamic supernatant (450 μg) was incubated in the dark with DTNB (30 ml, at 10 mM) and extraction buffer (pH 7.4) was used to reach a final volume of 1 ml. Final samples were measured at 412 nm of absorbance, and the results are expressed in mmol/mg protein.

Considering the normality of data distribution, the difference between the groups was evaluated by Student's t test and all data were expressed as mean and standard error of the mean (SEM). Considering significant only with $p<0.05$, the analyzes were conducted using the GraphPad Prism 6.0® software (GraphPad Software, Inc).

RESULTS

Oxidative stress biomarkers

The evaluation of protein and lipid oxidation, were performed in the hypothalamus of both groups (Figure 1). Our results demonstrate that in low-protein group no difference in lipoperoxidation (NP: 26.76±1.119 N=4 vs LP: 28.13±1.217 N=6, $p=0.4583$), although in protein damage we observed twice more damage in LP group (NP: 1.705±0.4592 N=4 vs LP: 4.091±0.6992 N=6, $p=0.0357$) than the NP group, Figure 1A and 1B, respectively.

Enzymatic antioxidant system

Was performed the activity of the antioxidant enzymes SOD, CAT and GST in hypothalamus (Figure 2) [28]. No difference was observed in SOD activity (NP: 67.16±6.149 N=4 vs LP: 95.78±12.10 N=7, $p=0.1261$) and CAT (NP: 413.6±173.6 N=3 vs HP: 512.5±34.33 N=4, $p=0.5409$). However, in GST activity we observed a significant decrease in LP group (NP: 0.2108±0.05301 N=4 vs LP: 0.05185±0.03226 N=6, $p=0.0257$) in this same group.

Non-enzymatic antioxidant system

Additionally to the enzymatic system we evaluate the non-enzymatic antioxidant system. Thus, reduced glutathione and total thiol groups are the key non-enzymatic molecules that act to reduce oxidative stress. Our data showed (Figure 3), that protein malnutrition induce in the hypothalamus a reduction in the REDOX state (NP: 2.663±0.09223 N=5 vs LP: 1.727±0.3725 N=5, p=0.0406), and total thiol levels (NP: 0.0362±0.004329 N=5 vs LP: 0.0230±0.002017 N=6, p=0.0166), corroborating with the establishment of oxidative stress.

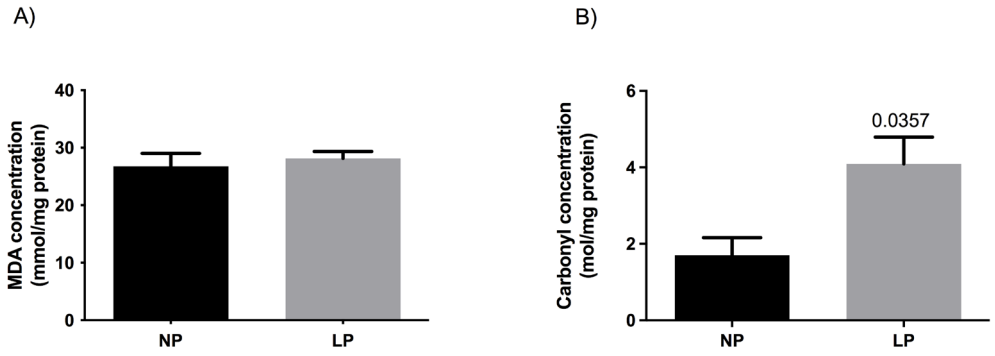

Figure 1 – Malondialdehyde and Carbonyl concentration.

Note: Oxidative stress biomarkers in animals from norm nourished and undernourished groups. A) Lipidiperoxidation (malondialdehyde – MDA) and B) Proteins oxidation (carbonyl content). Data expressed as mean and SEM. NP: control group; LP: malnourished group. N=4-6 per group.

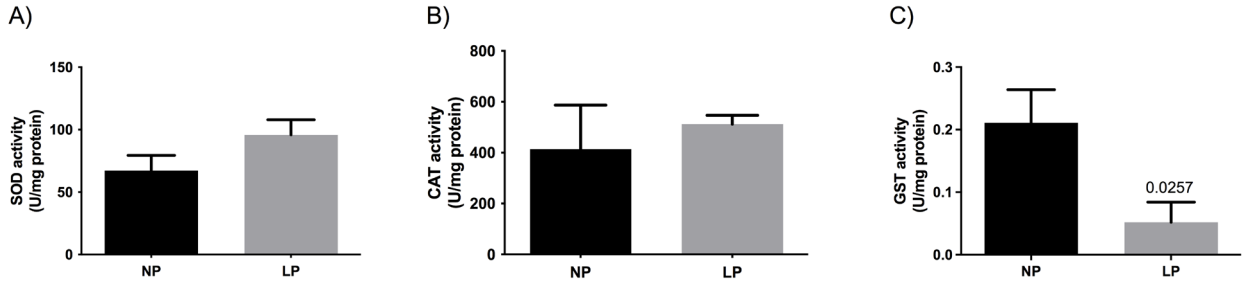

Figure 2 – Activity of superoxide dismutase, catalase and glutathione-S-transferase enzymes.

Note: Antioxidant enzymatic system: A) Superoxide dismutase (SOD); B) Catalase (CAT) and C) Glutathione-S-transferase (GST). Data expressed as mean and SEM. NP: control group; LP: malnourished group. N=4-7 per group.

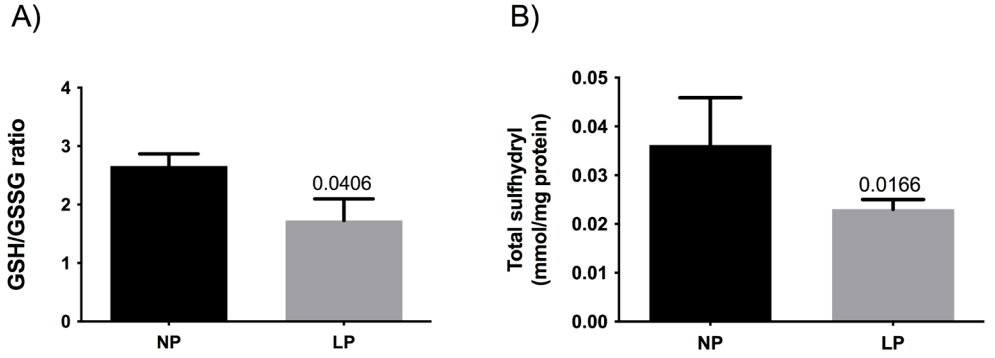

Figure 3 – REDOX state and Total thiols groups.

Note: Non-enzymatic system: A) REDOX state (GSH/GSSG); and B) Total thiols (sulfhydryl content). Data expressed as mean and SEM. NP: control group; LP: malnourished group. N=3-7 per group.

DISCUSSION

Previous experimental data in literature have shown that maternal protein restriction is associated with altered food intake and disease in the adult offspring [29-31]. Here we demonstrate that maternal protein restriction disturbs the offspring's hypothalamic oxidative balance at 60 days of life, which is observed through the increase in carbonyl content, reduction in GST activity, in the GSH/GSSG ratio and in the levels of total thiols.

In general, the brain of offspring from mothers submitted to protein malnutrition is subject to several negative modulations, including reticulum stress, oxidative damage, and downregulation of the growth factors [32,33]. Knowing the important relationship between clock genes and the regulation of energy metabolism [34], Crossland et al. [35] demonstrated a time-effect difference in the expression of Per 1, Clock, and Per 2 in the hypothalamus of rats submitted to a low-protein maternal diet. The results together show that maternal protein restriction during the developmental period can negatively influence several aspects of the hypothalamic milieu.

Our data show increased protein oxidation, with no difference in lipid oxidation. Similar to what we found here for biomarkers of oxidative stress, Ferreira et al. [15] using a similar experimental design with low-protein diet in *Wistar* rats during pregnancy and lactation, also found increased carbonyl content and no change in MDA levels in brainstem from LP animals when compared to their normoproteic counterparts. The preferential oxidative damage to proteins, instead to lipids, is not fully understood, but previous data in literature showed damage to the amino acids tyrosine and tryptophan in CNS regions from rats with 60-day-old submitted to a low-protein diet [36,37]; these studies can support the hypothesis that proteins can be more sensitive to oxidative damage depending on the amino acids that compose them [38].

In addition, we observed that in the LP group a strong reduction in GST activity, without any significant changes in SOD and CAT activity. Once again, data from Ferreira et al. (2016) showed an similar result, where animals submitted to a low-protein diet exhibited lower GST activity, without any change in SOD, CAT, Glutathione peroxide (GPx) and Glucose-6-Phosphate Dehydrogenase (G6PDH) [15]. Considering, therefore, the ability of GST to detoxify lipid peroxidation products and electrophilic compounds [39-41], it can be suggested that the LP diet results in an impairment of hypothalamic function, due to the modulation in the ROS production and an impair in antioxidant system.

Related to non-enzymatic defense, the ratio between reduced and oxidized glutathione (GSH/GSSG) is one of the main indicators of the REDOX state of the cell, since this molecule is the main thiols related to intracellular antioxidant capacity [40,42]. Considering the similarity of different brain regions, such as high O2 consumption and polyunsaturated fatty acid content, this organ is particularly vulnerable to oxidative stress and allows a comparison between the hypothalamus and the brainstem [43]. In this sense, data published previously in brainstem [14,15] demonstrate that perinatal protein malnutrition impairs, at different stages of life, mitochondrial bioenergetics, increases in oxidative stress markers and reduces the antioxidant capacity, which was justified by the reduction of the GSH/GSSG ratio – corroborating with our result, where we observed a reduction in malnourished animals when compared to their normonourished controls.

Finally, in line with the reduction in the REDOX state, we observed that the total thiol content was significantly reduced in animals from malnourished mothers. In the same sense, in terms of brain tissues (brainstem), was previously demonstrated that maternal protein restriction reduce both the level of Reduced Glutathione (GSH) in male *Wistar* rats and the content of total thiols in females

at 22 days old [15,44]. This effect in female rats, varying according to age, seems to be established due to the protective role of female estrogens, found in higher levels at the reproductive ages of rats [44,45]. Thus, the increased oxidative stress biomarkers and reduced antioxidant systems may be the initial mechanisms of neurodegenerative disease observed in adulthood, although initiated during the development due to the perinatal protein restriction.

Regardless of our manuscript demonstrating that perinatal protein deficient induces an increase in the key markers of oxidative stress in the hypothalamus, and this oxidative stress could be related to negative consequences in the tissue functionality, our study has the limitation of not having performed behavioral studies and performing a direct correlation between increased levels of oxidative stress with possible negative modulations in behaviors linked to hypothalamic function, mainly related to satiety eating behavior, among others effect in hypothalamic function.

CONCLUSION

In conclusion, we observed that a low-protein diet during development is closely associated with boosted oxidative stress and impair in antioxidant system in the hypothalamus of sixty-day-old rats. Thus, considering the central role of this tissue in energy homeostasis and, therefore, in metabolic regulation, greater focus should be given to the control of malnutrition, making more studies necessary to investigate this condition in other stages of life in order to better preventive and therapeutic interventions can be implemented. In addition, the need for additional work is justified due to the scarcity of data in the scientific community regarding the study of the hypothalamus under protein restriction conditions.

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