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Hyperhomocysteinemia and Gestational Programming of Genes Involved in Alzheimer's Disease Pathogenesis

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Abstract

Maternal hyperhomocysteinemia (hHcy) induced by a high methionine diet delays brain maturation and leads to impairment of learning performance in the offspring. Because methionine-homocysteine metabolism is related to the regulation of gene expression through one-carbon metabolism, we investigated whether maternal supplementation with methionine affects the expression of *Adam10*, *Bace1*, *Bace2*, *Ps1*, *Ps2*, *Tace*, *App*, *Il-1α*, *Il-1β* and *Tnf-α*, genes related to Alzheimer's disease (AD) risk, in the offspring. Thirteen female mice were distributed into the following groups: a) standard diet and b) standard diet supplemented with 1% methionine in water. After birth, the offspring were organized into Control (CT) and Supplemented Diet (SD) groups, and at Postnatal Day (PND) 90, all animals were weighed and then euthanized. The homocysteine (Hcy) concentration in plasma was measured, and the whole brain was weighed and dissected, and the hippocampus used for gene expression analyses. A decrease in total body weight was found in SD group, but no differences in brain weight were observed. The maternal diet did not seem to affect the Hcy concentration of PND 90 offspring. No differences were found in the expression of AD related genes in the hippocampus ($p>0.05$). In conclusion, hHcy induced by methionine supplementation during pregnancy and lactation did not affect the expression of genes related to AD risk in the offspring.

Keywords: Maternal nutrition; Fetal development; Mice; Methionine supplementation; Hyperhomocysteinemia

Abbreviations: SAM: S-Adenosylmethionine; AD: Alzheimer's Disease; CT: Control; DCT: Dams in Control Diet; DSD: Dams that received the methionine Supplement; Hcy: Homocysteine; hHcy: Hyperhomocysteinemia; PND: Postnatal Day; SD: Supplemented Diet

Introduction

Gestational programming is defined as a process whereby a stimulus or a stress, occurring in a critical period of development, can permanently change the structure, physiology, and metabolism of the offspring, predisposing individuals to disease in adult life [1]. A review published by Ross et al. [2] proposed the gestational programming of obesity as a potential contributor to AD. Maternal nutrition during pregnancy plays a key role in the growth of the fetus and placenta, and experimental studies in animals have shown that parental diet can impair fetal health through changes in DNA methylation patterns [3]. This process is one of the epigenetic mechanisms that regulate gene expression and is sensitive to one-carbon metabolism, which is influenced by the methionine-homocysteine flow.

Hcy is a sulfur-containing amino acid derived from the metabolism of methionine [4,5]. Hcy concentration may vary considerably among individuals according to genetic, dietary and environmental factors, and elevated plasma concentrations have been identified as a risk factor for a wide range of pathological conditions [6] such as cardiovascular disease [7,8] and AD [9,10].

AD is a progressive neurodegenerative disorder and is the most common cause of dementia in the elderly. Amyloid-beta (Aβ) brain deposits are one of the hallmarks of the neuropathological degeneration observed in AD patients, and Aβ is produced by two sequential proteolytic cleavages of amyloid precursor protein (APP). APP can be processed by α-secretases (PS1 and PS2) and γ-secretases (ADAM10 and TACE) producing non-amyloidogenic peptides, or by α-secretases and β-secretases (BACE) producing the Aβ fragments [11-13]. PS1 and BACE are regulated by methylation and decreased folate and vitamin B12 in culture media can cause a decrease in S-adenosylmethionine (SAM), a universal donor of methyl groups, and an increase Aβ concentration [14]. Conversely, anti-inflammatory non-steroidal therapy alters APP processing and decreases Aβ deposition in an animal model of AD [15].

Additionally, *in vitro* and animal studies suggest that increased Hcy concentrations promote the synthesis of several pro-inflammatory cytokines [16-18]. Increased production of cytokines such as Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) increases the risk of AD by approximately two-fold [19]. In the brain, pro-inflammatory cytokines may interfere with APP expression and processing [20,21], and evidence suggests a probable relationship between genes associated with inflammation and AD risk. Aside from these studies, little is known about the effects of hHcy on methionine-homocysteine metabolism in fetuses and neonates or the subsequent consequences to the brain. Considering the roles of nutrition and stress in gestational programming, we aimed to evaluate the consequences of maternal hHcy during pregnancy/lactation in male offspring. To this end, we supplemented the dams with 1% methionine in water during the period described above and analyzed the gene expression of *Adam10*, *Bace1*, *Bace2*, *Ps1*, *Ps2*, *Tace*, *App*, *Il-1α*, *Il-1β* and *Tnf-α* in the hippocampi of offspring on PND 90.

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Materials and Methods

Animal treatment protocols

Animal experiments were performed on Swiss mice, conducted according to the Guide for the Care and Use of Laboratory Animals (8th edition, National Academy Press, Washington D. C., 2011) and approved by the Institutional Animal Care and Use Committee of the Universidade Federal de São Paulo (#1169/08). Adult female mice were maintained under standard laboratory conditions on a 12-hour light/dark cycle, with food and water available *ad libitum*. One month before pregnancy, thirteen female mice were distributed into the following groups: a) standard diet (AIN-93M) and b) standard diet (AIN-93M) supplemented with 1% methionine in water. After twenty days of treatment, blood samples were collected to evaluate the Hcy concentration. Blood was collected using a lancet at the submandibular vein, which allows 0.2-0.5 ml of blood to be quickly drawn without the use of anesthesia. Following this procedure, male mice were placed in the females' home cages for mating, and gestational day zero was determined by confirming the presence of sperm in the content of a vaginal smear.

After birth, offspring were distributed into two groups: control (CT) and methionine supplement diet during pregnancy and lactation (SD). The dams of CT and SD offspring received standard and supplemented diet, respectively, during pregnancy and lactation. After weaning (PDN 28), all offspring groups were placed on a standard diet. Only males were utilized in this experiment.

Analytical procedures

Male mice were euthanized by decapitation at PND 90, and blood was collected in tubes (Becton Dickinson, New England, UK) containing ethylenediaminetetraacetic acid (EDTA) or heparin. Immediately after collection, total blood aliquots in heparin were used for glucose, total cholesterol and triglycerides levels, using photometric measurement of reflection by Accutrend Plus (Roche). Blood samples in EDTA were stored on ice for up 90 minutes and then centrifuged at 3000 rpm for 10 minutes at 4°C, and plasma aliquots were taken stored at -80°C for Hcy measurement. The whole hippocampus was collected, rapidly harvested and stored at -80°C for subsequent gene expression analyses by real-time PCR.

Biometric evaluation

All animals were weighed before euthanasia. An analytical balance (Bioprecisa, Model-FA2104N, Curitiba, Paraná, Brazil), accurate to 0.001 g, was used to evaluate the mass of the brain. The calculation of the relative mass of tissue was performed based on the percentage of brain tissue, taking into account the total weight of the animal.

Plasma

Plasma Hcy was analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection and isocratic elution. The method developed by Pfeiffer et al. [22] was used, with slight modifications: column C18 Luna (5 µm, 150 mm x 4.6 mm), mobile phase (0.06 M sodium acetate, 0.5% acetic acid, pH 4.7 (adjusted with acetic acid), 2% methanol) and flow rate 1.1 mL/min. The retention time was 3.6 minutes for Hcy [23].

RNA extraction and Real-time PCR

TRIzol® reagent (Invitrogen, Carlsbad, CA) was added to the hippocampus samples, and total RNA was isolated according to the manufacturer's protocol. Agarose gel (1%) electrophoresis was performed to evaluate the integrity of the molecules. After DNAse (Promega, Madison, USA) treatment, total RNA was reverse-transcribed using ImProm-IITM Reverse Transcriptase (Promega, Madison, USA). Diluted cDNA was added to 2× SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) together with the respective primers for *Adam10, Bace1, Bace2, Ps1, Ps2, Tace, App, Il-1α, Il-1β* or *Tnf-α*. The expression of the target genes was normalized using *Glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) as the endogenous control. The quantitative real-time PCR assays were performed using a 7500 Real-Time PCR instrument (Applied Biosystems). Relative expression analysis was performed using a standard, dilution curve-based method for relative real-time PCR data processing, based on the $^{\Delta\Delta}$CT method [24]. The results were expressed in arbitrary units.

Statistical analyses

All the variables were checked for normality (Shapiro-Wilk) and/or homogeneity (Levene's) and transformed when necessary, using the Z-score to normalize the data. Analyses of dams and offspring were performed using *t test* for independent groups. The data were presented as mean ± standard error, and the level of significance was $p \leq 0.05$. The program STATISTICA 8.0 was used to perform the analysis.

Results

Dams

The dams that received the methionine supplement (DSD) showed, after twenty days of treatment, an increase in Hcy concentration of approximately 100% (8.71 µmol/L) compared to the control group (DCT) (4.45 µmol/L) ($t_{(11)}$=5.59, p<0.001) (Figure 1).

Offspring biometric

As demonstrated in Table 1, the animals showed differences in body weight ($t_{(29)}$=2.81, p=0.009), with lower values in the SD group

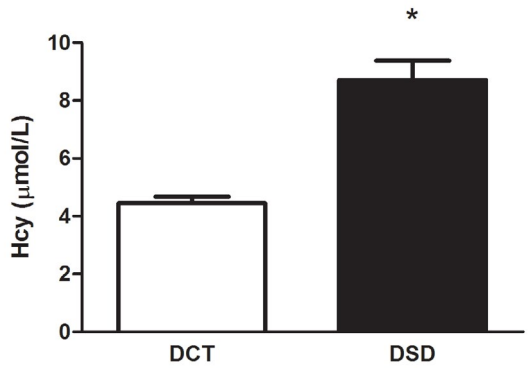

Figure 1: Hcy concentration compared to the control group (DCT) methionine supplement (DSD).

	CT	SD
Body weight total (g)	47.39 ± 0.8	43.97 ± 0.04*
Brain (g)	0.56 ± 0.00	0.56 ± 0.00
Brain (%)	1.19 ± 0.02	1.28 ± 0.02*

CT=control offspring (N=15); SD=Methionine supplement diet during pregnancy and lactation offspring (N=12). *Different between the groups. The values are presented as the mean ± S.E.M.

Table 1: Offspring body weight, brain weight and relative brain (%) weight in offspring PND 90 from dams on supplemented diet during pregnancy/lactation.

when compared to CT group. No differences were observed in total brain weight ($t_{(25)}$=0.11, p=0.91); however, relative brain weight (as a percentage of total body mass) was lower in the SD group ($t_{(25)}$=-2.82, p=0.009) (Table 1).

Total blood and plasma

Results concerning Blood total dosage at PND 90 are summarized in Table 2. The concentrations of blood glucose ($t_{(25)}$= 0.34, p=0.73), cholesterol ($t_{(25)}$= -2.04, p=0.051) and triglycerides ($t_{(25)}$= 1.20, p=0.24), did not change in male at PND 90. Figure 2 represents the effects of maternal methionine supplementation during pregnancy and lactation on plasma Hcy concentration in offspring on PND 90. No significant differences were found ($t_{(22)}$= -0.71, p=0.48).

Gene expression

No differences were found in the expression of the following genes related to AD risk in the hippocampus (Table 3): *Adam10* ($t_{(12)}$=-0.07, p=0.94), *Bace1*($t_{(12)}$= -0.01, p=0.99), *Bace2* ($t_{(12)}$= 0.06, p=0.95), *Ps1* ($t_{(12)}$= -0.15, p=0.90), *Ps2* ($t_{(12)}$= 0.02, p=0.98), *Tace* ($t_{(12)}$= 0.13, p=0.90), *App* ($t_{(12)}$= -0.09, p=0.92), *Il-1α* ($t_{(12)}$=0.01, p=0.99), *Il-1β* ($t_{(12)}$=-0.25, p=0.80) and *Tnf-α* ($t_{(11)}$=-0.05, p=0.96).

	CT	SD
Glucose (mg/dL)	183.06 ± 6.96	178.66 ± 10.00
Cholesterol (mg/dL)	162.60 ± 1.00	165.50 ± 0.86
Triglycerides (mg/dL)	238.40 ± 12.97	216.08 ± 11.59

CT=control offspring (N=15); SD=Methionine supplement diet during pregnancy and lactation offspring (N=12). The values are presented as the mean ± S.E.M.

Table 2: Blood glucose, cholesterol and triglycerides in offspring PND 90 from dams on supplemented diet during pregnancy/lactation.

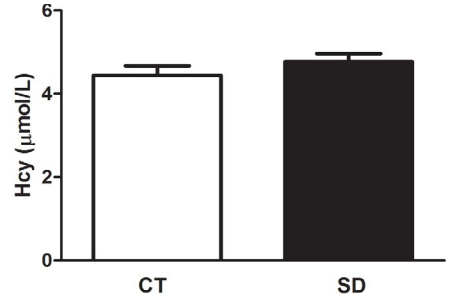

Figure 2: effects of maternal methionine supplementation during pregnancy and lactation on plasma Hcy concentration in offspring

	CT	SD
Adam10	1.03 ± 0.08	1.04 ± 0.13
Bace1	1.03 ± 0.08	1.03 ± 0.11
Bace2	1.18 ± 0.24	1.15 ± 0.26
Ps1	1.02 ± 0.07	1.04 ± 0.12
Ps2	1.02 ± 0.07	1.02 ± 0.08
Tace	1.04 ± 0.10	1.02 ± 0.09
App	1.01 ± 0.05	1.02 ± 0.10
Il-1α	1.05 ± 0.12	1.05 ± 0.14
Il-1β	1.06 ± 0.14	1.13 ± 0.20
Tnf-α	1.05 ± 0.12	1.06 ± 0.15

CT=control offspring (N=8); SD=methionine supplement diet during pregnancy and lactation offspring (N=6). The values are presented as the mean ± S.E.M.

Table 3: Gene expression in offspring PND 90 from dams on supplemented diet during pregnancy/lactation.

Discussion

A significant effort has been made to discover the role of genetic factors in the etiopathogenesis of AD, especially those factors related to Aβ processing. However, only recently has attention turned to evaluating the contribution of epigenetic alterations acquired during fetal and neonatal development. This research effort has been motivated by the recognition of the influence maternal nutrition has on fetal programming and by the fact that its consequences generally arise later in life [25].

During pregnancy, the methionine pathway is crucial for embryonic development, participating in many processes, including DNA and RNA synthesis, DNA methylation, and antioxidant defense [26]. Moreover, alterations in this pathway have been widely shown to be related to congenital malformations [27]. Studies on prenatal manipulation of this pathway and its consequences for the offspring have analyzed behavioral, biometric, biochemical or gene expression parameters [28-31]. In our study, we aimed to assess the impact of hHcy on male offspring by methionine supplementation during pregnancy/lactation in mice.

The supplemented diet increased the Hcy concentration in the dams by approximately 100% but did not seem to affect the methionine-homocysteine metabolism in the offspring in a long-lasting manner, as no differences were observed in plasma Hcy concentrations at PND 90. However, this treatment altered the total body weight of these animals. A decrease in total body weight was found in SD group, but no differences were observed in brain weight. When the brain weight was analyzed as a percentage of total body weight, an increase in relative mass of brain was observed. However, this difference could be attributed to the low body mass. Considering that Hcy increases the risk associated with hyperlipidaemia [32], we investigated if an early exposition to high Hcy concentrations alters blood cholesterol from adult males. No significant changes were observed in blood cholesterol. Additionally, the difference in total body weight between CT and SD groups does not seem to be associated with blood glucose and triglycerides.

In a study from Baydas et al. [33], it was shown that maternal hHcy induced by high methionine diet delays brain maturation and leads to an impairment of learning performance in the offspring. It was also observed that maternal hHcy alters the expression pattern of neural cell adhesion molecules. Therefore, we conjectured that low learning performance could be associated with alterations in the expression of genes involved in AD. However, no differences were observed in *Adam10*, *Bace1*, *Bace2*, *Ps1*, *Ps2*, *Tace*, and *App* expression in the hippocampi of the SD group. An *in vitro* study proposed that genes considered risk factors for AD are regulated by methylation [14]. The expression of BACE, as well as PS1, increased after the reduction of folate and vitamin B12 in the culture medium, with a consequent increase in Aβ production [14]. Another study published by the same group showed similar results when evaluating the effect of vitamin B deficiency in an animal model of AD [34]. Because an increase in SAM concentration is a known effect of a methionine-supplemented diet [35], which in turn could alter the availability of methyl groups, we expected a decrease in gene expression due hypermethylation. However, the evaluated AD risk genes do not appear to have been affected in our study. However, we could not exclude the possibility that these genes were affected early in life and that these marks were subsequently lost during development. On the other hand, genes related to metabolic control can be influenced by methionine supplemented diet because adult animals showed alterations in total body weight. These data are corroborated by another study published by Amaral et

al. [36] that found decreased body weight in 6-week-old rats subjected to a methionine supplemented diet.

Considering the relationship between Hcy and inflammation, we hypothesized that a gestational environment influenced by hHcy could up-regulate inflammatory response genes and consequently affect APP expression and processing. Accordingly, Sudduth et al. [37] found neuroinflammation in hHcy mice that was defined by elevated IL-1b, TNF-a, and IL-6 levels in brain tissue. However, no alterations were observed in *Il-1a*, *Il-1β* and *Tnf-α* gene expression in the hippocampi of offspring on PND 90 in our study.

In conclusion, hHcy induced by methionine supplementation during pregnancy and lactation did not affect the expression of genes related to AD risk in offspring (PND 90). However, as a decrease in body weight was observed in this group, it is possible that genes related to energy metabolism were affected by the supplemented diet during gestational and lactation periods.

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