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Methionine concentration in the diet has a tissue-specific effect on chromosomal stability in female mice

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ABSTRACT

Inadequate nutrient intake can influence the genome. Since methionine is an essential amino acid that may influence DNA integrity due to its role in the one-carbon metabolism pathway, we were interested in whether methionine imbalance can lead to genotoxic events. Adult female Swiss mice were fed a control (0.3% DL-methionine), methionine-supplemented (2.0% DL-methionine) or methionine-deficient (0% DL-methionine) diet over a 10-week period. Chromosomal damage was assessed in peripheral blood using a micronucleus test, and DNA damage was assessed in the liver, heart and peripheral blood tissues using a comet assay. The mRNA expression of the mismatch repair genes *Mlh1* and *Msh2* was analyzed in the liver. The frequency of micronucleus in peripheral blood was increased by 122% in the methionine-supplemented group ($p < 0.05$). The methionine-supplemented diet did not induce DNA damage in the heart and liver tissues, but it increased DNA damage in the peripheral blood. The methionine-deficient diet reduced basal DNA damage in liver tissue. This reduction was correlated with decreased mRNA expression of *Msh2*. Our results demonstrate that methionine has a tissue-specific effect because methionine-supplemented diet induced both chromosomal and DNA damage in peripheral blood while the methionine-deficient diet reduced basal DNA damage in the liver.

1. Introduction

Several food components can modulate DNA methylation in mammals, including methionine, choline, folic acid, and vitamins B6 and B12. Methionine is also a key component in protein synthesis and is necessary for normal growth and development (Finkelstein et al., 1988). Methionine is an essential amino acid that can only be obtained from the diet and is considered the major donor of methyl groups for the methylation of cytosine (Waterland, 2006). As part of the one-carbon metabolism cycle, methionine is converted to S-adenosylhomocysteine by an ATP-dependent process that transfers the adenosine to the methionine by adenosyltransferase (Cantoni, 1975). Thus, excessive or low methionine intake can induce alterations in DNA methylation (Niculescu and Zeisel, 2002), and emerging evidence suggests that alteration to DNA methylation may promote genomic instability (Arai and Kanai, 2010; Calvisi et al., 2007; Kanai, 2010; Sawan et al., 2008).

Methionine intake can be increased with the use of dietary supplements. Gahche et al. (2010) reported that more than half of the adult population of the United States consumes dietary supplements. Methionine intake can also be increased through diet. Chambers et al. (1999) demonstrated an increase in the plasmatic concentration of homocysteine, a methionine metabolite that plays a pivotal role in one-carbon metabolism, as a result of high methionine intake from eating a meal high in animal protein. In addition, a methionine supplementation study reported increases in iron and lipid peroxide levels in rat livers (Mori and Hirayama, 2000). Conversely, it has been suggested that methionine restriction simulates protein restriction, which is associated with lowered oxidative stress and increased life span (Sun et al., 2009). Thus, the low-methionine content of vegan diets has been proposed as a strategy for increased life expectancy in humans (McCarty et al., 2009).

It is widely accepted that inadequate dietary levels of nutrients result in genomic instability (Ferguson, 2010). Micronucleus frequency has been extensively used as a biomarker to assess chromosomal damage in organisms exposed to nutrient deficiency or excess (Thomas et al., 2011). Micronucleus formation occurs when

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chromosomal fragments or whole chromosomes that have lagged during anaphase are not incorporated into the nucleus of the daughter cells after nuclear division (Fenech, 2007). DNA damage can be evaluated by means of the comet assay (Ostling and Johanson, 1984). In this technique, the intensity of DNA damage is measured by the extent of DNA migration in cells immobilized in agarose under electrophoresis (Azqueta and Collins, 2013).

Some studies have reported the effects of an imbalanced intake of folic acid and vitamin B12, which play a critical role in the one-carbon metabolism cycle, on genomic instability *in vitro* and *in vivo* (Bull et al., 2012; Duthie et al., 2000; Fenech, 2012; Lazalde-Ramos et al., 2012; Swayne et al., 2012). Uccella et al. (2011) demonstrated that the use of supplements containing folic acid and vitamins B2, B6, and B12 was associated with an increased risk of type II endometrial cancer in women. Wang and Fenech (2003) reported that lymphocytes in women showed an increased frequency of micronuclei when cell cultures were treated with low concentrations of folic acid. In addition, Razzak et al. (2012) demonstrated that methionine intake was inversely associated with a risk of cancer in women. However, there have been no comprehensive studies performed in female mice on the influence of methionine content in the diet on chromosomal instability.

Methyl-deficient diets can induce DNA damage (Pogribny et al., 1995). It was recently demonstrated that diet can alter the methylation status of the mutL homolog 1 (*Mlh1*) DNA repair gene, and this alteration was accompanied by a significant correlation between the occurrence of DNA strand breaks and DNA methylation level at promoter *Mlh1* (Switzeny et al., 2012). Sidelnikov et al. (2010) reported as well that expression of *MLH1* and the mutS homolog 2 (*MSH2*) can be influenced by dietary factors. It was also reported that genomic instability can increase when DNA repair genes are abnormally expressed (Khanna and Jackson, 2001).

The effects of methionine supplementation have been investigated extensively not only in liver and peripheral blood cells, but also in heart tissue, due to the well-known toxic effect of its metabolite, homocysteine, on the heart (Humphrey et al., 2008). To assess the effects of different concentrations of methionine in the diet on genomic instability, we analyzed chromosomal damage in peripheral blood and DNA damage in the peripheral blood, liver, and heart tissues of female mice fed diets supplemented with or deficient in methionine for 10 weeks. We also investigated the mRNA expression of two mismatch repair genes, *Mlh1* and *Msh2*, in liver tissue. Our results suggest that methionine has a tissue-specific effect. Compared to the control diet, the methionine-supplemented diet induced genomic instability in peripheral blood, while the methionine-deficient diet reduced basal DNA damage in the liver.

2. Materials and methods

2.1. Chemical agents and reagents

Acridine orange (CAS 10127-02-3), Trypan blue (CAS 72-57-1), ethylenediaminetetraacetic acid (EDTA, CAS 60-00-4), Triton X-100 (CAS 9002-93-1), and Tris (CAS 77-86-1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Low melting agarose (CAS 9012-36-6), normal melting agarose (CAS 9012-36-6), TRIzol® and Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (CAS 67-68-5) was purchased from Merck Chemicals (Rio de Janeiro, Brazil). GelRed™ nucleic acid gel stain 10,000× was purchased from Biotium (Hayward, CA, USA).

All other reagents were of analytical grade and the purest quality available. All experiments were carried out in minimal indirect light.

2.2. Animals

Healthy female Swiss albino mice (*Mus musculus*), 4 weeks old and weighing approximately 15 g, were obtained from the "Prefeitura do Campus de Ribeirão Preto" Center at the University of São Paulo, Brazil. The animals were housed in polypropylene cages (six per cage), and the environmental controls were set to maintain conditions of 19–23 °C and 40–70% relative humidity, with a 12-h light–dark cycle. The mice were allocated randomly to each experimental group, and food and fresh water were given *ad libitum*. The diets were provided by Rhoster Industria e Comercio LTDA (São Paulo, Brazil) and were formulated in accordance with AIN-93 recommendations (Reeves et al., 1993). The methionine-supplemented diet was produced by increasing the DL-methionine content of the AIN-93 diet to 2%. The methionine-deficient diet was made by excluding DL-methionine from the mix of amino acids. All other components of the AIN-93 diet were increased proportionally to reach the same caloric content. The 2% test supplementation concentration of methionine was selected based on studies that reported inducing hyperhomocysteinemia in rodents with this dietary concentration of methionine (Jiang et al., 2007; Park et al., 2008). The experimental protocols for this study were approved by the local Ethics Committee for Animal Use (CEUA) of the University of São Paulo, Ribeirão Preto, Brazil (register number 10.1.270.53.0).

2.3. Experimental design

The animals were divided into three groups of six for each treatment: the control group received a diet containing 0.3% DL-methionine; the methionine-supplemented group received a diet containing 2% DL-methionine; and the methionine-deficient group received a diet lacking methionine (0% DL-methionine). The mice were fed the specified diets for 10 weeks, and their body weights were recorded weekly. At the end of the tenth week, the mice were euthanized. They were given access to their food until just before euthanasia. A micronucleus test in peripheral blood and a comet assay with the liver and heart tissues and peripheral blood were performed immediately. Liver tissues (50 mg) were immediately frozen and stored in liquid nitrogen for mRNA expression analysis.

2.4. Micronucleus test of peripheral blood cells

The micronucleus test of the peripheral blood cells was performed according to the protocol described by Hayashi et al. (2000). Blood smears were prepared with 5 μL of peripheral blood. The coded slides were fixed in absolute methanol for 10 min and stained with acridine orange, according to the protocol described by Celik et al. (2005). One thousand polychromatic erythrocytes (PCEs) were scored per animal under 1000× magnification, using a fluorescence microscope (Axiostar Plus®, Carl Zeiss, Gottingen, Germany) equipped with a 488-nm excitation filter.

2.5. Comet assay

The comet assay was performed under pH > 13 alkaline conditions, according to the method of Singh et al. (1988). The absence of possible cytotoxic effects on DNA migration was confirmed using the trypan blue dye exclusion method, which measures the viability of cells (Tice et al., 2000). The liver and heart tissues (0.2 g) were minced in 1 mL of Hank's solution. The peripheral blood (10 μL) or cell suspensions (10 μL) were mixed with 100 μL of 0.5% low melting agarose dissolved in phosphate buffer saline and spread onto microscope slides precoated with 1.5% normal melting agarose. The slides were immersed in freshly prepared lysis solution consisting of 2.5 M NaCl, 100 mM EDTA, 10% dimethyl sulfoxide, 1% Triton X-100, and 10 mM Tris, pH 10, for 60 min at 4 °C. Prior to electrophoresis, the slides were immersed in alkaline buffer (4 °C, pH > 13) for 20 min, to allow unwinding of the DNA. Electrophoresis was performed for 20 min at 0.78 V/cm (25 V and 300 mA) at 4 °C. The slides were then washed in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min. Each slide was randomly coded, stained with a 1:10,000 (v/v) solution of GelRed™ and water, and immediately analyzed. A total of 100 nucleoids per animal were randomly examined at 400× magnification under the fluorescence microscope, equipped with 515–560-nm excitation filter and a 590-nm barrier filter. The software Comet Assay IV™ version 4.3 (Perceptive Instruments, Bury St. Edmunds, Suffolk, UK) was used to measure DNA damage, using the Tail Intensity parameter, which represents the percentage of fragmented DNA in the comet tails. The data average was expressed as a percentage of the DNA damage.

2.6. Isolation of total RNA from liver samples

Total RNA was isolated using TRIzol® according to the manufacturer's instructions. Briefly, 50 mg of frozen liver tissue was powdered with liquid nitrogen, homogenized in 1 mL TRIzol®, and incubated for 10 min at room temperature (RT) to allow complete dissociation of the nucleoprotein complexes. After the addition of 200 μL of pure chloroform, the mixture was vigorously shaken for 15 s and allowed to stand for 5 min at RT. After 15 min of centrifugation at 16,000 g and 4 °C, the upper phase was mixed with 1.5 volumes of 100% ethanol. The purification of extracted total RNA was performed with an SV Total RNA Isolation System® (Promega, Madison, WI, USA) according to the manufacturer's instructions, including a treatment with DNase. RNA was eluted in a final volume of 100 μL RNase free water for qualitative and quantitative analyses, using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).