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NUTRITION

Effects of zinc deficiency and zinc supplementation on homocysteine levels and related enzyme expression in rats

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ABSTRACT

Methionine synthase (MS) and betaine-homocysteine methyltransferase (BHMT) are both zinc (Zn)-dependent methyltransferases and involved in the methylation of homocysteine. The objective of this study was to investigate the effects of dietary Zn supply on homocysteine levels and expression of the two enzymes in growing rats. Male weanling Sprague-Dawley rats were assigned randomly to four dietary groups ($n = 8$/group) for 3 weeks: Zn deficient (ZD; <1 mg Zn/kg); Zn control (ZC; 30 mg Zn/kg); Zn supplemented (ZS; 300 mg Zn/kg); pair fed (PF; 30 mg Zn/kg) to the ZD group. Serum and femur Zn concentrations were 83% and 58% lower in ZD, and 49% and 62% higher in ZS compared to ZC ($P < 0.001$), respectively. The ZD rats had lower feed intake (37%), body weight gains (45%), liver (43%) and kidney (31%) weights than those of ZC ($P < 0.001$), but these parameters in ZD were not significantly different from the PF controls. Serum homocysteine concentrations were 65% higher in ZD compared to PF ($P < 0.05$), and there was no significant difference in serum folate levels between ZD and PF groups. The mRNA expression of liver and kidney *MS* was 57% and 38% lower in ZD than PF ($P < 0.001$), respectively. Hepatic and renal *BHMT* mRNA levels were not altered in ZD compared to controls. The aforementioned measurements were not significantly different between ZS and ZC groups, except Zn levels. These results demonstrated that homocysteine homeostasis appeared to be disturbed by Zn deficiency but not Zn supplementation, and elevated serum homocysteine might be due to reduced expression of MS during Zn deficiency.

Introduction

Zinc (Zn) is an essential micronutrient for humans and animals, playing a critical role in numerous enzyme reactions [1]. Zn deficiency results in anorexia and growth retardation in animals [2]. In addition, an inverse association between heart health and Zn status has been demonstrated in humans [3].

Homocysteine (Hcy) is a sulfur containing amino acid and normally present in human plasma at low micromolar concentrations. However, elevated plasma Hcy concentrations have important implications for human health and disease. Clinical studies have shown that hyperhomocysteinemia (HHcy) is strongly associated with increased risk of cardiovascular disease, Alzheimer's disease and other neurological diseases [4,5]. Hcy is metabolized through transsulfuration and remethylation pathways. In the remethylation pathway, cobalamin-dependent methionine synthase (MS; EC 2.1.1.13) is an important metalloenzyme responsible for the biosynthesis of methionine. It catalyzes a methyl transfer from 5-methyltetrahydrofolate to Hcy by using a Zn ion to activate the Hcy substrate. Another alternate remethylation reaction is regulated by betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5) which is also a Zn metalloenzyme. It catalyzes a methyl transfer from betaine to Hcy forming dimethylglycine and methionine, respectively. Remethylation via MS occurs in all mammalian cells, however, remethylation reaction via BHMT is not traceable in all the tissues. In humans, BHMT mRNA is primarily isolated to liver and kidney [6]. BHMT and MS exert important effects on the cellular and plasma levels of Hcy in mammals [7].

There are few studies reporting the influence of Zn status on the metabolism of Hcy. Increased hepatic MS activity and decreased plasma Hcy and folate concentrations were found in Zn-deficient rats compared with controls [8]. A recent study suggested that Zn

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supplementation did not alter plasma Hcy and red blood cell folate concentrations in aging people [9]. Taken together, further studies are required to clarify the exact role of zinc in the metabolism of Hcy and the mechanisms involved in the reactions. Given the fact that MS and BHMT are involved in the remethylation of Hcy, and they are both zinc-dependent metalloenzymes, it could be hypothesized that zinc can participate in the regulation of the Hcy pathway via mediating MS and BHMT. As a result, the objective of the present study was to investigate the effects of dietary Zn supply on serum Hcy and folate concentrations, and mRNA expression of *MS* and *BHMT* in liver and kidney of growing rats.

Materials and methods

Animals and diets

Male weanling Sprague-Dawley rats (3 weeks old; Charles River Laboratories, St Constant, PQ, Canada), were housed individually in stainless steel hanging cages under a controlled environment (21–23 °C, 55% humidity, 14 h light–10 h dark cycle) with free access to distilled-deionized water in Zn-free plastic bottles. After a 5–7 day acclimatization period on the control diet, the rats were randomized into four dietary groups ($n = 8$/group): Zn control diet (ZC; 30 mg Zn/kg), Zn deficient diet (ZD; <1 mg Zn/kg), Zn supplemented diet (ZS; 300 mg Zn/kg) which were all provided ad libitum, and a pair-fed (PF) control that received the ZC diet in amounts consumed by a ZD weight-matched partner on the previous day. The PF group was designed to control for the effects of undernutrition commonly seen in Zn deficiency. The basal diet was a semi-synthetic, nutritionally complete diet based on the AIN-93G formulation with minor modifications [10]. The composition (per kg diet) of the ZC diet was as follows: dextrose, 600 g; egg albumin, 212.5 g; cellulose, 50 g; mineral mix, 35 g (AIN-93G-MX, Zn free); vitamin mix, 10 g (AIN-93-VX); soybean oil, 70 g; biotin premix, 10 g (contained 200 mg biotin/kg dextrose); choline, 2.5 g; Zn premix, 10 g (contained 5.775 g $ZnCO_3$/kg dextrose). The ZD and ZS diets contained 610 g and 510 g dextrose, respectively, and 0 and 100 g Zn premix, respectively. All ingredients were purchased from Dyets (Bethlehem, PA, USA). Rats were fed for 3 weeks, during which time feed intake and body weight were recorded.

After a 3-week feeding period, rats were killed by CO_2 asphyxiation and decapitation. Trunk blood was collected to obtain serum, and stored at −80 °C until analysis. The liver and kidney samples were excised and weighed. The liver, kidney and femur samples were flash frozen in liquid nitrogen and stored at −80 °C prior to Zn analysis. Additionally, approximately 100 mg samples of liver and kidney were collected into RNA*later*® solution (Life Technologies Inc., Burlington, ON, Canada) for later gene expression analysis. All animal care and treatment protocols received approval from the University of Manitoba's Protocol Management and Review Committee, and followed the guidelines established by the Canadian Council on Animal Care (1984) [11].

Determination of Zn, folate and Hcy concentrations

Serum Zn concentrations were analyzed by atomic absorption spectroscopy using a Perkin Elmer 3100 flame atomic absorption spectrometer. Serum folate concentrations were measured by a gamma counter system (2480 Automatic Gamma Counter, PerkinElmer, Woodbridge, ON, Canada) with a commercial radioimmunoassay kit (SimulTRAC-S Radioassay Kit Vitamin B_{12} [^{57}Co]/Folate [^{125}I] (MP Biomedicals, Orangeburg, NY, USA). Serum Hcy concentrations were measured by reverse-phase HPLC with fluorescence detection [12,13]. For tissue Zn analysis, femurs, and portions of liver and kidney, were placed a drying oven at 85 °C for 48 h to obtain dry weight. Dried samples were digested with 70% trace-metal grade nitric acid for 1 h at room temperature and then 2 h at 85 °C in a DigiPREP Jr. block digestion system (SCP Science, Baie D'Urfe, QC). After appropriate dilution, Zn concentrations were measured using simultaneous inductively coupled plasma-optical emission spectrometry (Varian ICP, Model-VISTA-MPX, CCD, Lexington, MA).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the liver and kidney using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada). RNA quantity and purity were checked by employing the relative UV absorbance expressed by the A_{260}/A_{280} ratios on a DU800 Spectrophotometer (Beckman Coulter Canada Inc., Mississauga, ON, Canada). Subsequently, the RNA samples were treated with the Ambion® TURBO DNA-free™ kit (Life Technologies Inc., Burlington, ON, Canada), in order to remove genomic DNA contamination. cDNA was prepared from 1 μg total RNA using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies Inc., Burlington, ON, Canada) and diluted with nuclease-free water (1:10, v/v), and then used for the detection of gene expression. The mRNA expression of the genes was analyzed by SYBR green real time PCR. Primers for the *BHMT*, *MS* and *Actb* (encoding β-actin) were purchased from QuantiTect Primer Assays (Qiagen, Mississauga, ON, Canada), and the catalog numbers were QT01830885, QT00194901 and QT00193473, respectively. The amplicon length (bp) of *BHMT*, *MS* and *Actb* was 174, 88 and 145, respectively. Conditions for the qRT-PCR were 95 °C for 20 s and 40 cycles of denaturation at 95 °C for 3 s, and combined annealing and extension at 60 °C for 30 s, then followed by one three-segment cycle of product melting (95 °C/15 s, 60 °C/1 min, 95 °C/15 s) which confirmed the specific amplification of the genes. Samples were run in duplicate in 20-μL reactions using a StepOne™ Real-Time PCR System (Life Technologies Inc., Burlington, ON, Canada). Data were normalized to *Actb* controls, and results were analyzed using the $\Delta\Delta Ct$ method to calculate relative quantities. ZC served as a control in the calculation. The expression stability of *Actb* was checked in the present study. Based on technical validation criteria [14], the average ΔCt from the mean should be lower than 1 among samples. Our results showed that the average ΔCt from the mean was 0.54 in the all analyzed samples, demonstrating the stability of *Actb* under dietary treatments and across tissues.

Statistical analysis

Data were analyzed by one-way ANOVA using the SPSS 13.0 software for Windows (SPSS Inc., USA). Significant differences between groups of means were determined using Duncan's multiple range test. Pearson correlation coefficients (r) were used to examine the correlation between each of the two variables. A probability of $P < 0.05$ was considered significant [15]. When evidence of unequal variances was present, data were log transformed before analysis, but non-transformed means are presented.

Results

Feed intake, body and organ weights

After 3 weeks of feeding, daily feed intake, body weight gains, liver and kidney weights were 37%, 45%, 43% and 31% lower in ZD, and 39%, 44%, 49% and 36% lower in PF ($P < 0.001$), respectively, compared to ZC. When adjusted for body weight, the ZD group had 10% ($P < 0.001$) lower relative liver weights (%), and 11% ($P < 0.01$) greater relative kidney weights (%) than the ZC group. The aforementioned observations were not altered by ZS compared to ZC. When compared to PF group, feed intake, body weight gains and