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Oxidative damage and antioxidant defense in thymus of malnourished lactating rats

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ABSTRACT

Objective: Malnutrition has been associated with oxidative damage by altered antioxidant protection mechanisms. Specifically, the aim of this study was to evaluate oxidative damage (DNA and lipid) and antioxidant status (superoxide dismutase [SOD], glutathione peroxidase [GPx], and catalase [CAT] mRNA, and protein expression) in thymus from malnourished rat pups.
Methods: Malnutrition was induced during the lactation period by the food competition method. Oxidative DNA damage was determined quantifying 8-oxo-7, 8-dihydro-2'-deoxyguanosine adduct by high-performance liquid chromatography. Lipid peroxidation was assessed by the formation of thiobarbituric acid-reactive substances. Levels of gene and protein expression of SOD, GPx, and CAT were evaluated by real-time polymerase chain reaction and Western blot, respectively. Antioxidant enzyme activities were measured spectrophotometrically.
Results: Oxidative DNA damage and lipid peroxidation significantly increased in second-degree (MN-2) and third-degree malnourished (MN-3) rats compared with well-nourished rats. Higher amounts of oxidative damage, lower mRNA expression, and lower relative concentrations of protein, as well as decreased antioxidant activity of SOD, GPx, and CAT were associated with the MN-2 and MN-3 groups.
Conclusions: The results of this study demonstrated that higher body-weight deficits were related to alterations in antioxidant protection, which contribute to increased levels of damage in the thymus. To our knowledge, this study demonstrated for the first time that early in life, malnutrition leads to increased DNA and lipid oxidative damage, attributable to damaged antioxidant mechanisms including transcriptional and enzymatic activity alterations. These findings may contribute to the elucidation of the causes of previously reported thymus dysfunction, and might explain partially why children and adults who have overcome child undernourishment experience immunologic deficiencies.

Introduction

Malnutrition is a complex syndrome composed of multiple nutritional deficiencies [1]. It is defined as an imbalance between food intake (protein and energy) and the ingested amount that the body requires to ensure the most favorable growth and function [2]. Childhood malnutrition is a major global health

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problem, contributing to an increased morbidity and mortality rate. Globally, about one in four children age <5 y is malnourished [3]. More than 100 million children worldwide suffer from undernourishment [4], and it is the underlying cause of death in one-third of all children age <5 y [5]. The interaction between malnutrition and infection creates a potentially vicious cycle of worsening illness and deteriorating nutritional status and immune response [3,6].

It has been proposed that severe malnutrition is associated with differential increased levels of DNA damage, depending on each tissue's structural and functional characteristics [7]. Severe infection and drug treatments are other factors related to increased DNA damage in malnourished children [8,9]. Additionally, malnutrition has been associated with decreased DNA repair capacity [10]. Moreover, severe malnutrition has been linked with increased lipid peroxidation (LPx) in children's erythrocyte and serum [11], as well as in rat's brain, liver, kidneys, lungs, and heart [12]. Some of the factors that have been associated with increased LPx are high levels of nonheme iron and polyunsaturated fatty acids in red cell membranes [13], as well as decreased plasma antioxidants concentrations [14].

In studies related to childhood malnutrition, decreased levels of free-radical scavenging molecules such as vitamins E and C have been found, as well as ceruloplasmin, superoxide dismutase (SOD), and glutathione peroxidase (GPx) [15,16], which correlated with increased oxidized proteins levels [17].

It is well known that malnutrition causes changes in lymphoid organs, with the thymus being one of the most vulnerable. For this reason, the thymus is regarded as a malnutrition barometer [18]. In malnourished organisms, the thymus suffers a variety of alterations such as severe atrophy, mainly associated to mature thymocytes depletion due to a hormonal imbalance involving decreased leptin levels that results in elevated serum corticosterone. Additionally, histologic and morphologic changes have been observed by increased fibronectin, laminin, and collagens [19,20], and the distinction between cortical and medullar areas were lost [21]. In relation to thymic atrophy, abnormally low levels of thymulin hormone production and proliferation have been observed [19,22], along with cell subsets modifications, increased apoptosis levels [23], and alterations in antioxidant defense mechanisms [24]. All these factors suggest that the functions of the thymus, and therefore the continuous T-cell supply, might be affected by malnutrition.

Hence, the aim of this study was to analyze the oxidative stress status in lactating malnourished rat thymus by evaluating DNA and lipid oxidative damage as well as classic antioxidant enzymes (SOD, GPx, and catalase [CAT]) levels and activity. Our results showed that thymus redox state is severely compromised in malnourished rat pups, mainly in third-degree malnourishment (MN-3) but also in second-degree (MN-2). This data might explain why children and adults who have overcome child undernourishment experience immunologic deficiencies.

Materials and methods

Animals

Wistar albino nursing rats from the closed breeding colony at the Universidad Autónoma Metropolitana-Iztapalapa (UAM-I) were maintained under standard conditions (12-h light/12-h dark, temperature 22°C ± 3°C, with 45% relative humidity). Nursing rats were fed with a rodent's balanced diet (Purina Mills International 5001, Richmond, VA, USA) and filtered water ad libitum. Animals were bred in acrylic boxes with beds (Betachips, Northeastern Products Corp, Warrensburg, NY, USA). All procedures with animals were strictly carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Principles of the Mexican Official Ethics Standard 062-ZOO-1999.

Experimental malnutrition

Experimental malnutrition was induced during lactation by the food competition method, which was based on reduction of the quantity of milk per pup by increasing the number of pups per nursing mother [25]. One-d-old Wistar rats from different litters were randomly assigned either to the control or to the experimental group. In the control group, five pups were assigned to nursing mothers. In the experimental group, each nursing mother fed 15 pups. The same proportion of male and female pups per litter was assigned to each group. Pups were weighed every 3 d, and mean body weight was estimated for each litter from day 0 until weaning (day 21). At the end of weaning, according to a previously proposed classification [26], rats were considered as first degree (MN-1) when body-weight deficit was between 10% and 24%, MN-2 body-weight deficit was between 25% and 39%, or MN-3 when body-weight deficit was >40% compared with age-matched well-nourished (WN) pups. These rats also had other physical signs of malnutrition, such as sparse hair, bone fragility, and low activity levels.

At day 21, pups were sacrificed by cervical dislocation; the thymus was removed and stored at −70°C until analysis.

8-oxo-7, 8-dihydro-2'-deoxyguanosine analysis by HPLC/electrochemical detection

Thymus DNA was isolated using a previously modified chaotropic-NaI method [27,28]. This technique avoids DNA oxidation during the isolation process. DNA concentration was determined spectrophotometrically at 260 nm and its purity was assessed by ensuring that the A_{260}/A_{280} ratio was >1.75. DNA was enzymatically digested with nuclease P1 and *Escherichia coli* acid phosphatase. One hundred micrograms of digested DNA was injected into a high-performance liquid chromatography/electrochemical (EC) system consisting of a Waters 600 C pump (Waters, Milford, MA, USA) connected to a Supelcosil LC-18 (Supelco, Bellefonte, PA, USA) reverse-phase column (250 × 4.6 mm i.d., particle size 5 μm). The isocratic eluent was 50 mM potassium phosphate buffer, pH 5.5, with 8% methanol at 1 mL/min flow rate. EC detection was performed by an INTRO detector (Antec Leyden, Leiden, The Netherlands) operated at 290 mV. Elution of unmodified nucleosides was simultaneously monitored by a 486 Waters UV spectrometer set at 254 nm. The molar ratio of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodGuo) to desoxyguanosine (dGuo) in each DNA sample was determined based on EC detection at 290 mV for 8-oxodGuo and absorbance at 254 nm for dGuo.

Lipid peroxidation assay

Lipid peroxidation (LPx) was assessed by the thiobarbituric acid-reactive substances (TBARS) formation, according to a previous report [29]. For these assays, 100 mg of thymus tissue was processed with an OMNI TH Homogenizer in NaCl 0.9% 1 mL, and 200 μL of this solution were immediately mixed with 2 mL thiobarbituric acid (TBA) reagent (containing TBA 0.075 g + trichloroacetic acid 2.25 mL + HCl 397.8 μL, in a 15-mL final volume), and incubated in a boiling water bath (94°C) for 20 min. Samples were kept on ice for 5 min and centrifuged at 3000*g* for 15 min. The optical density was estimated in supernatants at 532 nm. Protein concentration was determined using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA) [30], and a 1.41 mg/mL standard of bovine serum albumin (BSA). Malondialdehyde (MDA) concentrations, expressed as μmol of MDA/mg of protein, were calculated by interpolation on a standard curve.

Gene expression analysis

Thymus samples obtained from organisms recently sacrificed were lysed in a buffer containing β-mercapto-ethanol, and RNA purification was performed using the commercial kit SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA obtained was fractionated into aliquots and stored at −70°C until further use. RNA was spectrophotometrically quantified (Thermo Electron Corporation, Madison, WI, USA), and 5 μg RNA were separated on a 1% agarose gel containing ethidium bromide in tris-acetate-EDTA buffer of total RNA revealed that all RNA samples were intact and suitable as templates for reverse transcription. To prevent trace amounts of DNA contamination, RNA samples were treated with amplification grade DNase I (Invitrogen, Carlsbad, CA, USA) before reverse transcription. 0.5 μg of RNA were used for reverse transcription with oligo-d(t) primers in 20 μL reaction volumes using Superscript III reverse transcriptase (Life Technologies, Rockville, MD, USA), and reactions were performed in an Eppendorf Mastercycler thermocycler (Eppendorf Scientific, Inc., Westbury, NY, USA). The amplified cDNA was quantified on a spectrophotometer at 260 nm. The cDNA samples were stored at −70°C until use. Real-time polymerase chain reaction (PCR) was carried out using Rat Universal ProbeLibrary (Roche