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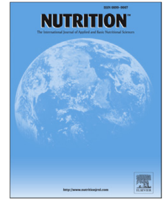

Basic nutritional investigation

Oxidative damage and antioxidant defense in thymus of malnourished lactating rats

Graciela Gavia-García M.Sc. [a,b], Haydeé González-Martínez Ph.D. [c], Ángel Miliar-García Ph.D. [d], Edmundo Bonilla-González Ph.D. [a], María de los Ángeles Rosas-Trejo B.Sc. [a], Mina Königsberg Ph.D. [a], Oralia Nájera-Medina Ph.D. [e], Armando Luna-López Ph.D. [f], María Cristina González-Torres Ph.D. [a,*]

[a] Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, México City, Mexico
[b] Posgrado en Biología Experimental, Universidad Autónoma Metropolitana-Iztapalapa, México City, Mexico
[c] Centro de Nanociencias y Micro y Nanotecnologías, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, México City, Mexico
[d] Sección de Estudios de Posgrado, Escuela Superior de Medicina, Instituto Politécnico Nacional, México City, Mexico
[e] Departamento de Atención a la Salud, Universidad Autónoma Metropolitana-Xochimilco, México City, Mexico
[f] Departamento de Investigación Básica, Instituto Nacional de Geriatría, México City, Mexico

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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.
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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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* Corresponding author. Tel.: +52 55 5804 4729; fax: +52 55 5804 4727.
E-mail address: mcgt@xanum.uam.mx (M. C. González-Torres).

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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

Diagnostics GmbH, Mannheim, Germany). Specific oligonucleotide primers were generated by using the online software (ProbeFinder; http://www.roche-appliedscience.com/sis/rtpcr/upl/index) [31]; the primer sequence for each gene is shown in Table 1. The gene expression levels were determined using real-time PCR. The 20 μL reaction mixture contained 1 × LightCycler Taqman Master reaction mixture (Roche Diagnostics), 200 nM of each primer, 100 nM of Universal ProbeLibrary probe, 0.5 U LightCycler Uracil DNA Glycosylase (Roche Diagnostics), and 2 μL of standard DNA in appropriate dilutions. The thermal cycling parameters were as follows: one cycle at 95°C for 60 sec to denature the template and to perform a "hot start," followed by 40 three-segment cycles to amplify the specific PCR product (95°C for 10 sec, the primer-specific annealing temperature for 30 sec, and product elongation at 72°C for 18 sec) and was terminated with one cycle at 40°C for 30s. The amplification was performed in borosilicate glass capillaries (Roche Diagnostics). The real-time PCR assay included serial log dilutions of the cDNA to generate a standard curve for each gene. The mean crossing threshold (Ct) of each gene was normalized to the mean Ct of the housekeeping gene β-actin. The data were analyzed using LightCycler® Software version 4.0 (Roche Diagnostics) [32].

Western blot analysis

One hundred mg of thymus tissue were processed with an OMNI TH Homogenizer. Proteins were extracted from it using a T-PER® Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) with protease inhibitor complete, Mini (Roche Diagnostics). The homogenates were incubated at 4°C for 10 min and centrifuged at 11 200g, 4°C, for 20 min. Total protein concentration in the supernatant was determined spectrophotometrically at 595 nm using a commercial Bradford reagent and standard curve of BSA.

Proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA) and probed with anti-SOD, anti-GPx, or anti-CAT (Santa Cruz Biotechnology, Santa Cruz, CA, USA) dilution (1:1000). Membranes were washed three times with TBS–Tween and incubated with a horseradish peroxidase-conjugated secondary antibody dilution (1:1000; Santa Cruz Biotechnology) for 1 h. After three consecutive washes, the blots were developed using a commercial chemiluminescence reagent (Merck KGaA, Darmstadt, Germany). The proportion of these proteins was quantified by densitometric analysis, using Kodak Molecular Imaging Software (v.4.5.1).

Antioxidant enzyme activity

To evaluate antioxidant enzyme activity, 100 mg of thymus of each rat were processed with an OMNI TH Homogenizer. SOD activity was evaluated spectrophotometrically at 560 nm, through the xanthine/xantine oxidase system, based on protocols previously proposed [33,34]. GPx activity was assessed spectrophotometrically at 340 nm through nicotinamide adenine dinucleotide phosphate monitoring of protocol previously described [35]. CAT activity was quantified using a previously established protocol that evaluates H_2O_2 decrease at 240 nm [36].

Statistical analysis

In all cases, statistically significant differences among groups were established using the statistical test Duncan. P values ≤0.05 were considered significant differences. Data are presented as mean ± SE and samples were prepared in triplicates for each treatment. To determine the association among the obtained data the Spearman correlation coefficient was used. All analyses were performed using the statistical program NCSS 2001 for Windows Version 2.0 software (NCSS Statistical Software, Kaysville, UT, USA).

Table 1
Detailed primers and conditions used for real-time PCR assays

Gene	Primer name	Primer sequence	Annealing temp (°C)	Product Amplicon size (bp)
SOD1	SOD-L	GGTCCAGCGGATGAAGAG	59	78
	SOD-R	GGACACATTGGCCACACC	60	
GPx	GPx-L	CGACATCGAACCCGATATAGA	59	60
	GPx-R	ATGCCTTAGGGGTTGCTAGG	60	
CAT	CAT-L	ATCAGGGATGCCATGTTGTT	60	74
	CAT-R	GGGTCCTTCAGGTGAGTTTG	59	
β-Actin	β-Actin-L	CCCGCGAGTACAACCTTCT	60	72
	β-Actin-R	CGTCATCCATGGCGAACT	60	

SOD1, copper/zinc-superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase

Results

Animal's physical characteristics

Table 2 shows body weight and thymus weight data of the organisms used in the present study. In malnourished groups, rats exhibited a higher weight deficit compared with WN animals and therefore were considered malnourished. Weight deficits of malnourished rates were established in comparison with the mean body weight of the WN group.

DNA and lipid oxidative damage

The DNA oxidative damage (DOD) and LPx were assessed in WN, MN-1, MN-2, and MN-3 rats ($n = 5$ for each group). Figure 1A illustrates 8-oxodGuo/dG ratios in all groups. No differences in DOD were observed in MN-1 group compared with WN rats, whereas significant increases of 57% and 70% were observed for the MN-2 and MN-3 groups, respectively ($P < 0.05$). The correlation between body and thymus weights showed that decreased body and thymus weights were associated with increased oxidative DNA damage values ($r = -0.755$; $P < 0.01$ and $r = -0.819$; $P < 0.01$, respectively). Figure 1B shows the results obtained for the LPx assay. Again, LPx in the MN-1 group was the same as WN rats, whereas a significant increase was found in the MN-2 and MN-3 groups (63% and 70%, respectively; $P < 0.05$). Once again, the correlation values between body and thymus weights showed that both correlate with increased LPx ($r = -0.657$; $P < 0.01$ and $r = -0.729$; $P < 0.01$, respectively), suggesting that malnourishment might promote oxidative damage in biomolecules.

Antioxidant enzymes mRNA expression

To evaluate the effect of malnutrition on antioxidant enzymes in the thymus, mRNA expression was performed by real-time PCR. The mRNA expression results were standardized with respect to the reference β-actin gene. SOD mRNA expression levels (Fig. 2A) were very low in MN-1 (70% less), MN-2 (94% less), and MN-3 (98% less) rats compared with the WN group ($P < 0.05$). In the MN-1 group, GPx (Fig. 2B) and CAT mRNA expressions (Fig. 2C) remained unchanged with respect to WN rats (control group), whereas GPx mRNA levels in the MN-2 and MN-3 groups decreased 83% and 96%, respectively, compared with WN rats ($P < 0.05$). CAT mRNA expression in both MN-2 and MN-3 groups was 46% less than the control group values ($P < 0.05$).

Table 3 shows the correlation values between DNA and lipid oxidative damage, against SOD, GPx, and CAT mRNA expression levels. Table 3 shows a higher correlation between DOD and SOD mRNA expression ($r = -0.92$; $P < 0.01$); GPx ($r = -0.79$; $P < 0.01$) and CAT ($r = -0.79$; $P < 0.01$) correlation values were similar. Also, Table 3 shows the correlation between LPx and mRNA expression, a high correlation was observed in all cases: *SOD* ($r = -0.819$; $P < 0.01$), *GPx* ($r = -0.708$; $P < 0.01$), and *CAT* ($r = -0.724$; $P < 0.01$). These results suggest that *SOD*, *GPx*, and *CAT* mRNA expression decreased during malnourishment and that this fact is associated with increased DNA and lipid oxidative damage.

Table 2
Characteristics of well-nourished and malnourished rat groups

Weights and deficits	Well-nourished (n = 25)	First-degree malnourished (n = 25)	Second-degree malnourished (n = 25)	Third-degree malnourished (n = 25)
Body weight (g)	54.96 ± 3.41	45.97 ± 1.51*	37.85 ± 1.25*	28.22 ± 1.86*
Weight deficit (%)		16.35	31.13	48.65
Thymus weight (g)	0.25 ± 0.04	0.22 ± 0.05	0.17 ± 0.04*	0.11 ± 0.03*
Thymus deficit (%)		12	32	56

* Statistical significance with respect to control (well-nourished) ($P < 0.05$).

SOD, GPx, and CAT Western blots

SOD, GPx, and CAT protein levels were determined using the total proteins obtained from the thymus of WN, MN-1, MN-2, and MN-3 rats. Representative blots and densitometry data are presented in Figure 3; data were normalized against WN rats. SOD (Fig. 3A), GPx (Fig. 3B), and CAT (Fig. 3C) protein levels decreased 57%, 80%, and 40%, respectively, in the MN-3 group compared with the WN group ($P < 0.05$). Interestingly, and in agreement with the previous data, the MN-3 group presented the lowest antioxidant proteins content compared with the other malnourished groups.

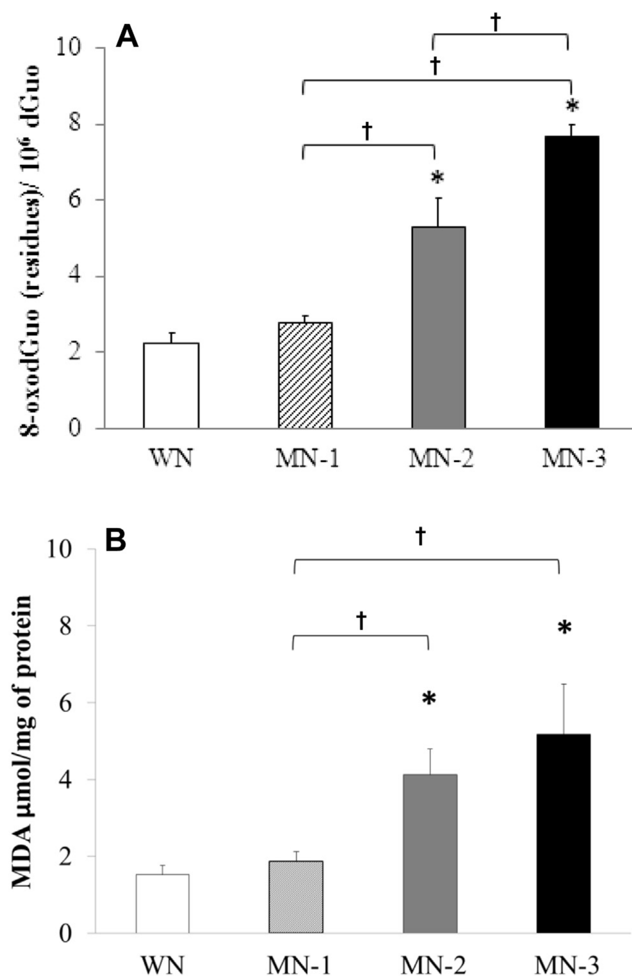

Fig. 1. Oxidative damage in thymus of well-nourished (WN; white bars), first-degree malnourished (MN-1; shredded bars), second-degree malnourished (MN-2; gray bars), and third-degree malnourished (MN-3; black bars) rats. Oxidative DNA damage (A) was quantified as the molar ratio 8-oxodGuo/dGuo, and lipid peroxidation (B) was determined as concentrations of malondialdehyde (MDA)/mg of protein. Each point represents the mean ± SE of five determinations performed in triplicate. *Statistical significance with respect to control (WN) or † between malnourished groups ($P < 0.05$). n = 5.

Antioxidant enzyme activity

SOD, GPx, and CAT activity was quantified in WN, MN-1, MN-2, and MN-3 rats; the results are shown in Figure 4. SOD activity in MN-2 and MN-3 groups (circle in the Figure) diminished (43% and 84%, respectively) compared with the WN group ($P < 0.05$). GPx activity (square in the Figure) also decreased in the MN-2 and MN-3 groups (54% and 81%, respectively) compared with the WN group ($P < 0.05$). Additionally, CAT activity (triangle in the Figure) decreased in all malnourished groups MN-1, MN-2, and MN-3 (44%, 53%, and 73%, respectively) compared with the WN group. In summary, all enzymatic activities analyzed diminished in malnourished organisms and, as expected, the MN-3 group showed the lowest enzymatic activities for all antioxidant enzymes. Interestingly, CAT was the most affected, enzyme followed by GPx and then SOD. Table 3 shows the correlation between DOD and the enzymatic activities of SOD ($r = -0.78$; $P < 0.01$), GPx ($r = -0.43$; $P < 0.01$), and CAT ($r = -0.83$; $P < 0.01$). SOD and CAT enzymatic activities showed a high correlation, whereas GPx presented a moderate correlation in relation to DOD. Table 3 shows correlations between LPx and enzymatic activities of SOD ($r = -0.59$; $P < 0.01$), GPx ($r = -0.27$; $P < 0.01$), and CAT ($r = -0.75$; $P < 0.01$). These results suggest that a diminished antioxidant activity is related to an increased oxidative damage.

To determine whether the decreased enzymatic activity detected was due to a reduction in the enzymatic protein expression, a correlation between both parameters was performed. The results indicated that there is a high positive correlation between GPx enzymatic activity and protein expression ($r = 0.805$), a moderate correlation for SOD ($r = 0.624$), and a weak correlation for CAT ($r = 0.302$). These data support the idea that the lower enzymatic activity observed is associated with a diminished protein expression.

Discussion

To evaluate the effects of malnutrition on children's health, several studies have been performed at different levels. Children and experimental models have been used with this purpose. Malnutrition is related with diminished lymphocyte activation capacity [37] and with alterations in the proportion of immunologic cell subpopulations [38,39]. Additionally, it correlates with greater DNA damage as determined by increased structural chromosomal aberrations and single-strand breaks in peripheral blood lymphocytes [8,40], and in liver and testis of severely malnourished rats during lactation period [7]. Furthermore, it has been reported that different cell types and organs related to the immunologic system, such as spleen and bone marrow, are more susceptible to DNA damage [41].

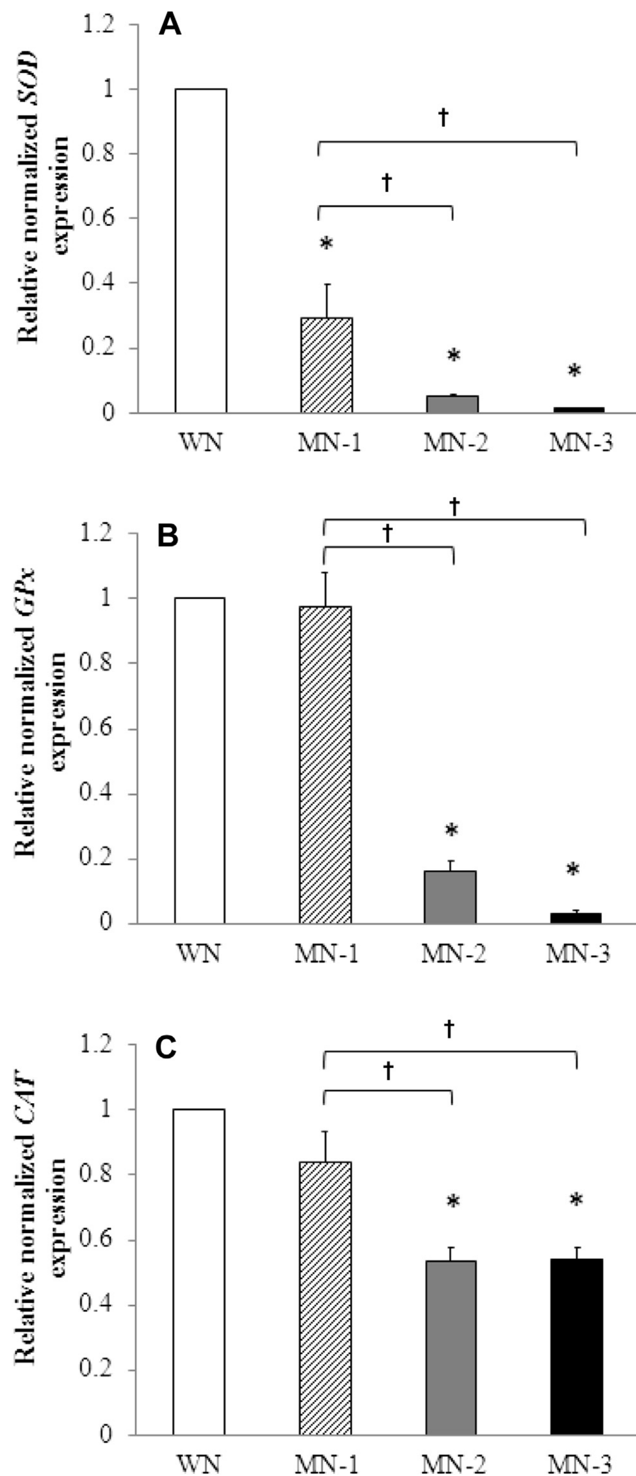

Fig. 2. Antioxidant enzymes mRNA expression levels in malnourished rats' thymus. (A) *SOD*, (B) *GPx*, and (C) *CAT* mRNA expression levels were quantified by real-time polymerase chain reaction in thymus obtained from well-nourished (WN; white bars), first-degree malnourished (MN-1; shredded bars), second-degree malnourished (MN-2; gray bars), and third-degree malnourished (MN-3; black bars) rats. The *SOD*, *GPx*, and *CAT* mRNA relative expression levels were determined after normalization against *β-actin*. Determinations were made in triplicate, and the points in the graph represent the mean ± SE. *Statistical significance with respect to control group (WN) or † among malnourished groups ($P < 0.05$). n = 5. CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase.

Table 3
Correlation between oxidative damage and mRNA expression levels and enzymatic Activity of SOD, GPx, and CAT for all groups

	DNA oxidative damage			Lipid peroxidation		
	SOD	GPx	CAT	SOD	GPx	CAT
mRNA expression levels	−0.92	−0.79	−0.79	−0.81	−0.70	−0.72
Enzymatic activity	−0.78	−0.43	−0.83	−0.59	−0.27	−0.75

SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase $P < 0.01$

Oxidative stress is most likely involved in malnutrition pathophysiology as a result of an imbalance in the production of free radicals and their elimination [42]. It is well known that overproduction of reactive oxygen species (ROS) and/or decreased antioxidant defenses result in molecular oxidative damage [11]. Thus, the idea that oxidative damage (especially DNA damage) might be an outcome of malnourishment was proposed previously [10], but to our knowledge, this is the first report where this is confirmed. DOD determination in malnourished organisms complements previous cellular and cytogenetic damage studies. Here we showed that malnutrition is clearly related with increased DOD in thymus, and that this injury was associated with both reduced gene–protein expression and decreased antioxidant enzymes activity. Oxidative DNA damage was evaluated by 8-oxodGuo adduct quantification, which is an excellent oxidative stress biomarker [43]. In the MN-2 and MN-3 groups, the adduct frequency increased 57% and 70%, respectively compared with the control group. Concentrations of 8-oxodGuo increased depending on the degree of malnutrition, thus confirming that this molecule is a biological oxidative stress marker in malnutrition. Oxidative DNA damage is perhaps the most detrimental consequence of oxidative stress, which might generate harmful consequences in the cells, such as a wide range of chromosomal abnormalities that may block DNA replication, inducing cytotoxicity and cell death [44].

It is known that child malnutrition results in increased LPx in blood, serum [11,45], plasma, brain, liver, kidney, and lungs [46], as well as in different rat cerebral regions [47]. This damage is attributed to a deficiency in trace elements [48], which are required for various enzyme functional activities. It is recognized that even a mild deficiency of these micronutrients is related to failures in antioxidant protection and increased oxidative stress damage [49]. For example, zinc and copper deficiencies are associated with a concomitant LPx increase [45]. LPx products could modify cell membrane ionic permeability by direct lipid damage or by membrane protein damage [13], which is known to be associated with the edema pathogenesis during kwashiorkor malnutrition [17].

The results of the present study demonstrated that malnutrition is clearly related with increased thymus LPx, and in the same manner with DOD; these alterations were associated with both decreased gene–protein expression and antioxidant enzymes activity. MDA levels increased in MN-2 and MN-3 by 63% and 70%, respectively; whereas no significant difference in the MN-1 group was found compared with the WN group. It should be noted that both oxidation products 8-oxodGuo and MDA, augmented in the same percentage (70%) for the MN-3 group compared with WN rats. A negative relationship between body weights and oxidative damage was observed, suggesting that a lack of ≥25% of the body weight have severe repercussions in DNA and lipid oxidative damage, and that oxidative damage increased in relation to weight deficits.

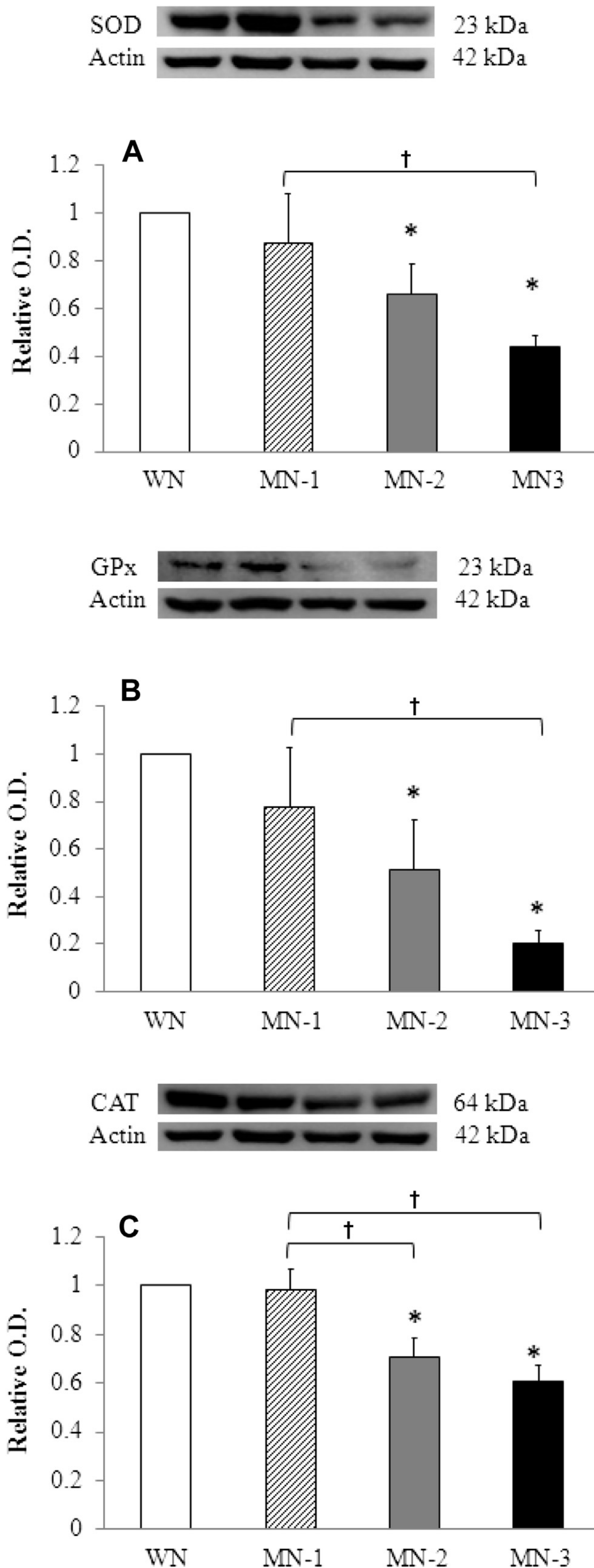

Fig. 3. Antioxidant enzymes in thymus obtained from well-nourished (WN; white bars), first-degree malnourished (MN-1; shredded bars), second-degree malnourished (MN-2; gray bars), and third-degree malnourished (MN-3; black bars) rats. The figure shows representative blots performed for (A) SOD, (B) GPx, and (C) CAT. Relative optic density (O.D.) was normalized against control (WN). Each point represents the mean ± SE of five determinations performed in independent experiments. *Statistical significance with respect to WN or † among malnourished groups ($P < 0.05$). n = 5. CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase.

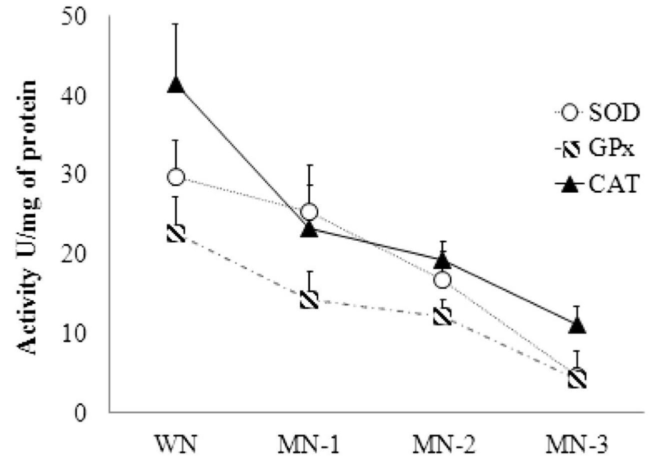

Fig. 4. Antioxidant enzymatic activities assessed in thymus of first-degree malnourished (MN-1), second-degree (MN-2), and third-degree (MN-3) rats. The figure shows the enzymatic activity of (A) SOD (circle), (B) GPx (square), and (C) CAT (triangle). Each point represents the mean ± SE of five determinations performed in independent experiments. MN-2 and MN-3 showed statistical significance with respect to control (WN) ($P < 0.05$). n = 5.

To advance in the understanding of malnutrition effect in the thymus with regard to oxidative stress generation, the expression and activity of three representative enzymatic antioxidants were analyzed (SOD, GPx, and CAT). *SOD* mRNA was decreased in all malnourished groups. These results concur with a similar modulation in the expression of this gene reported in malnourished offspring goat's thymus [24]. Additionally, *GPx* and *CAT* gene expression decreased in the MN-2 and MN-3 groups. Similar results were reported for *CAT* expression in pancreatic islets of malnourished rats during pregnancy [50]. This shows that gene expression modulation is affected by malnutrition (mainly in the MN-2 and MN-3 groups). Likewise, it has been suggested that gene expression modulation may be affected by oxidative stress [44].

To verify whether decreased mRNA expression correlated with diminished protein levels, expression of *SOD, GPx,* and *CAT* were determined by Western blot. Our results showed a significant decrease in SOD, GPx, and CAT protein levels in MN-2 and MN-3 rats compared with the WN group. These results confirmed that a decrease in mRNA expression is indeed associated with a decrease in protein expression in the same groups, which in turn, can be linked to the augmented oxidative damage. Regarding the MN-1, group no differences in SOD levels were observed compared with the control group, which contrasts with the gene expression results obtained for the same degree of malnutrition. This may be due to the individual variability observed or to posttranscriptional and posttranslational splicing or translational modifications that may affect the final mRNA amount and the synthesis of this protein [51].

The long-term significance of thymus oxidative damage might be associated with adverse effects observed in malnourished organisms as a deficient immune response because the thymus is a major site of T-cell maturation and differentiation [19]. Therefore, malnourishment-associated thymus damage

might not only have direct consequences on the thymus, but also may affect other secondary lymphoid organs involved in the immune response [52], thereby enhancing the susceptibility of malnourished children to multiple infectious diseases such as parasitic diseases, diarrhea, and acute respiratory infections [53, 54]. Hence, oxidative damage in this organ could be an underlying factor in the high mortality rates associated with malnutrition [54].

As expected and in concordance with previous studies [24,45], SOD and GPx enzyme activities were significantly decreased in the MN-2 and MN-3 groups compared with the control group. CAT activity was affected in all grades of malnutrition. Based on the above, it can be suggested that malnutrition causes an imbalance in the superoxide radical ($O_2^{-\bullet}$) inactivation systems mediated by SOD and removal of hydrogen peroxide (H_2O_2) mediated by GPx and CAT in the MN-2 and MN-3 groups. This could result in increased generation of more toxic molecules such as the hydroxyl radical ($^{\bullet}OH$) via the Fenton or the Haber-Weiss reactions [55] and possibly be the source of the higher oxidative damage rates observed in MN-2 and MN-3 rats.

The decreased activity of these enzymes could be attributed not only to the low protein levels found, but also to a direct damage by increased ROS production, and/or a trace elements deficiency due to malnutrition. For example, SOD-decreased enzymatic activity is associated with zinc and copper deficiencies [45]. A decline of selenium in plasma is associated with decreased GPx activity in malnourished children [15].

The results of diminished SOD and GPx enzymatic activity correlate with decreased protein levels in MN-1 and MN-2, whereas CAT enzymatic activity and protein levels in the MN-1 group showed no association, suggesting that the activity reduction of this enzyme might not be related due to the protein expression, and might be explained as the result of post-translational modifications [56]. Decreased CAT activity in the MN-1 group is probably being compensated by GPx activity because this enzyme is the major scavenger of H_2O_2 [57] and therefore confers greater protection against oxidative stress that may coincide from the results observed in this degree of malnutrition (MN-1) where no significant differences were found in DNA and lipid oxidative damage compared with the WN group.

It is important to note that many studies have been performed to evaluate the effects of severe malnutrition (MN-3, weight deficits >40%) at cellular and cytogenetic levels [7,9,58], probably because the consequences in children's health are more serious and persistent in this stage (MN-3). However, many of these studies have not yet explored the effect of MN-1 or MN-2, which also might be very important. For this reason, another original contribution of this study was the contribution to the understanding of the antioxidant/oxidant state during MN-1, MN-2, and MN-3 in rats, with MN-2 and MN-3 being the most affected. Further studies are needed to determine which transcription factors activated by ROS may be involved in the malnutrition pathophysiology. Furthermore, the thymus histologic evaluations are of special importance during malnourishment, as they will relate all malnutrition alterations studied so far.

Conclusion

The present research demonstrated for the first time that malnutrition in the early stages of life is strongly associated with increased DNA and lipid oxidative damage, which can be attributed to a decreased enzymatic antioxidant defense status, starting from transcriptional level to enzymatic activity. These alterations may lead to an inability to eliminate ROS, contributing to thymus dysfunction and might be a factor that compromises the immune response in malnourished organisms.

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