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Effects of protein malnutrition on oxidative status in rat brain

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Abstract

Objectives: This study evaluated the effects of protein malnutrition on oxidative status in rat brain areas.

Methods: We investigated various parameters of oxidative status, free radical content (dichlorofluorescein formation), indexes of damage to lipid (thiobarbituric acid-reactive substances assay), and protein damage (tryptophan and tyrosine content) in addition to total antioxidant reactivity levels and antioxidant enzyme activities of superoxide dismutase, glutathione peroxidase, and catalase in different cerebral regions (cortex, hippocampus, and cerebellum) from rats subjected to prenatal and postnatal protein malnutrition (control 25% casein and protein malnutrition 7% casein).

Results: Protein malnutrition altered various parameters of oxidative stress, especially damage to macromolecules. Free radical content was unchanged by protein malnutrition. There was an increase in levels of thiobarbituric acid-reactive substances, the index of lipid peroxidation, in the cerebellum and cerebral cortex ($P < 0.05$) from protein-malnourished rats. Moreover, significant decreases in tryptophan and tyrosine in all tested brain structures ($P < 0.05$) were observed. Catalase activity was significantly decreased in the cerebellum ($P < 0.05$). In addition, a significant decrease in total antioxidant reactivity levels ($P < 0.05$) was observed in the cerebral cortex from protein-malnourished rats.

Conclusions: The present data indicated that protein malnutrition increased oxidative damage to lipids and proteins from the studied brain areas. These results may be an indication of an important mechanism for changes in brain development that are caused by protein malnutrition.

Keywords: Protein malnutrition; Brain; Free radical; Oxidative damage; Antioxidant capacity

Introduction

Malnutrition corresponds to worldwide, severe health and socioeconomic problems. Nutritional inadequacy, including maternal malnutrition, is a major non-genetic factor that leads to disturbances in development of the brain [1]. Some studies have demonstrated that malnourished children have more learning and behavioral problems than do well-nourished children [2].

Many investigators have found biochemical alterations in nervous systems in experimental models of malnutrition, especially those related to neurotransmitter systems [3,4]. Histologic changes also have been demonstrated by decreased neuronal and glial cell density in the cerebral cortex and cerebellum [5] and decreased dendritic spines in the cingulated cortex and hippocampus of malnourished rats [6,7].

It has been suggested that oxidative damage to macro-

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molecules occurs in clinical manifestations of kwashiorkor, a severe form of protein-energy malnutrition that is clinically characterized by edema [8,9]. As first proposed by Golden and Ramdath [10], oxidative stress, an imbalance between free radical generation and tissue antioxidant capacity, is involved in the pathophysiology of kwashiorkor.

Free radicals such as superoxide, hydrogen peroxide, and hydroxyl radicals and alkoxyl and peroxyl radicals are continually generated during oxidative metabolism. An overload of free radicals may oxidize critical biological molecules, producing membranous lipid peroxidation, specific protein damage, and DNA strand breaks [11]. The lipid oxidative damage, lipid peroxidation, is accompanied by generation of aldehyde products such as malondialdehyde and 4-hydroxy-2-nonenal; these are chemically reactive and may covalently modify cellular macromolecules, especially proteins. A highly reactive free radical attacks amino acid residue proteins and an oxidative modification may lead to structural alteration and generally to functional inactivation of enzymes. Oxidation of amino acid side chains greatly depends on their structure. Oxidative modifications occur at specific amino acid residues, such as arginine, lysine, proline, histidine, tryptophan, methionine, tyrosine, and phenylalanine residues [12].

The antioxidant defense system includes enzymes such as superoxide dismutase (SOD), which converts superoxide radicals into H_2O_2, catalase (CAT), which has been shown to be responsible for detoxification of H_2O_2, and glutathione peroxidase (GPx), which breaks down peroxides, notably those derived from the oxidation of membrane phospholipids. Moreover, there are also nonenzymatic antioxidants (carotenoids, vitamin E, and glutathione) with important roles in defense mechanisms [11]. Cellular damage occurs when there is oxidative stress, namely an imbalance between free radical generation and scavenging systems.

The brain may be particularly vulnerable to free radicals due to its high rate of oxidative metabolic activity, high content of polyunsaturated fatty acids, regions rich in iron concentration, and moderate levels of antioxidant [13].

We hypothesized that an increase in free radical content and/or its interaction with macromolecules, especially proteins, could be a potential mechanism for changes in brain development that are related to protein malnutrition. To verify a relation between brain oxidative damage and protein malnutrition, we investigated various parameters of oxidative status, namely free radical content and indexes of damage to macromolecules, i.e., lipid and protein damage, in addition to total antioxidant reactivity (TAR) levels and antioxidant enzyme activities of SOD, GPx, and CAT in different cerebral regions (cortex, hippocampus, and cerebellum) from rats subjected to prenatal and postnatal protein malnutrition.

Materials and methods

Chemicals

Thiobarbituric acid and Trolox were obtained from Merck (Rio de Janeiro, Brazil); 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) was obtained from Wako Chemicals (Richmond, VA, USA); and 2′-7′-dichlorofluorescein diacetate, 2′-7′-dichlorofluorescein (DCF), trichloroacetic acid, phenyl methyl sulfonyl fluoride, 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol), and H_2O_2 stock solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Albino Wistar rats were maintained under standard conditions (12-h light/12- h dark, temperature 22 ± 2°C); food and water were given ad libitum. The experimental protocol was developed according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

Diets

Animals had free access to isocaloric diets (Table 1) that contained 25% or 7% protein (casein), salts, and vitamins as recommended by the Association of Official Analytical Chemists (Horwitz W. Official methods of analysis of the Association of Official Analytical Chemists. Association of

Table 1
Nutritional composition of the diets*

	25% Protein diet	7% Protein diet
Casein (87% protein)† (g/kg diet)	287	80.50
Fat (soybean oil) (g/kg diet)	150	150
Carbohydrate (corn starch) (g/kg diet)	501.5	708
Salt mix‡ (g/kg diet)	40	40
Vitamin mix§ (g/kg diet)	10	10
Non-nutritive fiber (g/kg diet)	10	10

* Salt and vitamin compositions are according to Horwitz (Horwitz W. Official methods of analysis of the Association of Official Analytical Chemists. Association of Official Analytical Chemists, Washington, D.C. 1980). Energy for both diets was 4.3 kcal/g of diet.

† Casein, purity 87% (from Farmaquímica, Porto Alegre, Brazil), supplemented with 0.15% L-methionine (from Merck, Rio de Janeiro, Brazil).

‡ Mineral mixture (from Roche, São Paulo, Brazil; mg/100 g of ration): NaCl, 557; KCl, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4 \cdot 7H_2O$, 108; $MnSO_4 \cdot H_2O$, 16; $ZnSO_4 \cdot 7H_2O$, 2.2; $CuSO_4 \cdot 5H_2O$, 1.9; $CaCl_2 \cdot 6H_2O$, 0.09.

§ Vitamin mixture (from Roche, São Paulo, Brazil; mg/100 g of ration): vitamin A, 4; vitamin D, 0.5; vitamin E, 10; menadione, 0.5; choline, 200; *p*-aminobenzoic acid (PABA), 10; inositol, 10; niacin, 4; pantothenic acid, 4; riboflavin, 0.8; thiamin, 0.5; pyridoxine, 0.5; folic acid, 0.2; biotin, 0.04; vitamin B12, 0.003.

Official Analytical Chemists, Washington, D.C. 1980) and previously described by our group [3].

Malnutrition model

Prenatal and lactational malnutrition was induced by restricting to 7% the protein content of the mothers' diet (control group, 25% protein) during the entire gestation and lactational periods, whereas malnutrition up to 60 d was induced by maintaining the same 7% protein diet. Both diets were isocaloric and were given ad libitum. Litter size was adjusted to eight pups per mother on the first postpartum day and they were maintained at 22°C on a 12-h light/12-h dark cycle until they were of experimental age.

Tissue preparation

Rats were killed by decapitation, and hippocampi, cerebral cortices, and cerebella were dissected out and instantaneously placed at −70°C until biochemical measurements, when they were homogenized in 10 vol of ice-cold phosphate buffer (0,1 M, pH 7.4) containing 140 mM of KCl, 1 mM of ethylenediaminetetraacetic acid, and 1 mM of phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 960*g* for 10 min and the supernatant was used. All steps were carried out at 4°C.

Free radical levels

An aliquot of the sample was incubated with 2′-7′-dichlorofluorescein diacetate (100 μM) at 37°C for 30 min. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000; Hitachi, Tokyo, Japan). The formation of reactive oxygen species was quantified by using a DCF standard curve and results were expressed as picomoles of DCF formed milligram of protein [14].

Lipid peroxidation assay

Sample aliquots were incubated with 10% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was heated on a boiling water bath for 30 min, an equal volume of *n*-butanol was added, and the final mixture was centrifuged; the organic phase was collected for fluorescence measurements at excitation and emission wavelengths of 515 and 553 nm, respectively [15]. We used 1,1,3,3-tetramethoxypropane as the standard. Results are reported as picomoles of malondialdehyde per milligram of protein.

Degradation of protein tryptophan residues

Sodium dodecylsulfate was added to sample aliquots (final concentration 0.1%). The tryptophan content within solubilized proteins was determined fluorometrically at excitation and emission wavelengths of 280 and 345 nm, respectively [16].

Degradation of protein tyrosine residues

Sodium dodecylsulfate was added to sample aliquots (final concentration 0.1%). The tyrosine content within solubilized proteins was determined fluorometrically at excitation and emission wavelengths of 277 and 320 nm, respectively [17].

TAR assay

The TAR assay is based on luminol-enhanced chemiluminescence induced by an azo initiator [18,19]. The reaction mixture contained 2 mM of ABAP and 6 mM of luminol in glycine buffer. TAR values were determined by assessing the initial decrease of luminescence, which was calculated as the ratio of chemiluminescence in the absence of additives to the chemiluminescence after the addition of 20 nM of Trolox or samples (1 μL). TAR values were expressed as equivalents of Trolox concentration per milligram of protein.

SOD activity

Superoxide dismutase activity was determined with a RANSOD kit (Randox Laboratories, San Diego, CA, USA). This method employs xanthine and xanthine oxidase to generate superoxide radical, which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye that is assayed spectrophotometrically at 505 nm at 37°C. Inhibition of production of the chromogen is proportional to the activity of SOD present in the sample.

CAT activity

The homogenate was incubated with ethanol (10%) and Triton (10%). Activity was assayed at 25°C by determining the rate of degradation of H_2O_2 at 240 nm in 10 mM of potassium phosphate buffer (pH 7.0). The extinction coefficient of 43.6 mM/cm was used for calculation. One unit is defined as 1 pmol of H_2O_2 consumed per minute and the specific activity is reported as units per milligram of protein [20].

GPx activity

The reaction was carried out at 25°C in 600 μL of solution containing 100 mM of potassium phosphate buffer, pH 7.7, 1 mM of ethylenediaminetetraacetic acid, 0.4 mM of sodium azide, 2 mM of glutathione, 0.1 mM of nicotinamide adenine dinucleotide phosphate, and 0.62 U of glutathione reductase. Activity of selenium-dependent GPx was measured taking *tert*-butyl-hydroperoxide as the sub-

strate at 340 nm. The contribution of spontaneous nicotinamide adenine dinucleotide phosphate oxidation was always subtracted from the overall reaction rate. GPx activity was expressed as nanomoles of nicotinamide adenine dinucleotide phosphate oxidized per minute per milligram of protein [21].

Protein assay

Total protein concentration was determined according to the method described by Lowry [22] with bovine serum albumin as the standard.

Statistical analysis

Data were analyzed statistically by Student's *t* test, with the level of statistical significance set at $P < 0.05$. Results are expressed as mean ± standard error of the mean.

Results

Prenatal and postnatal protein malnutrition decreased body and brain weights by about 65% and 40%, respectively (data not shown).

No differences on DCF levels were found in any brain region (Fig. 1A). In contrast, protein malnutrition altered various parameters of oxidative stress, specifically damaged macromolecules. There was an increase in levels of thiobarbituric acid-reactive substances (Fig. 1B), the index of lipid peroxidation, in the cerebellum ($F_{12} = 2{,}22$, $P = 0.049$) and cerebral cortex ($F_{14} = 3.19$, $P = 0.007$) in protein-malnourished rats. Moreover, protein malnutrition significantly decreased levels of tryptophan and tyrosine in all tested brain structures (Fig. 2); decreased tryptophan content was observed in the hippocampus, cerebellum, and cerebral cortex ($F_{15} = 2{,}34$, $P = 0.044$; $F_{13} = 2{,}26$, $P = 0.043$; $F_{12} = 4{,}77$, $P = 0.001$, respectively), as was tyrosine content ($F_{15} = 2{,}83$, $P = 0.020$; $F_{13} = 2{,}25$, $P = 0.044$; $F_{12} = 4{,}71$, $P = 0.001$). It is important to note that the amounts of tyrosine and tryptophan were normalized by total protein level.

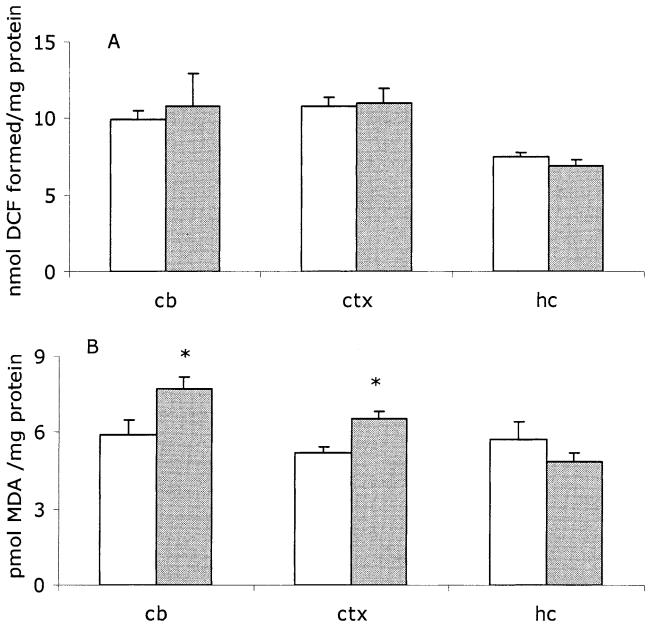

Fig. 1. Effects of protein malnutrition (gray bars) versus control (white bars) on free radical content (A) and lipid peroxidation (B) in studied brain areas. Data are expressed as mean ± standard error of the mean of seven to eight animals per group. Student's *t* test, $^{*}P < 0.05$. cb, cerebellum; ctx, cortex; DCF, 2′-7′-dichlorofluorescein; hc, hippocampus; MDA, malondialdehyde.

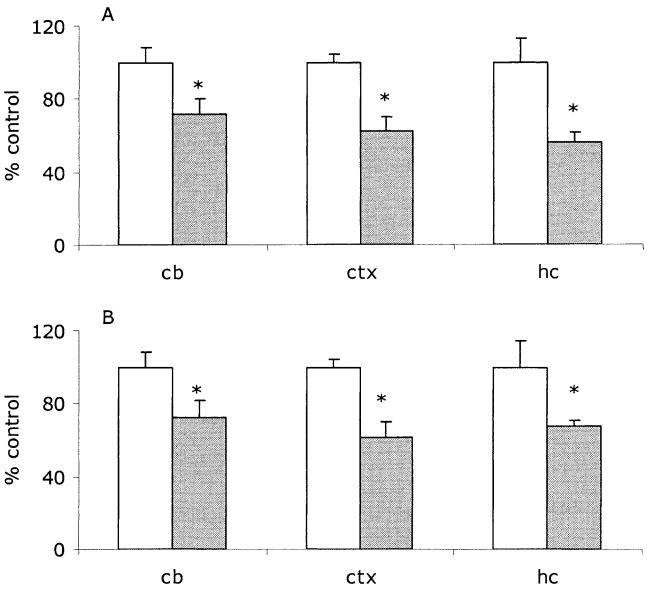

Fig. 2. Contents of tryptophan (A) and tyrosine (B) in studied brain structures from control (white bars) and protein malnutrition (gray bars). Data expressed as mean ± standard error of the mean of seven to eight animals per group. Values from control animals were considered as being 100% in each experiment (Student's *t* test, $^{*}P < 0.05$). cb, cerebellum; ctx, cortex; hc, hippocampus.

The effects of protein malnutrition on the antioxidant brain enzymes studied are presented in Fig. 3. SOD and GPx activities were unchanged in all studied structures, whereas CAT activity was significantly decreased only in the cerebellum (~35%, $F_{13} = 2.43$, $P = 0.032$). In addition, a significant decrease in TAR levels ($F_{13} = 2{,}43$, $P = 0.032$) was observed in the cerebral cortex of protein-malnourished rats (Fig. 4).

Discussion

The present results confirm the working hypothesis and support the idea that oxidative damage may be related to brain changes induced by protein malnutrition. There are only a few studies about oxidative stress in brain tissue of rats subjected to protein malnutrition. Caloric and/or protein

prenatal malnutrition has been reported to cause oxidative stress in extracerebral tissues by changes in antioxidant enzyme activity and/or antioxidant compounds in humans [23–25] and rats [26].

Severe protein malnutrition provoked long-lasting oxidative deleterious effects on macromolecules because it increased lipid peroxidation levels and significantly decreased tyrosine and tryptophan contents. Oxidative damage to lipids and proteins may be associated with numerous alterations in membrane structure, biochemical parameters, and functional activities such as membrane fluidity and mitochondrial dysfunction [11].

Although protein malnutrition can interfere with protein synthesis and structure and, hence, alter enzyme activity [1], our findings suggest that cerebral effects of malnutrition are not simply caused by protein deficiency. However, there is involvement of other altered parameters, including accumulated oxidative damage and decreased antioxidant activity.

We found that CAT activity was significantly decreased in the cerebellum of malnourish rats; this fact is important

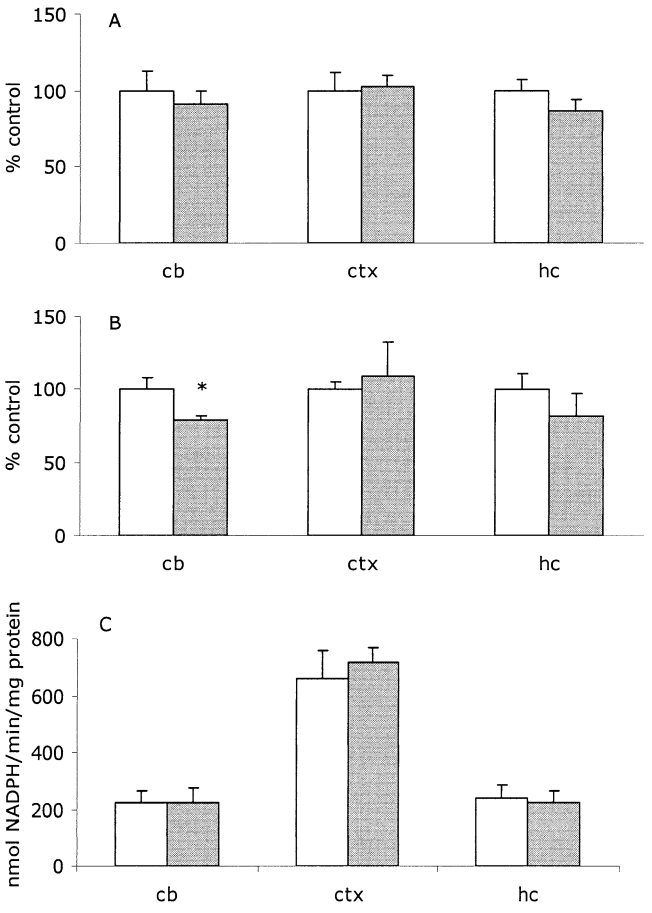

Fig. 3. Effects of protein malnutrition on activities of the antioxidant enzymes superoxide dismutase (A), catalase (B), and glutathione peroxidase (C) of studied brain areas of control (whited bars) and protein-malnourished (gray bars) rats. Data expressed as mean ± standard error of the mean of seven to eight animals per group (Student's t test, $^{*}P < 0.05$). cb, cerebellum; ctx, cortex; hc, hippocampus; NADPH, nicotinamide adenine dinucleotide phosphate.

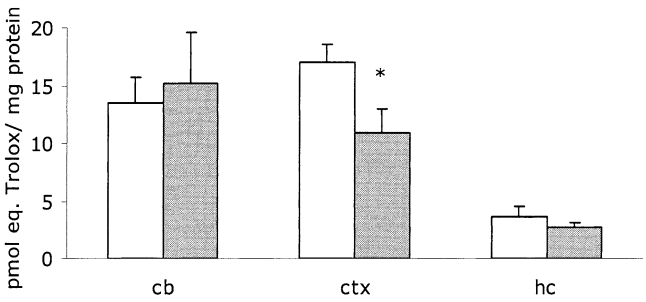

Fig. 4. Effects of protein malnutrition on total antioxidant reactivity levels from the studied brain areas of control (white bars) and protein-malnourished (gray bars) rats. Data expressed as mean ± standard error of the mean of seven to eight animals per group (Student's t test, $^{*}P < 0.05$). cb, cerebellum; ctx, cortex; hc, hippocampus.

because accumulated H_2O_2 might generate hydroxyl radicals by the iron-catalyzed Fenton reaction or by the Haber-Weiss reaction [27].

In addition, TAR levels were decreased in the cerebral cortex. TAR is an index of the capacity of a given sample to modulate the damage associated with enhanced production of free radicals [18]. Alterations in TAR levels cannot be attributed to any known antioxidant because this index reveals the existence of unidentified and specifically unmeasured antioxidant molecules [19]. The lower TAR levels in the cerebral cortex of nourished rats may produce sensitivity to oxidative events in this brain area.

It is important to note that the cerebral cortex and cerebellum, which showed at least one altered antioxidant parameter, exhibited increased levels of lipid peroxidation as evaluated by testing with thiobarbituric acid-reactive substances. Brain lipid peroxidation affects membrane integrity and stability; therefore, cell membranes from malnourished animals, already altered under basal conditions, may be more sensitive when exposed to oxidative alterations. We used amino acid quantification methods as an index of protein damage and found a decrease in the content of tyrosine and tryptophan.

Interestingly, Manary et al. [8] suggested that oxidative stress is prevalent in children with kwashiorkor, based on the finding that urinary levels of oxidized amino acids, dityrosine and orthotyrosine, normalized to their precursors, tyrosine and phenylalanine, were increased in children with kwashiorkor. They further suggested that oxidatively damaged cytosolic or membrane-bound proteins could be the source of oxidized amino acids in urine. Our data show that the decreased amino acid residue content may result from significant oxidation in all tested brain areas, which might contribute to blood and urinary levels.

Because malnutrition, although it did not affect brain free radical content as assessed by DCF levels, changed antioxidant capacity in the cerebral cortex and CAT activity in the cerebellum, we propose that it induces impairment in the protein repair system.

In general, oxidative stress status leads to chemical al-

terations in amino acid residues from proteins, whose function might be compromised. Oxidation of amino acid residues from proteins increased susceptibility to proteolytic degradation. Another possibility is that oxidative damage promotes formation of protein aggregates, which are resistant to removal by proteinases. We hypothesize that the higher levels of oxidized proteins caused by severe malnutrition might occur through a decline in the proteolytic machinery, namely the proteasome, which is an important system responsible for the degradation of damaged proteins; alternatively, oxidized proteins strongly tends to aggregate and form covalent cross-links, preventing proteasome action and leading to accumulation of oxidized proteins [28]. Yin [29] suggested that the final step of the crosslinking process is the formation of a water-insoluble self-fluorescent material, which accumulates in aging cells.

In conclusion, our results show the existence of oxidative stress, an imbalance between free radical content and tested scavenging systems, in the cerebral cortex and cerebellum caused by protein malnutrition. In addition, decreased amino acid residue levels seem to be important in all tested brain areas, which may indicate an impairment of protein damage repair systems. Our data support the idea of prophylactic and therapeutic strategies to improve antioxidant status as coadjuvants in protein deficiency. More studies are necessary to determine the exact mechanism of malnutrition-induced protein damage.

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