

Protein Energy-Malnutrition: Does the In Vitro Zinc Sulfate Supplementation Improve Chromosomal Damage Repair?

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Abstract Protein-energy malnutrition (PEM) is originated by a cellular imbalance between nutrient/energy supply and body's demand. Induction of genetic damage by PEM was reported. The purpose of this study was to determine the genetic effect of the in vitro zinc sulfate ($ZnSO_4$) supplementation of cultured peripheral blood lymphocytes from children with PEM. Twenty-four samples from 12 children were analyzed. Anthropometric and biochemical diagnosis was made. For the anthropometric assessment, height-for-age index, weight-for-age index, and weight-for-height index were calculated (WHO, 2005). Micronutrient status was evaluated. A survey for assessed previous exposure to potentially genotoxic agents was applied. Results were statistically evaluated using paired sample t test and χ^2 test. Each sample was fractionated and cultured in two separate flasks to performed two treatments. One was added with 180 $\mu g/dl$ of $ZnSO_4$ (PEMs/ $ZnSO_4$) and the other remains non-supplemented (PEMs). Cytotoxic effects and chromosomal damage were assessed using the cytokinesis-block micronucleus assay (CBMN). All participants have at least one type of

malnutrition and none have anemia, nor iron, folate, vitamin A, and zinc deficiency. All PEMs/ $ZnSO_4$ samples have a significant reduction in the micronucleus (MNI) frequency compared with PEMs ($t=6.25685$; $p<0.001$). Nuclear division index (NDI) increase in PEMs/ $ZnSO_4$ ($t=-17.4226$; $p<0.001$). Nucleoplasmic bridge (NPBs) frequency was four times smaller in PEMs/ $ZnSO_4$ ($\chi^2=40.82$; $p<0.001$). No nuclear buds (NBuds) were observed. Cytotoxic effects and chromosomal damage observed in children suffering from PEM can be repaired in vitro with zinc sulfate supplementation.

Keywords Protein-energy malnutrition · Chromosomal damage · Zinc sulfate · Children

Introduction

Growth is defined as a continuous process resulting from the complex interaction between inheritance and environment [1]. In this context, Protein energy-malnutrition (PEM) results from food insufficiency as well as from poor social and economic conditions [2, 3]. Malnutrition is originated by a cellular imbalance between nutrient/energy supply and body's demand in order to ensure growth and maintenance [4]. The term protein-energy malnutrition applies to a group of related disorders that develop in children and adults whose consumption of protein and energy (measured by calories) is insufficient to satisfy the body's nutritional needs (primary PEM). This problem affects approximately one third of children worldwide [5], and approximately 60 % of deaths that occur in children under five are directly related to malnutrition and associated diseases. The total number of underweighted and stunted children has not dropped significantly since 1980 [6]. Malnutrition is a serious public health problem, and then the adequate nutritional support of severe PEM in infants represents a great challenge [7].

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Many micronutrient deficiencies were described in PEM, for example, zinc, iron, and vitamin A [6, 8–10]. It has long been recognized that vitamins and minerals play a crucial role in health and disease [11–14]. Such essential micronutrients are required for the synthesis of DNA maintenance factors and repair mechanisms [14, 15]. In fact, not only nutritional deficiencies or excess can greatly alter physical function neither inadequate intake of micronutrients contributes significantly to DNA damage and cancer risk [16–21].

In these sense, in previous works, we studied the relationship between PEM and genetic damage [10, 22, 23]. We found that children aged 1–60 months with PEM exhibit increased frequencies of chromosomal aberrations (dicentric chromosomes, gaps, isogaps, breaks, and telomeric association) in peripheral blood lymphocytes. Similar results in peripheral blood lymphocytes and bone marrow cell cultures have been found in another work, abnormalities persist even after the children have attained normal height and weight [24]. In addition, it was reported that low-protein diets induce chromosome breaks and deletions in bone marrow cells and lymphocytes of rats and female mice [25, 26].

Zinc (Zn) is an essential component for more than 1,000 proteins including copper/Zn super oxide dismutase (SOD) as well as a number of other Zn finger proteins [27, 28]. The function of Zn involves a wide range of biological processes including cell proliferation, immune function, and defense against free radicals [27–32]. In addition, Zn appears to regulate key physiological processes such as cellular response to oxidative stress, DNA repair, cell cycle regulation, and apoptosis [29, 30, 33–48].

There are various methods to measure genome stability but to date the cytokinesis blocked micronucleus assay (CBMN) appears to be the best validated tool for measuring DNA damage as a result of nutrient deficiency [13]. This method can provide data on cytotoxic effects as well as chromosomal damage. In the CBMN assay, a cytokinesis blocking agent (cytochalasin B) is used to produce once-divided binucleated cells that can express micronuclei (MNi) which are biomarkers of whole chromosome loss and/or breakage. In addition, it is also possible to measure nucleoplasmic bridges (NPBs), which arise from dicentric chromosomes and nuclear buds (NBuds), a biomarker of gene amplification, replication stress, or unresolved DNA repair-DNA protein complexes [13, 49–51]. The presence of these DNA damage endpoints are a strong indicator of chromosomal damage and instability within a cell [49].

Taking into consideration that *in vivo* and *in vitro* supplementation with vitamins and minerals reversed the damage caused by different genotoxic agents [13, 52–58], the purpose of this study was to determine the genetic effect of *in vitro* zinc sulfate (ZnSO_4) supplementation of cultured peripheral blood lymphocytes from children suffering from PEM without associated infections.

Materials and Methods

Experimental Procedure

Twenty-four samples obtained from twelve primary malnourished infants attending to Primary Health Care Rooms, La Plata, Argentina, were included in this analysis (Table 1). Children aged 1–60 months. Anthropometric were performed to assess nutritional condition. Biochemical evaluation was made to determined micronutrients status. Prior to blood collection, we interviewed each individual's parent to complete a semi-structural survey specifying age, dietary habits, viral or bacterial diseases, previous exposure to diagnostic X-rays, and use of therapeutic drugs. All children were disease-free, and had not been exposed to X-rays, drug therapy (including vitamins or minerals), or viral infections in 1 month prior to the study. Also, children with anemia or signs of vitamin deficiency were not included. Specific written information about the aims of the study was provided to all participants. Written informed consent was obtained from the participant's parents. The "Comité de Bioética del Hospital de Niños Sor María Ludovica" (La Plata, Argentina) provided the IRB approval.

Cytogenetic Analysis

Heparinized venous blood samples were used to obtain lymphocytes from the participants and set up two flasks, each one containing 0.5 ml of total blood in 5 ml RPMI 1640 (Gibco-Invitrogen Buenos Aires, Argentina) containing 1 % phytohemagglutinin (Gibco-Invitrogen), 50 IU penicillin, and 50 $\mu\text{g/ml}$ streptomycin (Sigma, St. Louis, MO, USA). One culture was supplemented with ZnSO_4 (PEMs/ ZnSO_4) while the other remains non-supplemented (PEMs); by this way, each child was its own control. ZnSO_4 concentration was 180 $\mu\text{g/dl}$; it is in the middle of the normal physiological range established in children and was able to reduce the genetic damage induced by zinc deficiency in preliminary studies in our laboratory with *in vitro* cultured human lymphocytes (manuscript in preparation). Cytotoxic effects and chromosomal damage were assessed by CBMN assay according to the method of Fenech [49]. Briefly, after 6 days in culture, cells were resuspended in fresh culture medium in the presence of cytochalasin-B (3 $\mu\text{g/ml}$ final concentration) (Sigma, St. Louis, MO, USA) for 24 h. At the seventh day, cultures were harvested. The cell suspension was centrifuged and the pellet was resuspended in 5 ml of fixative (methanol:acetic acid 3:1). The cells were washed three times with fresh fixative; they were later resuspended, dropped onto clean slides, and finally stained with 5 % of Giemsa for 10 min. The chromosome damage biomarkers scored are micronuclei (MNi), nucleoplasmic bridges (NPBs), and nuclear buds (NBuds). One thousand binucleated cells (BN)

Table 1 Sex, age, and anthropometric data of the malnourished children

Samples	Sex	Age (year)	Weight (kg)	Height (cm)	WAZ	HAZ	WHZ
1	M	1.631	9.45	78	-1.57	-2.08	-0.39
2	M	4.255	13.54	94.9	-1.72	-2.35	-0.2
3	M	3.764	13.12	88.1	-1.53	-3.33	1.14
4	M	0.906	8.86	71	-0.52	-1.46	0.46
5	M	3.847	14.28	95.6	-0.93	-1.62	0.21
6	M	1.42	9.86	70.5	-0.76	-4.06	2.41
7	M	2.039	12.2	83.9	-0.03	-1.18	1
8	M	2.983	12.12	95.5	-1.38	-0.12	-2.08
9	M	2.39	9.85	81.5	-2.42	-2.83	-0.86
10	F	1.428	9	75	-0.91	-1.68	0.15
11	F	2.736	12.2	87.5	-0.66	-1.45	0.35
12	F	1.225	10.47	73.5	0.75	-1.34	2.08

were analyzed per experimental point. Fenech's scoring criteria for MNi, NPBs, and NBuds determinations were used [49]. Cytostatic effects were analyzed through the nuclear division index (NDI), estimated by the ratio of mono-, bi-, and multinucleated cells in 500 viable cells, using the formula $= (M_1 + 2M_2 + 3M_3 + 4M_4) / N$; where M_{1-4} represent the number of cells with 1–4 nuclei and N is the total number of viable cells scored [49].

Anthropometric Evaluation

Height (H) and weight (W) were measured. Height-for-age index (HA), weight-for-age index (WA), and weight-for-height index (WH) were calculated. Z-scores for height-for-age (HAZ), weight-for-age (WAZ), and weight-for-height (WHZ) were assessed with the WHO standards using the ANTHRO 2005 program [59]. To determine the prevalence of stunting (HA), underweight (WA), and wasting (WH), the cut-off was -1.1 . Malnutrition degrees were established according to Torun and Chew [60].

Micronutrients Status

Part of the blood samples were taken for hemogram, ferritin, intra-erythrocyte folates, vitamin A, and zinc determinations. Samples were collected in aluminum foil-wrapped tubes with EDTA for hemogram determinations and with heparin to determine the other parameters. Samples were then transported to the Children's Hospital laboratory within 2 h after extraction. Hemogram samples were processed immediately. The separated plasma is being kept frozen at -70 °C and will be analyzed when all samples were collected to avoid inter- and intra-assay variations. Another aluminum foil-wrapped tube is being kept for vitamin A dosage. Hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) were analyzed

with an automated system. Ferritin and folate will be assessed by automated chemiluminescence immunoassay. Serum retinol concentration will be determined by HPLC and zinc by atomic absorption spectrophotometry at 213.9 nm (Shimadzu AA 6200).

Indicators and cut-off points. Anemia (Hb value <11 g/dl) [61]; iron deficiency (blood ferritin <12 ng/ml) [61]; folate deficiency (intra-erythrocyte folates <140 ng/dl) [62]; vitamin A deficiency (plasma retinol <20 μ g/dl) [63]; and zinc deficiency (blood zinc levels <70 μ g/dl [64].

Statistical Analysis

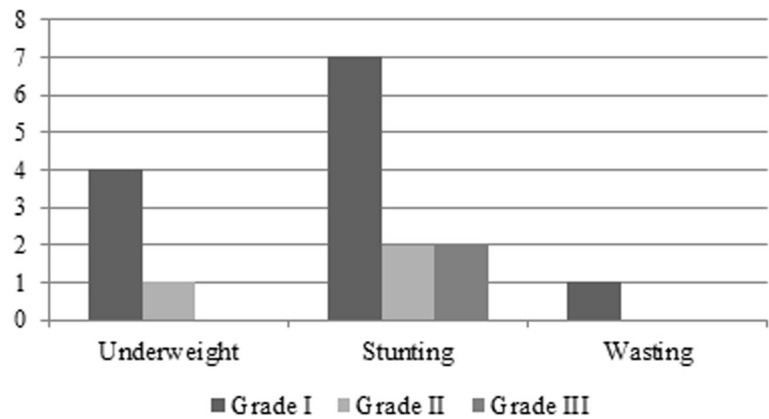
Data were statistically analyzed with the paired sample t test with the Statgraphics® 5.1 software and χ^2 test [65] with $p < 0.05$ considered statistically significant.

Results

Table 1 summarizes anthropometric data of the sample. All participant have at least one type of malnutrition (stunting, underweight, and/or wasting). Stunting is the prevalent form of PEM (92 %). Figure 1 shows that most of the children have mild malnutrition (grade I).

Hematological and biochemical variables are summarized in Table 2. None of the children have anemia nor iron, folate, vitamin A, or zinc deficiency.

Table 3 shows the results of CBMN assay for each paired sample (MNi and NDI). All PEMs/ZnSO₄ samples have a significant reduction in the frequency of micronucleus when compared with PEMs ($t=6.25685$; $p < 0.001$). Zn concentration also bring about impact on the nuclear division index, with significant increase for this variable in PEMs/ZnSO₄ ($t=-17.4226$; $p < 0.001$). Figure 2 summarizes the DNA-damage

Fig. 1 Type and degree of PEM in the malnourished children

endpoints (MNi, NPBs, and NBuds) and NDI. The global MNi frequency was five times smaller in supplemented samples with regard to non-supplemented ($\chi^2=77.95$; $p<0.001$). Similar trend was observed for NPBs ($\chi^2=40.82$; $p<0.001$). No NBuds were observed in both groups.

Discussion

PEM is originated by a cellular imbalance between nutrient/energy supply and body's demand in order to ensure growth and maintenance [4]. It must be considered that energy and amino acids are required for protein synthesis and DNA repair [66–68]. In this sense, we recently carried out a research in order to study the occurrence of structural chromosomal aberrations in children suffering from PEM and we found that chromosomal damage could be attributed to several factors such as a severe deficiency of essential nutrients and energy required for the synthesis of DNA maintenance factors; deterioration of repair mechanisms; and/or the lack of specific

factors that are needed to protect the cell against oxidative DNA damage.

Results presented here showed a significantly increased frequency of chromosomal damage and cytotoxic effects in lymphocytes of malnourished children. These results agree with those of other authors performed in children with severe PEM [10, 24, 69] and our own previous research conducted with lymphocytes of malnourished children in the same range of age (1–60 months) who exhibit increased frequencies of structural chromosomal aberration (dicentric chromosomes, gaps, isogaps, breaks, and telomeric association). Likewise, Cervantes-Rios and coworkers [70] reported the induction of micronuclei in reticulocytes of children with malnutrition and infections but Celik and coworkers [71] did not found DNA damage increase in marasmus cases. In addition, some results were reported in mice or rats fed with low-protein diets who exhibit high rates of chromosomal damage [24, 72–74] and citomolecular damage showed by the alkaline single cell electrophoresis assay [75].

In this work, the increased frequency of chromosomal damage and cytotoxic effects observed in lymphocytes of

Table 2 Hematological and biochemical variables

Samples	Ht (%)	Hb (g/dl)	MCV (μm^3)	MCH (g/dl)	Ferritin (ng/ml)	Folate (ng/dl)	Retinol ($\mu\text{g}/\text{dl}$)	Zinc ($\mu\text{g}/\text{dl}$)
1	40	12.6	79.8	25.5	26.7	359.0	29.4	129.4
2	41	14.0	77.0	26.2	13.0	376.0	30.7	118.7
3	34	11.1	76.9	25.8	29.1	299.0	26.3	101.5
4	38	12.8	83.0	27.5	48.9	364.7	27.9	102.1
5	39	13.4	83.0	28.3	14.0	554.6	32.3	84.0
6	34	11.3	72.0	23.5	15.8	298.5	21.9	83.0
7	33	11.1	74.0	24.6	27.4	467.3	31.6	80.25
8	34	11.5	74.0	25.1	52.7	306.4	27.1	85.75
9	35	11.7	78.0	26.4	63.7	345.6	25.7	113.4
10	34	11.4	67.0	22.3	25.3	372.9	22.8	97.2
11	37	12.3	81.0	27.0	12.1	345.2	28.1	102.6
12	36	11.7	72.0	23.3	19.1	355.8	24.7	105.7

Table 3 Results of CBMN assay for each paired sample (MNI and NDI)

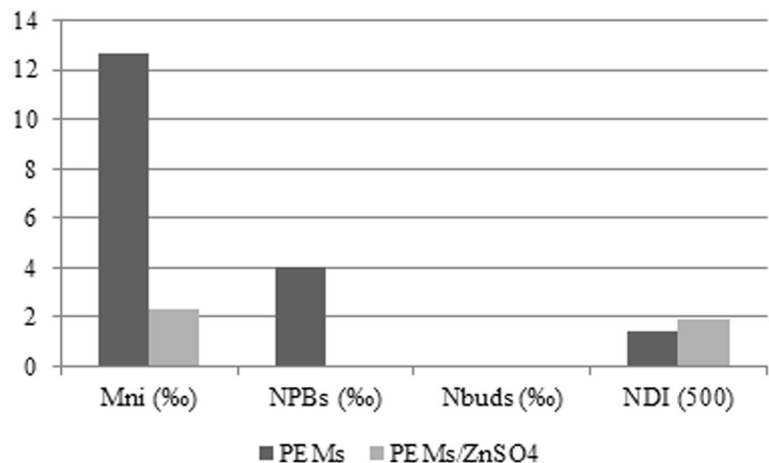
Samples	MNI (‰)		NDI (500)	
	PEMs	PEMs/ZnSO ₄	PEMs	PEMs/ZnSO ₄
1	8	0	1.5	2.1
2	22	2	1.2	1.88
3	2	0	1.66	2.21
4	5	2	1.55	1.95
5	25	9	1.12	1.6
6	17	3	1.4	1.85
7	6	0	1.52	2.18
8	10	2	1.49	1.89
9	20	5	1.36	1.74
10	11	1	1.51	1.98
11	19	4	1.3	1.7
12	7	1	1.48	1.9

PEMs was significantly minimized with zinc sulfate supplementation. Zinc plays an important role in children growth. The association of zinc deficiency with growth retardation and hypogonadism was first described in 1963 from Iran [76] and is now well established from animal and human studies, demonstrating a critical role of this micronutrient in cellular growth, cellular differentiation, and metabolism [77]. Despite it is known that growth retardation, immune dysfunctions, and cognitive impairment are major effects of zinc deficiency [78] also is recognized that these effects are reversible by zinc supplementation [78–80]. The function of Zn involves a wide range of biological processes including cell proliferation, immune function, and defense against free radicals [27–32]. In addition, Zn appears to regulate key physiological processes such as cellular response to oxidative stress, DNA repair, cell cycle regulation, and apoptosis [29, 30, 33–48]. The role of zinc in modulating oxidative stress has been recognized [13]. Most of the beneficial effects displayed by zinc might be related to its action as an

“hormetin”, i.e., by the induction of a mild pro-oxidant activity that improve the anti-oxidative defensive responses leading ultimately to an improved capacity of the cell to respond to additional stressors or harmful agents [81]. In this sense, Sliwinski and coworkers conducted a study in normal human lymphocytes and human myelogenous leukemia K562 cancer cells treated with zinc sulfate and hydrogen peroxide; they analyzed the induction of cyto- and genotoxicity and reported that zinc may protect normal cells against DNA-damaging action of hydrogen peroxide and on the contrary amplify genetic damage induced by this agent in cancer cells [82].

The development or identification of nutrient-dense foods and ingredients that are rich in micronutrients required for DNA replication and repair and for the prevention of genome-damaging events is essential for individuals to achieve their daily nutrient requirements for genome health maintenance [15]. At present day, there is an international increasing interest to redefine recommended dietary intakes (RDIs) of minerals and vitamins to prevent developmental abnormalities and degenerative diseases of old age as well as optimizing cognition [14]. In this sense, prevention of DNA damage is an important parameter for the definition of new dietary reference values because increased loss of genome integrity has been repeatedly demonstrated to be prospectively associated with increasing risk of degenerative diseases [14, 15]. In this context, our results become important if we consider that all participants in the present study showed blood zinc levels within the normal physiological range established for children.

We conclude that, in our study, cytotoxic effects and chromosomal damage observed in children with PEM can be repaired in vitro with zinc sulfate supplementation. Moreover, the normal physiological range for this important micronutrient must be revised for DNA damage prevention. The strength of this work is, as far as the authors know, to be the first in which the role of the in vitro zinc sulfate supplementation of peripheral blood from children suffering from PEM is evaluated. Future investigations in this field should focus on

Fig. 2 Frequency of DNA-damage endpoints (MNI, NPBs, and NBuds) and NDI

elucidating the underlying mechanisms of the nutritional modulation of DNA repair, studying redox, as well as epigenetic regulation of DNA repair pathways and changes in gene expression. In addition, further studies should analyze the protective genotoxic effect of Zn in children with severe malnutrition and compare MNI, NPBs, and NDI frequencies, in healthy, well-nourished children.

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Conflict of Interest The authors declare that they have no conflict of interest.

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