Journal
of Negative & No Positive Results

Original
Artículo inglés

Effect of nutritional recovery on the serum concentration of lipid peroxide in children with primary and severe protein energy malnutrition.

Efecto de la recuperación nutricia en la concentración sérica de lipoperoxidos en niños con desnutrición proteínico-energética primaria grave.

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Abstract
Objective. The purpose is to show lipid peroxide's serum concentration trend during a four-week nutritional recovery period in children with primary and severe protein energy malnutrition (PEM).
Methods. In a clinical intervention 12 primarily and severely malnourished children (three to 48 months of age) were included. *Dependent variable:* Serum lipid peroxide (LPO) concentration (nmol/mL). *Independent variables:* non lactose starting infant formula (200 kcal/kg/d and proteins 4 g/kg/d). Age, sex, nutritional recovery, weight/age, length/age and weight/length indices calculated and expressed as Z scores were included. For statistical analysis a repeated measure ANOVA model was applied. A non-parametric Mann Whitney U-Test was used to compare groups. Null hypothesis was rejected with a p value ≤ 0.05.
Results. Throughout the study the LPO concentration was higher in subjects with PEM than in the control group ($p< 0.001$). There was a decrease in the LPO concentration (nmol/mL) between basal vs. two weeks (12.9 vs. 7.3, $p = 0.06$) and basal vs. four weeks (12.9 vs. 8.16, $p = 0.08$).
Conclusion. LPO concentrations were significantly higher in children with severe PEM at the beginning and end of the four-week nutritional recovery period. This finding was probably associated with increased metabolism of the cellular tissue and/or the high consumption of energy and nutrients compared to a control group. The null hypothesis of basal-end differences in LPO serum concentrations could not be rejected due to the great variability in serum lipoperoxides in these children with severe primary protein-energy malnutrition.

KEYWORDS
Severe malnutrition, lipoperoxides, nutritional recovery

Resumen
Objetivo. El propósito es mostrar la tendencia de la concentración sérica de lipoperoxidos durante un período de cuatro semanas de recuperación nutricia en niños con desnutrición proteínico-energética primaria grave (DPE).
Métodos. En un estudio de intervención se incluyeron 12 niños desnutridos graves (tres a 48 meses de edad). Variable dependiente: Concentración de lipoperoxidos en suero (LPO) (nmol/mL). Variables independientes: fórmula sin lactosa para lactantes (200 kcal/kg/d y las proteínas 4 g/kg/d). Edad, sexo, se estimaron los índices peso/edad, longitud/edad, peso/longitud y se expresaron como puntaje Z. Se utilizaron las pruebas ANOVA, pruebas de mediciones repetidas y U Mann Whitney. La hipótesis nula fue rechazada con un valor de $p\leq 0.05$.
Resultados. Durante todo el estudio la concentración LPO fue mayor en sujetos con DPE que en el grupo control ($p<0.001$). Hubo una disminución en la concentración de LPO (nmol / ml) entre la determinación basal vs. Dos semanas (12.9 vs. 7.3, p=0,06) y vs. Cuatro semanas (12,9 vs. 8,16, p=0.08).
Conclusión. Las concentraciones de LPO fueron significativamente mayores en los niños con DPE grave al inicio y al final del periodo de cuatro semanas de recuperación nutricia. Este hallazgo estuvo probablemente asociado al mayor metabolismo del tejido celular y/o al elevado consumo de energía y nutrimentos en comparación con un grupo de control. No pudo rechazarse la hipótesis nula de

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Recibido el 31 de julio de 2016; aceptado el 06 de agosto de 2016.

diferencia basal-final debido a la gran variabilidad en la concentración sérica de lipoperoxidos en los niños con desnutrición proteínico-energética primaria grave.

Palabras Clave
Desnutrición grave, lipoperoxidos, recuperación nutricia

Abbreviations:

ANOVA, analysis of variance
CAT: catalase
SOD: copper-zinc superoxide dismutase
GHS: glutathione
GPx: glutathione peroxidase
LPO lipoperoxides
MCDP: methylcarbamoy l-3,7-dimethylamine-10H-phenothiazin
PEM, protein energy malnutrition
PUFA´s, polyunsaturated fatty acids
ROS, reactive oxygen species
RNS, reactive nitrogen species
SD, standard deviation.

Contribution to scientific literature:

This work demonstrated that the concentration of lipoperoxides at the beginning and the end of a four-week period of nutritional recovery was higher in toddlers with severe malnutrition than in controls of similar age.

Introduction:

The combination of the nutritional demands typical in children less than 36 months and the presence of severe and primary protein energy malnutrition (PEM) have shown increased pro-oxidant production [1-4]. This oxidative stress is a steady state level of oxidative damage in a cell, tissue, or organ that is caused by the reactive oxygen species, such as free radicals and peroxides, within a biological organism. The oxidative stress is the result of an imbalance between pro-oxidant and antioxidant processes within that organism [5,6]. The pro-oxidant/antioxidant imbalance is linked to the substrate deficiency necessary for antioxidants formation [7-12]. As is known; water soluble (ascorbic acid) and lipid soluble (Vitamin E) nutrients comprise an important factor in the antioxidant defense system [13,14]. This pro-oxidant/antioxidant imbalance increases the risk of oxidative and nitrosative stress caused by higher amounts of reactive oxygen and nitrogen species (ROS, RNS), such as free radicals and peroxides, within the malnourished and potentially infected young organism [5, 9,15-18].

Reduced antioxidant defense status of serum and erythrocytes may result in increased peroxidation of all membrane lipids and enhanced concentrations of lipid per-oxidation [19]. When free radicals attack polyunsaturated fatty acids on the cell membrane of living organisms in the presence of molecular oxygen, a chemical cascade is triggered, eventually leading to the disintegration of fatty acids and formation of hydrocarbon gases (e.g. pentane) and aldehydes (e.g. malondialdehyde) [20]. This lipid peroxidation can occur due to increased oxidative stress or increased lipid levels [21]. Reactive oxygen species has been implicated in the pathogenesis of many conditions including anemia or edematous and severe PEM [8,17]. Normally, ROS-s are removed by a group of enzymes as copper-zinc superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [4,22,23]. An study has shown that erythrocyte and whole blood concentration of glutathione (GHS), the primary intracellular antioxidant, is low in children with edematous that with non-edematous PEM probably because a decreased synthesis of GSH due to a shortage of cysteine [24]. When children with PEM received a supplementation with cysteine had a faster resolution of edema (≈5 days less) than a control group, suggesting early restoration of cell integrity and function.

Recurrent infection, a common characteristic of PEM, promotes ROS formation disrupting the prooxidant/antioxidant balance increasing plasma antioxidant consumption. This is due in part to polyunsaturated fatty acids oxidation and the lipoperoxidation of cell membranes as the first mechanism of cell dysfunction and tissue damage [25]. In addition, prolonged exposure to high oxidative stress levels has been implicated in the development or acceleration of several dysfunctions and diseases, such as cardiovascular disease [5,26], inflammation [27,28], as well as type 2 diabetes [29] and breast, colon and prostate cancers [30,31].

Nonetheless, free radicals, like superoxide radicals, represent an important protection against the invasion of microorganisms and their production by activated polymorphic nuclear lymphocytes and other phagocytes represent an essential component of bactericide armamentarium [32,33]. Furthermore, superoxide generated by phagocytes is useful for stimulating fibroblasts which give rise to cell proliferation extending collagen fibers and forming scar tissue that close and seal the wound avoiding new infection [34]. Therefore, it could be hypothesized that severe and primary PEM produces higher amounts of free radicals and peroxides compared to controls and that a four week nutritional recovery period decreases them to a normal concentration. The purpose of this report is to show how lipid peroxide's serum concentration behaves during the four-week nutritional recovery period in children with primary and severe PEM compared with a control group.

Subjects and methods:

An intervention study with subjects acting as their own control was carried out in the pediatric metabolic ward of Guadalajara's Civil Hospital. Subjects were assigned by convenience until the intended sample was completed $(Z_{1-\alpha/2} + Z_{1-\beta})^2 . \sigma^2 / (\delta)^2$ where: $\alpha = 0.01 = 2.66$; $\beta = 0.80 = 0.842$; σ = standard deviation (0.939) of the LPO concentration (nmol/L) in a control group and $\delta = 1$ standard deviation (SD) from the true mean, n = 12. Only full term infants, between the ages of three and 48 months, with primary and severe PEM and a normal birth weight (>2500g) were included. They were admitted if the weight/age or weight/length indices were below –3 SD from the median or when the subject presented edema and a clinical picture of Kwashiorkor or Marasmus-kwashiorkor independent of the weight/age or weight/length score. PEM was defined as primary when the cause of malnutrition was an inadequate and insufficient diet commonly associated with repeated upper respiratory infectious disease and / or frequent episodes of diarrhea.

The clinical intervention was done in severely malnourished children once they were stabilized. They were admitted into the study only when clinically free of infection, diarrhea or any disease which might alter nutritional recovery. Subjects were fed infant milk based lactose free formula and corn syrup added to increase energy density to 0.8 kcal/mL. In all, 18 subjects were accepted. Six were excluded for the following reasons: technical lab difficulties (n=2), others for sepsis (n=1), milk protein allergy (n=2) and chronic pulmonary disease (n=1). These diseases were diagnosed during the nutritional recovery process and, one subject was voluntarily discharged. In order to compare the serum concentration of lipid peroxides, 12 healthy subjects, 3 to 48 months of age, from the hospital's outpatient clinic were included as a control group.

Administration of infant formulas: A starting infant formula (NAN non lactose, Nestle®) was used with nucleotides added, Selenium (0.08 μg/100 kcal) and n-3 PUFA's (90 mg/100 kcal). The infant formula was placed in a 500 mL feeding bag (Pisa®) then introduced into a feeding tube (D-731 o 732, Desvar de Mexico, S.A.) and given to infants using a continuous infusion pump (Braun®). After the fifth day the amount of formula was increased to provide an energy intake of 200 kcal/kg/d and proteins (4 g/kg/d). At the beginning of the third week, they were bottle fed *ad libitum* and children older than 12 months were started on complementary pap foods (cereal, and/or vegetables mixed with the same formula). Before and after each feeding the bottle and the container with the complementary foods were weighed on a triple beam balance (Ohaus ®). The total amount of formula, complementary foods, and protein and energy intake were calculated everyday.

The formula fulfilled the total requirements for water, energy, proteins and other nutrients during the first two weeks of enteral feeding, no other foods were offered during this period. From the first day, all subjects received 1 mL of oral vitamins daily (vitamin A 1500 mg RE, vitamin D 25 μg, vitamin C 50 mg, thiamin 1 mg, riboflavin 0.8 mg, niacin 6 mg) and folic acid (0.5 mg). From the sixth day on elemental iron was given in daily doses of 3 mg/kg.

Anthropometric measurements: All children were weighted and measured using the same procedure and observers. Weight: Subjects were weighted without clothes on a calibrated scale (® Bame model 440, México, with a minimum of 5 g). Length: each subject was measured with an infant measuring board read to the nearest 0.1cm. Weight/age, length/age and weight/length indices were calculated and expressed as Z scores using EPI Info 2000 Program, Version 3.3.2.

Laboratory: Before the beginning of the intervention blood samples were collected by antecubital veno-puncture at 07:00 am and then again after two and four weeks (two weeks of enteral feeding, and two weeks of the *ad libitum* feeding). The LPO measures were done in serum with the K-Assay ™ LPO-CC method (Kamiya Biomedical Company, Seattle, WA), which specifically and directly quantify LPO by colorimetry. In the presence of hemoglobin, lipid hydroperoxides are reduced to hydroxyl derivates and the methylcarbamoy l-3,7-dimethylamine-10H-phenothiazin (MCDP) chromogen is oxidatively cleaved to form methylene blue in an equal molar reaction. Subsequently LPO were quantified two times for each sample by colorimetrically measuring the methylene blue at 675 nm. Lipid peroxides are normally present in very low levels in normal human serum and plasma (0-1.3 nmol/mL).

Statistical analysis. After general descriptive statistics were estimated, the intra group changes of LPO serum concentration data (basal, at two and four weeks) were carried out with repeated ANOVA measures. Large SD for LPO was observed throughout the nutritional recovery period. Due to the great variability of LPO concentration, a non-parametric Mann Whitney U-Test was used to compare the initial, middle and final data between subjects vs. the control group. Excel, and SPSS-10 for Windows were used to process data and analyze the information; null hypothesis was rejected with a p value $\leq$ than 0.05.

Ethical considerations: Protocol for the clinical assay was submitted and approved by the Civil Hospital of Guadalajara "Dr. Juan I. Menchaca" Ethics Committee. Parents or legal guardians were informed of this protocol and once a consent form was signed candidates were admitted to the metabolic ward. Specialized personnel cared for the subjects during the study.

Results:

Table 1 shows the anthropometric indicators of subjects with primary and severe PEM including the different types of severe malnutrition. After a four week nutritional recovery period the weight/height index had recovered and most of the subjects showed resolution of edema between 7 to 10 days of the nutritional support. At start, 83% of subjects were anemic (hemoglobin < 110 g/L) and the hemoglobin concentration improved through the nutritional recovery period from 98.7 ± 22.0 g/L to 105.6 ± 7.2 g/L at the end of the study period. All subjects of the control group were non-anemic

(hemoglobin 128.6 ± 4.8 g/L). **Table 2** summarizes the energy and protein intake during the four-week period of nutritional recovery. It shows slight differences in energy and protein intake between the first two weeks (continuous enteral feeding) and the second two weeks (ad libitum feeding).

Table 1. Anthropometric indicators in children with protein energy malnutrition (n = 12) *

Variable	Mean	SD	Range	
Age	14.2	10.6	3.2	34.4
Weight/age (z) **				
Basal	-3.67	0.88	-5.01	-2.04
Two weeks	-2.64	0.76	-3.87	-1.30
Four weeks	-2.16	0.74	-3.64	-1.31
Length/age (z) †				
Basal	-2.87	1.02	-4.52	-1.41
Two weeks	-2.87	1.02	-4.92	-1.40
Four weeks	-2.73	1.09	-4.83	-1.22
Weight/length (z) ‡				
Basal	-2.34	0.73	-3.41	-0.90
Two weeks	-0.89	0.52	-1.56	0.38
Four weeks	-0.38	0.56	-1.62	0.35

Control group: Age (m) 26.0 ± 12.6; weight/age (z) -0.38 ± 1.13; Length/age (z) -0.47 ± 0.74; Weight/length (z) -0.05 ± 1.00. * Kwashiorkor (n = 6); Marasmus (n = 3); Marasmic-kwashiorkor (n = 3); ** Weight/Age Basal vs two weeks (p<0.001), two vs. four weeks (p=0.003); † Length/Age Basal vs two weeks (p=0.984), two vs. four weeks (p = 0.065); ‡ Weight/Length Basal vs two weeks (p<0.001), two vs. four weeks (p=0.013)

Table 2. Energy and protein intake during the four-week period of nutritional recovery (n=12)

Intake *	Marasmus & Marasmic-kwashiorkor (n = 6)		Kwashiorkor (n = 6)	
	Enteral feeding **	Ad Libitum †	Enteral feeding	Ad libitum
Liquids (mL/kg/d)	250	225	200	180
Energy (Kcal/kg/d)				
· Formula ‡	200	180	160	145
· Complementary food	-	30	-	40
Protein (g/kg/d)	4	3.6	3.2	2.9

* Rounded numbers; ** First two weeks; †3rd and 4th weeks; ‡ Non lactose infant formula

In **table 3** we can see that the control group's LPO serum concentration was 0.274 ± 0.271 nmol/mL; these values were significantly lower than those observed at the beginning in the treatment group (12.9 ± 4.5 nmol/mL), ($p < 0.001$). Two weeks after the start of intensive nutritional support with a formula rich in antioxidants, the serum levels of LPO children with severe and primary PEM decreased 1.7 times and 1.5 times by the end of the four-week period in relationship to the basal determination ($p = 0.06$ and $p = 0.08$ respectively). At the end of the nutritional recovery period, the LPO serum concentration continued to be much higher in the malnourished children than in the control group ($p < 0.001$) indicating the presence of some stress inducing factors that could not be controlled by the nutritional support with the amount of antioxidants provided in the formula diet.

Table 3. LPO serum concentration in children with severe and primary PEM during nutritional recovery and controls

	Malnourished (n = 12)		Controls (n = 12)	
LPO (nmol/L)	X	SEM *	X	SEM
Basal	12.90 ** †	4.59	0.274	0.271
Two weeks (n = 11)	7.32 ** †	2.43		
Four weeks	8.15 ** †	2.29		

* Standard Error of the mean; ** Cases (basal, two and four weeks) vs. controls $p < 0.001$;
† Cases: Basal vs. two weeks: $p = 0.06$; basal vs. four weeks: $p = 0.08$; Two vs. four weeks: $p = 0.727$

Discussion:

Even though all body cells are equipped with an antioxidant defense system they are exposed to oxidants from both, endogenous and exogenous sources [14]. During the recovery period the malnourished child has higher metabolic demands; as a consequence, oxidative stress is increased, and more oxidant production is seen due to the pro-inflammatory state of these children, especially when they are under threat of infection [32,33].

The clinical picture of PEM is already well known; however, its physiopathology is still poorly understood. It has been observed that changes in cell membrane structure caused by any microorganisms attack (fungi or bacteria) induce lipid per oxidation [35]. The cause of edema in the physiopathology of Kwashiorkor is not well established; however, there are studies that strongly support the hypothesis that oxidative and nitrosative stress play a role [8]. Oxidative stress produces cell dysfunction characterized by the inability to maintain balance and ionic gradient, especially the efflux of potassium and the influx of sodium/calcium that may be related to the physiopathology of Kwashiorkor [8]. In addition there are cell metabolism disorders, DNA structural modifications, tissue damage and an increase of the intestinal tract permeability to antigens [18,36]. In fact, susceptibility to high oxidative stress is more pronounced in children with Kwashiorkor than in those with Marasmus [37]. Manary et al [38] have shown that they can use the urinary levels of o,o'-dityrosine as damage markers for the tyrosyl radicals which is noticeably elevated in patients with Kwashiorkor, with or without infection. They speculate that cytosolic oxidative damage or membrane-bound proteins targeted for proteolytic degradation could be the possible source. In this case oxidative damage to membrane proteins, such as ion channels, might account for the abnormal ion gradients seen in the red and white cells of children with Kwashiorkor. Consequently, increased cation permeability across cell membranes damaged by oxidant stress could cause edema. Another study [19] verifies that weakened antioxidant defense systems and increased lipid peroxidation are important pathophysiologic events occurring in Marasmus. These findings suggest that a prooxidant/antioxidant imbalance might contribute to primary and severe PEM's physiopathology and that the reduced concentration of antioxidants [10,11] or its cofactors compromise their capacity to neutralize free radicals, which may eventually induce tissue damage [39-41].

In our study, six subjects had kwashiorkor, three marasmus and three marasmic-kwashiorkor. From the beginning to the end of the study period, the LPO concentration was higher in the latter type of PEM; however, it was not a significantly difference among them. Marasmic-kwashiorkor has a higher morbidity and mortality [24], probably because this type of malnutrition disrupts the adaptation to food deprivation maybe related to acute infectious diseases [42], and/or increased protein and energy demand due to cell damage provoked by the presence and persistence of oxidative stress state. It is known that in children with severe PEM the capacity to synthesize more protein may not be achievable because of a chronically poor dietary intake [43]. It has also been shown that the positive acute-phase proteins response, very important in the structure and function of the immune response, is weaker in the edematous PEM [44].

As it was pointed out above, in the present study was observed that oxidative stress, with a very high concentration of LPO (47 times higher than controls), decreased after two weeks of continuous enteral feeding. However, this high concentration of LPO persisted during the following two weeks in spite of the nutritional recovery. The LPO concentrations of malnourished children remained significantly higher than the control group even at the end of the recovery period. Still the prooxidant/antioxidant imbalance remained in spite of nutritional recovery and generous anti-oxidant supplementation, via infant formula and multivitamin drops. This is most likely due to the innate cellular immune response improvement [45] which has a defense mechanism against infection maintained ROS leukocyte production [33] and, phagocytes to stimulate fibroblast cell proliferation by producing superoxide to repair damaged tissue derived from the combination of both chronic diseases: infection and severe PEM [34]. This would mean that, in spite of the apparent clinical and anthropometrical recovery, they would have had an unsatisfactory cell function that would not have been sufficiently able to satisfy the need for high energy and nutrient intake during the last part of the nutritional recovery period.

Strengths and weaknesses:

The main strengths were: it was a study with the subject as their own control in toddlers with severe primary malnutrition treated with intensive nutritional support for four-weeks period. Another strength would be that both cases and controls were mainly toddlers between 12 to 24 months of age. The finding of a much higher concentration of lipoperoxides at the beginning and end of the nutritional recovery period in cases vs controls ($p<0.001$) confirmed the high oxidative stress that seriously affects severely malnourished children even at the end of the recovery period. We consider that there would exist certain weaknesses as the difficulty for rejecting the null hypothesis with a p-value of 0.05; that happens for a potential type II error caused by the wide variability in the concentration of lipoperoxides, especially in malnourished children at the beginning, at two weeks and at the end of the study ($p = 0.06$ and $p = 0.08$ respectively). Also, it was not possible to follow cases for a long time to set the time required for the disappearance of the excess of indicators of oxidative stress; and, finally, another possible weakness would be that the average age of severe malnutrition was significantly lower than in controls, although both groups were mainly toddlers.

Conclusion:

During nutritional recovery, a frank trend of improvement in the oxidative stress markers was observed particularly in the first two weeks of continuous enteral feeding. We were not able to ascertain if this development was due to nutritional support or to physiological changes that characterize the beginning of nutritional recovery. With these results it is difficult to speculate how many weeks or months would be needed, to eliminate oxidative stress and balance the prooxidant/antioxidant status in severely malnourished infants and children once they have recovered nutritionally. It would be necessary to carry out a follow up study to predict whether this potentially prolonged oxidative stress (30 times higher than controls at the end of the recovery period in the study) after weight for height catch up, might produce irreversible effects in cell function or in protective defense mechanisms and cell metabolism in children with primary and severe protein energy malnutrition.

Acknowledgement:

The authors gratefully acknowledge the nursing assistance and dedication shown by Isabel Ibarra Gutiérrez and María Martha Ruelas Buenrostro. We also thank to Mrs Joyce Jackson for the correction and grammatical editing of this document in English.

Conflict of interest:

We affirm that there are not financial or other contractual agreements that might cause conflicts of interest or be perceived as causing conflict of interest. All authors declare that there are not other sources of funding for research other than that reported in our manuscript with no potential conflicts of interest.

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