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Original/*Pediatría*

Immune response of severe malnutrition children treated according to the protocol of the World Health Organization

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Abstract

The aim of the study was to compare the innate immune system of severely malnourished children admitted to the Instituto de Medicina Integral Professor Fernando Figueira and treated according to the protocol of the World Health Organization (WHO) at admission and discharge. An experimental study was conducted with 20 children under two years of age. Ten of them had severe malnutrition and ten were a control group. The malnourished group consisted of hospitalized infants and it was submitted to WHO's protocol. Children with HIV and re-admitted during the study period were excluded. A blood sample was taken at admission and at discharge. Later, an analysis of blood leukocytes, adherence index, phagocytic capacity, production of free radicals superoxide and nitric oxide was performed. Patients with severe malnutrition at hospital discharge showed improved phagocytic function, release of oxygen radicals and reduction of the number of lymphocytes when compared to the time of admission. When compared to the control group, patients at hospital discharge had lower lymphocyte values and lower production of free radicals. Thus, it can be concluded that the duration of hospitalization was insufficient to restore cell-mediated immunity and microbicide activity.

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Key words: *Severe malnutrition. WHO Protocol. Immune system.*

RESPUESTA INMUNE DE NIÑOS CON DESNUTRICIÓN GRAVE TRATADA SEGÚN EL PROTOCOLO DE LA ORGANIZACIÓN MUNDIAL DE LA SALUD

Resumen

El objetivo del estudio fue comparar el sistema inmune innato de niños con malnutrición grave ingresados en el Instituto de Medicina Integral Professor Fernando Figueira, tratados de acuerdo con el protocolo de la Organización Mundial de la Salud (OMS), al ingreso y al alta hospitalaria. Se llevó a cabo un estudio experimental con 20 niños menores de dos años de edad, 10 con malnutrición grave y 10 niños del grupo de control. El grupo de malnutridos se compuso de lactantes hospitalizados y sometidos al protocolo de la OMS. Se excluyeron los niños afectados por el HIV y los readmitidos durante el período del estudio. Se recogió una muestra de sangre al ingreso y otra al alta, y posterioriormente se realizó el análisis del perfil leucocitario, y el índice de adherencia, la capacidad fagocítica y la producción de los radicales libres superóxido y óxido nítrico. Los pacientes con malnutrición grave en el alta hospitalaria mostraron mejoría de la función fagocítica, la liberación de radicales oxidantes y la reducción del número de linfocitos en comparación con el ingreso hospitalario. En comparación con el grupo de control, los pacientes en el alta hospitalario presentaron valores más bajos de linfocitos y de producción de radicales libres. Por lo tanto, se puede concluir que el tiempo de hospitalización fue insuficiente para restablecer la inmunidad mediada por células, así como para restaurar la actividad microbicida.

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Palabras clave: *Malnutrición grave. Protocolo OMS. Sistema inmune.*

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Abbreviations

AI: Adherence index.
HBSS: Hanks balanced salt solution.
HIV: Human Imunnodeficiency Virus.
IMIP: Instituto de Medicina Integral Professor Fernando Figueira.
NO: Nitric Oxide.
RPMI: Roswell Park Memorial Institute.
O_2^-: Superoxide Radicals'.
WHO: World Health Organization.

Introduction

Malnutrition occurs due to the deficiency, transport or use of nutrients, and it affects with a higher frequency infants and preschoolers due to their greater vulnerability[1]. Malnutrition, in turn, interferes with the growth and development of children, and compromise all organs and systems, including the immune system[2].

In malnourished children, the number of leukocytes[3], the mobilization of phagocytes to the inflammatory focus[2], the stage of phagocytosis[4] and intracellular digestion are affected[5], compromising the first line of defense and increasing susceptibility to infections. Thus, the majority of children with a low weight enters a cycle of malnutrition and infection[5] often difficult to reverse. This cycle repeats itself until the clinical deterioration becomes fatal or causes irreversible effects on the growth and development of the child[6].

This characterizes one of the most important factors by which child malnutrition is responsible for high rates of morbidity and mortality[7]. In 2010, a worldwide systematic review found that of 7.6 million deaths of children under five years of age, 64% (4,879 million) were caused by infectious diseases and 40.3% occurred in neonates, with a higher prevalence of pneumonia (14.1%) and diarrhea (9.9%)[8]. High mortality rates associated with malnutrition in developing countries results from potentiating effects of the disease, from inappropriate practices of diagnosis and from treatment management of malnourished children[8,9].

In order to reduce high mortality rates in hospitals, the World Health Organization (WHO) published in 1999 a Manual for the management of severe malnutrition. This protocol provides practical guidelines for treatment in order to promote the best available therapy to reduce the risk of death, shorten hospital stay and facilitate rehabilitation and full recovery[9].

Several countries have adopted the WHO protocol after its publication. Worldwide statistics show an average reduction of hospital mortality after its use[10,11,12,13]. In the Brazilian Northeast, specifically in Recife city, the Instituto de Medicina Integral Professor Fernando Figueira (IMIP) has implemented the Manual from December 2000. In the following year, there was a reduction in hospital mortality rate of children treated according to manual guidelines from 33.8% to 16.2%[13].

It is well documented in the literature that severe malnutrition in childhood affects immune activity. However, few studies report the time required to restore the immune system. Therefore, this study aims to compare innate immune systems of severely malnourished children treated according to WHO protocol guidelines on admission and discharge.

Methods

An experimental study with 20 children under two years old, evaluated up to 24 hours of hospital admission. As inclusion criteria were included: children who started the WHO 's nutritional rehabilitation protocol by malnutrition or related diseases, those that showed nutritional status classification lower score than Z- less than three standard deviations according to weight for height. As exclusion criteria: children rehospitalized during the study period, thus avoiding possible interference in the alternating exposure to the WHO protocol; and those living with Human Immunodeficiency Virus (HIV), to minimize immunological changes resulting from the disease. The control group consisted of healthy infants who had the same average age of the children in the study and were evaluated on the care of the childcare.

The study was approved by the Ethics committee in Research of IMIP n. 1437, in accordance with Resolution 466/12 of the National Health Council. Parents or guardians signed the informed consent form after agreeing to participate.

Blood collection

Blood collection was performed by specialists of the IMIP Laboratory upon request of the physician in charge at two moments: first, within 24 hours of the protocol onset and second, at the day of hospital release. There was no need for fasting to blood collection. Thus, about 4 ml blood sample was collected by venipuncture for each patient in vacuum *containers* containing 15% EDTA.

Leukogram

The leukogram was performed by the IMIP's Hematology laboratory, which were measured automatically by the cyanometahemoglobin using a SYSMEX - XT2000i mass hemoglobimeter.

Collection of peripheral blood monocytes

The blood was diluted at 1:1 in culture medium, Roswell Park Memorial Institute (RPMI) 1640, sterile, 8 °C. To the 8 mL solution were added 4 mL HISTO-

PAQUE (1077-SIGMA) being later centrifuged for 30 min at 3000 rpm. Plasma was aspirated and the layer formed by peripheral blood mononuclear cells was collected and transferred to another tube, and then centrifuged for 10 min at 1500 rpm. The supernatant was aspirated and discarded. Subsequently, two washes of sediment were performed with RPMI 1640, centrifuging for 5 min each time. The supernatant was discarded and the sediment re-suspended in 2 mL of RPMI 1640 complete culture medium, containing 3% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mcg/mL streptomycin). In the end, cell counting was performed by hemocytometer by placing the cell suspension and trypan blue dye at 0.3% concentration and diluted from 1:10. Cell concentration was adjusted in all experiments to $1x10^6$ cells/mL. For the experiments, monocytes were obtained after the time of cell adhesion, with removal of non-adherent cells, thereby isolating only monocytes.

Assessment of Adherence index (AI) in monocytes

Aliquots of the non-adherent cells suspension, after the first hour of culture, were added to the trypan blue and counted by hemocytometer. The AI was calculated using the formula AI = 100 - (number of non-adhered cells/mL ÷ initial number of macrophages) x 100.

Evaluation of the rate of phagocytosis

To assess the rate of phagocytosis, we used *Saccharomyces cereviseae* yeasts. Fungi were washed three times with Hanks balanced salt solution (HBSS). Subsequently, $1x10^8$ fungi/mL were mixed in suspension containing $1x10^6$ cells/mL using the RPMI 1640. Mononuclear cells and fungi were distributed on slides for microscopy and incubated at 37 °C, humid atmosphere, for one hour. Afterwards, the slides were washed with HBSS, dried at room temperature and stained with Diff-Quick set (Baxter Dade, Dudunen, Switzerland). The reading was carried out under optical microscope, 1,000 times magnification. The rate of phagocytosis was obtained as percentage by counting monocytes that phagocytosed the fungus in a total of 100 cells.

Analysis of nitric oxide (NO) release

The production of nitric oxide was determined from the supernatant of cultured cells and according to the method described by Feder et al., (1994). The cells were left at $2x10^6$ cells/ml per well of Falcon-type plate in incubator. After 24 hours at 37 °C in an oven with 5% carbon dioxide (CO_2) atmosphere, 500 μl of the supernatant of cell cultures was collected and added to 50 mL of Griess reagent (1.5% sulfanilamide in 5% H_3PO_4, 0.1% in naftiletilene diidrocloride diamine H_20). Subsequently, the reading was performed on a spectrophotometer at 540 nm. The nitrite concentration was calculated by the sodium nitrite standard curve ($NaNO_2$) and data expressed in μM/mL nitrite/nitrate.

Analysis of the superoxide radicals' (O_2^-) production

To evaluate the superoxide production formed, it was prepared a discontinuous analysis system examined evaluated every 1 hour during 2 hours. For preparing this system was used cultured monocytes (2ml/well with $1x10^6$ cells/ml RPMI 1640) plus cytochrome and PMA (phorbol-PMA myristate acetate, Sigma) prepared in a 3000μg/ml concentrated solution in sodium sulfoxide dimethyl (DMSO, SIGMA). The PMA used to stimulate the cells was diluted to 2μg/ml in 2145 ml of Hanks (HBSS balanced salt solution, GIBCO) and placed in wells of the culture plate. Subsequently, 700 μL aliquots were collected and placed in eppendorf tubes at time zero, which corresponded to the blank. Immediately after collection, aliquots were paralyzed in ice bath. Subsequent samples were hourly collected at under the same conditions. Afterwards, the reading was performed in spectrophotometer at 550nm. Final results were expressed as nmol/ml.

Statistical analysis

The Student t test was used for comparison among experimental groups. To compare the changes occurred after treatment with the WHO protocol, the Student t paired test was used. Results are presented as mean ± standard deviation. Statistical significance was considered by assuming a 5% critical level ($p < 0.05$) in all cases. Data were analyzed by the Graphpad Prism software version 5.1.

Results

Severely malnourished children had an average of 12.7 days of hospital stay, ranging from 6 to 26 days. The main diagnoses, in addition to severe malnutrition, were respiratory tract infection (54.5%), diarrhea (27.3%) and other (18.2%).

Figure 1 shows that patients with severe malnutrition showed no change in the number of total leukocytes if compared to the group of well-nourished children. This difference was not observed after introducing the WHO protocol in the hospital. Regarding the number of lymphocytes, these were significantly different only in malnourished patients at discharge if compared to well-nourished children, where lower numbers of these cells were shown after treatment using the WHO protocol. There was no difference between groups in

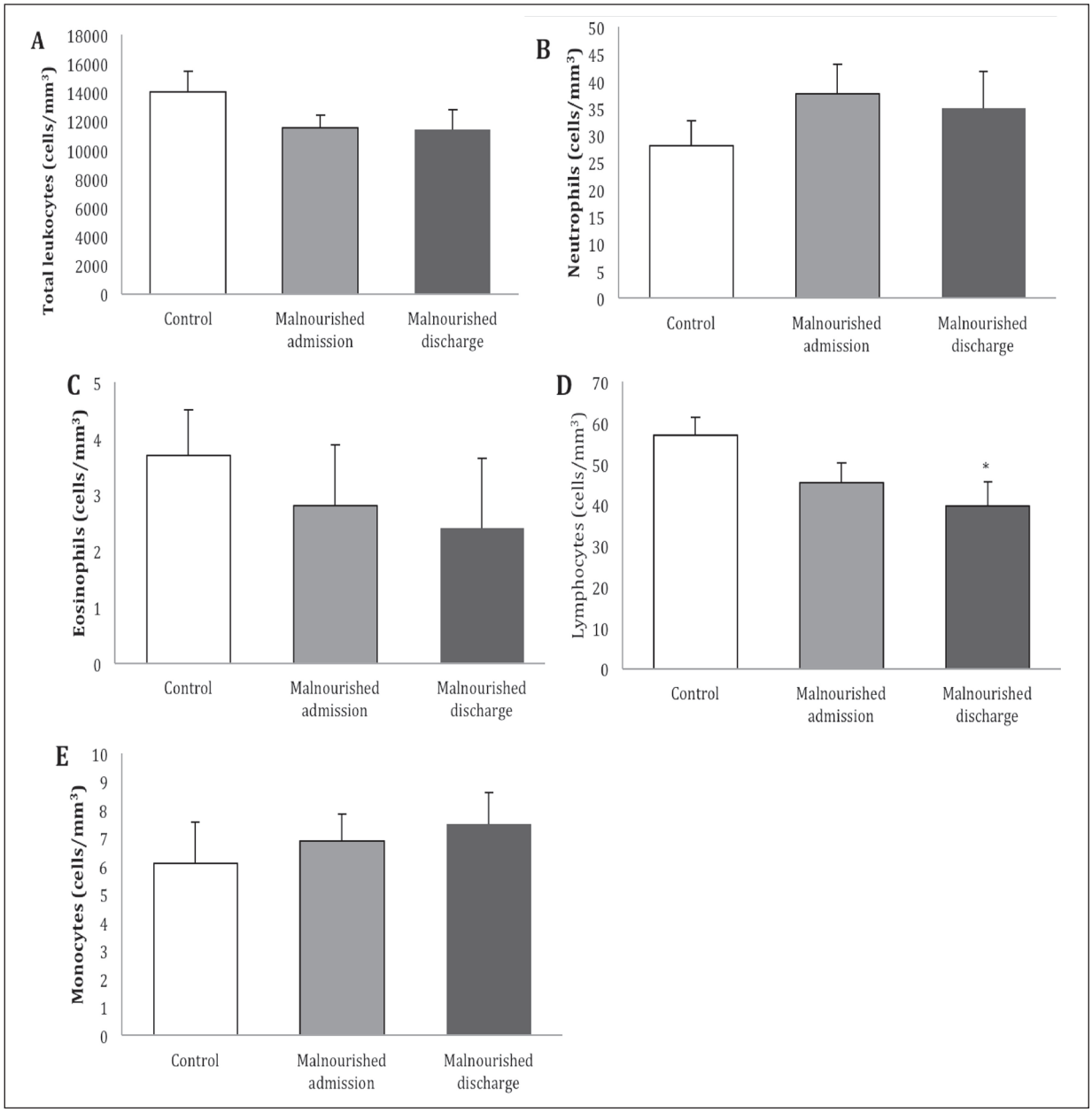

Fig. 1.—A-E *Leukogram of children under two years of age treated at IMIP, Recife - Brazil, 2010.*
**Significant difference if compared to the control group (Student t test), with p=0.0338.*

the number of segmented neutrophils, eosinophils and monocytes.

The percentage of cell adhesion of monocytes did not differ between the groups, as can be seen in Figure 2. The phagocytosis in response to *S. cerevisiae* was lower (p= 0.0236) in monocytes of malnourished children than in children from the control group. After treatment using the WHO protocol, these values showed a significant improvement in their activity (p=0.005), with a phagocytic function similar to that found in healthy children.

The production of NO and O_2^- in a macrophage culture supernatant (Fig. 2) demonstrated to be very low (p<0.05) in malnourished children if compared to healthy children after *in vitro* stimulation. At the time of hospital discharge, there was a significant increase in production when compared to the time of admission. However, these values remained lower (p<0.05) when compared to the control group.

Discussion

The impairment of the immune system has been observed in children with nutritional deficiencies, especially malnutrition[14], as nutrient deficiencies and

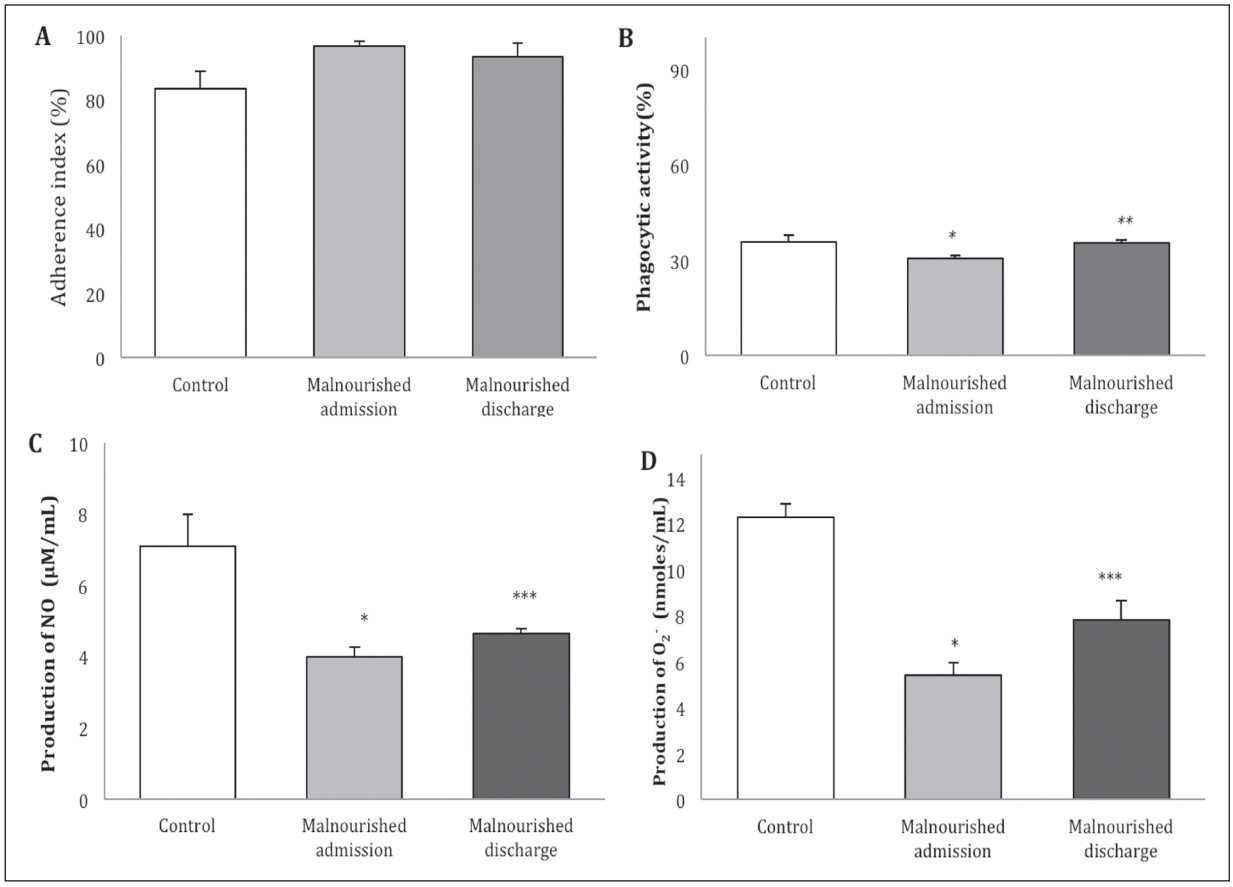

Fig. 2.—Immune response of children under two years of age treated at IMIP, Recife - Brazil, 2010.
(A) *adherence index,* (B) *phagocytic activity,* (C) *nitric oxide (NO) production,* (D) *superoxide (O_2^-) production.*
* *Significant difference with control group (Student t test), with p<0.005.* ** *Significant difference with malnourished admission (paired Student t test) with a* p<0.005. *** *Significant difference with control group (Student t test) and with malnourished admission (paired Student t test) with a* p<0.005.

changes in nutritional status can negatively influence the immune response and host resistance to infection[15]. Malnutrition increases susceptibility and severity of diseases caused by bacteria, viruses, parasites and other pathogens[16], probably due to an atrophy of central and peripheral lymphoid organs[17,18], with changes in leukocyte response to infections due to the reduction in the reserve compartment of the bone marrow[19].

No significant changes in the number of white blood cells were found in severe malnutrition, in accordance with Nájera et al., who evaluated malnourished and well-nourished children with gastrointestinal and respiratory infection and did not observe white blood cell count changes when compared to the control group[20]. On the other hand, an study with animals found a decrease in leukocyte numbers and leukocyte migration in undernourished infants resulting from hypocellularity in the bone marrow and in the peripheral blood[3]. There is a report of leukocytosis and leukopenia in malnourished patients. However, leukopenia is always present in situations where malnutrition is not accompanied by other diseases[19].

Regarding the assessment of the number of lymphocytes after treatment, according to WHO guidelines, there was no significant change in the number of cells. However, when compared to well-nourished children, the number of lymphocytes was reduced. It is suggestive, therefore, that malnourished children hospitalized after resolution of infection/inflammation showed decreased values of lymphocytes by the reduction in their recruitment cells. This finding may be explained by the fact that severe malnutrition during childhood affects the development of the thymus, which compromises immunity in children through a long-term reduction in lymphocyte count[21].

The thirteen-day hospital stay, on average, proved to be insufficient to maintain cellular levels of lymphocytes in the blood similar to well-nourished children, a damage that may be the result of protein deficiency or deficiency of elements such as iron, zinc and copper, or due to hormonal imbalance involving adrenaline, insulin, thyroxine or cortisol[22]. Added to this, the need for a period of approximately two months in nutritional treatment, so that there is a complete recovery of the thymus, and thus appropriate and ma-

tured levels of lymphocytes in the bloodstream, was reported[23,24].

There was no impairment of cellular adhesion between the groups, which may correspond to an efficient allocation to the infection site during the stage that follows macrophage adherence, i.e., phagocytosis. Morais et al. found similar results, suggesting that malnutrition may affect defense mechanisms that depend on the activation of macrophages and not necessarily before activation[25]. Divergent findings from other studies, where both a decrease in adhesion of leukocytes[26] as well as an increase [27] in the presence of malnutrition, are reported.

The phagocytic activity in malnutrition was reduced, consistent with literature findings[4,28]. It demonstrates that the phagocytic function is susceptible to malnutrition and suggests that functional alterations of the macrophages may be involved in the immune response failure[30], since, in response to infection, the immune system initially executes the innate mechanism[6], being the macrophages the most prominent cell derived from blood monocytes to migrate to the tissue[28]. After phagocytes reach the inflammatory site, the phagocytosis mechanism becomes the main line of defense against pathogens that exceeded mechanical barriers. After adhesion to the surface of phagocytes, the microorganisms are internalized and subsequently destroyed[29].

At hospital discharge, phagocytosis of patients showed an increased activity, with a significant similarity to the phagocytic ability of well-nourished children, in accordance with Vásquez-Garibay et al., in their work, twelve malnourished children were evaluated from three to eighteen months of age after four weeks of nutritional recovery. No significant improvement of the phagocytic ability of neutrophils was observed[27,28].

Concerning free radicals, NO and O_2^-, they showed a decreased production in severe malnutrition, a result also found by other researchers[5,25], suggesting higher deficits in the microbicide function of macrophages. The activation of macrophages after internalization of pathogens induces intracellular production of potent microbicide products, which are responsible for the destruction of the phagocytized microorganisms[31]. The evaluation of the synthesis of oxidant substances is considered sensitive and convenient to monitor the function of macrophages[32], since a low synthesis of these free radicals may result in bacterial resistance, leading to proliferation and compromising this defense stage[33].

Both NO and O_2^- showed a significant increase after treatment using the WHO protocol in hospital. However, the production of these free radicals was reduced when evaluating macrophages of well-nourished child, which may compromise the adequate microbicide activity against infectious agents. The patients may improve unfavorably due to infections, even if leukocytes contain pathogens that have been ingested by phagocytosis, if they are not destroyed effectively.

A study conducted by MacMurray et al. found that a four to six-week period is sufficient to the improvement of immunological parameters in malnourished children, demonstrating that the abnormality in the destruction of pathogens is due to malnutrition[34]. A similar result found by Vásquez-Garibay et al., in which the microbicide activity of studied phagocytes was successfully achieved after four weeks of nutritional recovery[28]. The hospital stay in our study, which consisted of about thirteen days, proved to be insufficient to reverse microbicide capacity.

During the treatment of severe malnutrition, immune recovery should be considered as a part of disease management, since the infection, among other factors, creates an additional demand of an already depleted nutritional status due to the need for rapid protein synthesis and cell proliferation for host defense[35]. The discharge criteria, successful treatment of the reason of admission and a 10g/kg weight gain for three consecutive days did not follow a full immunological recovery of these patients. Chevalier et al. suggest evaluation of arm circumference and thymus ultrasound as means to assess the full recovery of the patient, as well as one month of hospitalization, so that the child is able to safely face a pathogenic environment [24].

Conclusion

The data lead us to infer that severe malnutrition adversely affects the immunity of children, and the use of WHO protocol as a treatment for severe malnutrition proved to be a significant improving mechanism of the phagocytic function. However, the hospital stay was insufficient to restore cell-mediated immunity, as shown by evaluating the number of lymphocytes as well as restoring microbicide activity. Thus, it is necessary to evaluate other criteria in addition to those already used to ensure that children with severe malnutrition do perpetuate in the malnutrition-infection cycle.

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