Long-Term Effect of Early Protein Malnutrition on Growth Curve, Hematological Parameters and Macrophage Function of Rats

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Summary To evaluate the long-term effect of mild-early maternal protein malnutrition on weight gain, hematological parameters and macrophage function in rats at adult age, we compared rats whose dams were fed diets containing either 9.5% (low protein—LPD) or 23% protein (normal—NPD) for the first 12 d of lactation. At 80 d of age, the functions of spreading, phagocytosis and killing Candida albicans were determined in resident peritoneal macrophages, whereas leukocytes and red blood cells were counted in peripheral blood. The number of resident peritoneal macrophages from LPD was the same as from NPD, but the ability of spreading and phagocytosing opsonised yeast was impaired. Besides, they were not able to block the germ tube formation or kill C. albicans to the same extent as in the control group. The low protein diet produced a significant reduction in the pups’ growth and in hematological parameters although no difference was found in leukocyte counts. Taken together the data suggest that protein malnutrition during early lactation induces permanent alterations in macrophage function, body composition and hematological status, which are not restored completely even after a normal protein diet is supplied.

Key Words protein malnutrition, macrophages, phagocytosis, hematological parameters

Nutrition is a basic human need, and although malnutrition has been declining in some regions throughout the world, it remains high in many developing countries, thus affecting a vast number of children (1).

Severe protein energy malnutrition in early life can permanently change the growth and form of the body and a whole range of its structures and functions; however, the long-term effect of malnutrition depends on the stage and intensity of its occurrence (2). The alterations include linear growth retardation and a significant reduction in body weight, reduced cell number in tissues and organs leading to the modification of organ structure, selection of particular clones of cells and changes in metabolic differentiation (3, 4). Some researchers had suggested that undernutrition and low birth weight early in life was associated with increased susceptibility to chronic diseases in adulthood, e.g., impaired glucose tolerance, type 2 diabetes, and cardiovascular diseases (2, 3, 5).

In epidemiological, clinical and experimental models, it becomes evident that undernutrition, immunosuppression and infection frequently exist simultaneously.

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one augmenting the severity of others, predisposing to infection, particularly by intracellular pathogens (6–8). Undernutrition impairs various functions of the immune system, causing alterations in different components of both specific and nonspecific immunity (9–11). The consequences of malnutrition in the immune response during pregnancy and after birth are well documented, and the severity, malnutrition techniques, time of onset and duration of malnutrition and duration of nutritional recovery may determine the extent of the immune alteration (4). Malnutrition in early life can result in neuroendocrine immune dysfunction of the pups affecting profoundly the endocrine and immune functions in adulthood, which leads to immunosuppression (3). Protein malnutrition induces alterations in composition and fluidity of the cellular membrane with implications in signal transduction and cell functions, including cell permeability and expression of the receptors on the cell surface. These alterations may affect the fatty acid content of membrane phospholipids, in particular arachidonic acid release and PGE2 production. The known biological activities of PGE2 suggest several mechanisms by which it may modulate the immune system, inducing immunosuppression and progression of infectious agents such as Candida albicans (7–9). In kwashiorkor, a diet-associated elevation of PGE2 has been suggested as the main cause of infections in children by depressing cellular immunity (12, 13).

Fixed immune tissue macrophages play a central role...
in defence against microbial infection by their microbial activity and presentation of processed antigens to lymphocytes, forming an important link between the innate and acquired immune system (14). Macrophages express more than 40 cell-surface receptors and they release more than 100 soluble factors, most of which are involved in phagocytosis and in the regulation of the immune response (15, 16). Since macrophages initiate and orchestrate the immune response, and because identification of defective function following undernutrition is critical to the understanding of undernutrition-induced immunodeficiency (7–9), our aim was to evaluate the long-term effect of a mild early undernutrition on the weight gain, hematological parameters and macrophage function of rats.

MATERIALS AND METHODS

Animals and diet. Wistar rats were coupled and pregnant females placed in individual cages at 23°C on a 12 h light/dark cycle (7 am–7 pm). They were fed a 23%-protein commercial diet. After birth, females were divided into two groups, malnourished or low-protein diet (LPD) and control or normal-protein diet (NPD), each litter being reduced to six male rats to guarantee the same lactotrophic capacity. Dams of the NPD group were fed the normal protein diet (23% protein) during lactation. The normal diet consisted of sucrose 20%, cornstarch 47.4%, casein 23%, liquid vitamin mixture 1%, cod liver oil 1.6%, soya-bean oil 4.8% and a mixture of mineral salts 3.2%. Total protein mixture amounted to 23% (casein). Dams of the LPD group received a 9.5% protein diet (casein) during the first 12 d of lactation and then a 23% protein diet. The low protein diet consisted of sucrose 20%, cornstarch 59.9%, casein 9.5%, vitamin liquid mixture 1%, cod liver oil 1.6%, soya-bean oil 4.8% and a mixture of mineral salts 3.2%. Total protein mixture amounted to 9.5% (casein). Diets were isocaloric and the decrease in body weight in the LPD group amounted to 23% (casein). Dams of the LPD group received a 9.5% protein diet (casein) during the first 12 d of lactation and then a 23% protein diet. The low protein diet consisted of sucrose 20%, cornstarch 59.9%, casein 9.5%, vitamin liquid mixture 1%, cod liver oil 1.6%, soya-bean oil 4.8% and a mixture of mineral salts 3.2%. Total protein mixture amounted to 9.5% (casein). Diets were isocaloric and the decrease in casein was replaced by an increase in cornstarch. Weaned on the 21st day, male rats of both groups were separated from dams and were given water and the regular diet ad libitum till the 80th day. The same conditions of temperature and photoperiodic cycle were maintained. The protocol of this study was approved by the Ethical Committee of Universidade do Oeste Paulista (Unioeste), Presidente Prudente, Brazil.

Macrophages. Peritoneal resident macrophages were harvested from 80-d-old NPD and LPD rats. Animals were anesthetized with Pentobarbital 3% (Fon-toven, São Paulo, Brazil) and the peritoneal washing was performed with 8.0 mL RPMI 1640 medium supplemented with N-hydroxymethylpiperazine-N-ethanesulphonate (HEPES) 12 mM and with bovine albumin fraction V 1%. After centrifugation, peritoneal cells were re-suspended in 2.0 mL RPMI and counted in a Neubauer chamber. The macrophages were adjusted to 2×10^6 cells/mL and a 200 µL aliquot of this suspension was left to adhere to glass coverslips for 1 h at 37°C/5% CO_2 in a humidified atmosphere. Viability of macrophages was controlled with Tripan Blue and only those with viability higher than 95% were used. Reagents were obtained from Sigma Chemical Co (St. Louis, MO).

Candida albicans culture. C. albicans strain 577 was maintained in Sabouraud agar (Difco Laboratories, MI) at room temperature. A loopful of this culture was transferred to a tube containing Sabouraud/dextrose broth (Difco Laboratories) and cultured at 24°C overnight in a BOD. Yeast was washed three times by centrifugation (2,000 ×g for 5 min) in a phosphate buffer solution pH 7.0 (PBS) and re-suspended in RPMI buffered pH 7.0 with Heps 12 mM plus bovine albumin 1%. The same preparation was used for phagocytosis in all assays.

Hemogram. The hemogram was undertaken by a flow cytometer flux counter (Pentra 80, Horiba Diagnostics, Montpellier, France), and the leukocyte differential counting was compared to direct microscopic observation in blood smears, taken as the reference method.

Phagocytosis and germ tube formation. In vitro phagocytosis of C. albicans by macrophages was carried out in 3 different assays: one containing C. albicans/RPMI suspension; the second containing C. albicans/RPMI suspension and 2.5% heat-inactivated serum at 56°C for 30 min (yeast+HS); the third containing C. albicans/RPMI suspension with 2.5% fresh serum (yeast+FS). Sera were obtained from normal adult male Wistar rats bred in the same conditions as other experimental animals. Separately, 1 mL of each yeast suspension was added to the macrophages’ monolayer already adhering to the coverslip in a 1:10 macrophage/Candida ratio. Coverslips were then incubated for 30 min at 37°C/5% CO_2 in a humidified atmosphere. After incubation they were washed three times with PBS to remove non-adhering cells, fixed with methanol for 15 min and stained with Hematoxylin/Eosin. Phagocytic index was determined under a 100× optic microscope and calculated as the percentage of macrophages that phagocyted one or more C. albicans. Two hundred macrophages were counted per slide. Results were expressed by phagocytic index, which is expressed as the percentage of infected macrophages times the average number of yeasts per macrophage.

The C. albicans germ tube formation inside the macrophages was determined in yeast suspensions in RPMI, HS, and FS. One milliliter of each suspension was added to the macrophage-adhering coverslip (MO-Candida ratio 1:10) and incubated for 30 min at 37°C/5% CO_2 in a humidified atmosphere. After incubation the non-adhering cells were removed and the coverslips were fixed and stained with Hematoxylin/Eosin. The fields of the coverslips were randomly selected and the percentage of macrophages with germ tube was microscopically determined in 200 cells.

Killing of C. albicans. The ability of resident peritoneal macrophages to kill C. albicans was studied by coculturing 200 µL of a suspension of macrophages in RPMI (2×10^6/mL) with 200 µL of a suspension of non-opsonized C. albicans in RPMI, in a 96 well plate (MO-
Candida ratio 1:1) for 3 h at 37°C/5% CO₂ humidified atmosphere. The experiments were plated in triplicates. At the end of the incubation period the macrophages-Candida-containing medium of each well was vigorously pipetted to remove adherent macrophages. The content of each well was then transferred to eppendorf tubes. Afterwards, each well was supplemented with 200 µL of a washing solution (H₂O+Triton X-100, 0.05%), vigorously pipetted and the content transferred to eppendorf tubes (four times) to achieve a final volume of 1 mL. The suspensions were then vortexed for 30 s, plated in Agar Sabouraud and incubated for 24 h/37°C. To determine candidacidal activity of macrophages, the value obtained for CFUs was subtracted from control tubes containing only C. albicans.

**Spreading technique.** Macrophage spread was evaluated according to Rabinovitch et al. (17). Two hundred microliters of macrophages (2×10⁶/mL) was placed in coverslips and incubated for 15 min at 37°C/5% CO₂ in a humidified atmosphere. The non-adherent cells were removed and the coverslip containing macrophages cultured in 199 culture medium (Sigma, St. Louis, MO, USA) at 37°C/5% CO₂ in a humidified atmosphere for 60 min. The medium was removed and adherent cells fixed in 2.5% glutaraldehyde, stained with hematoxylin-eosin, and examined under a light microscope. The percent of macrophage spread was determined in 100 cells.

**Statistical analysis.** Data are expressed as mean±SE of at least three experiments, and statistical analysis was performed using GraphPad Software (San Diego, CA). Student’s t test was used when comparing two groups and ANOVA/Bonferroni test when comparing more than two groups. A p value of less than 0.05 was considered statistically significant.

**RESULTS**

**Body weight and food intake**

As expected, dams on the 9.5% protein diet (LPD) had a significant reduction in food intake and body weight (Fig. 1A and B, respectively) compared to those on the diet containing 23% protein (NPD). After 3 wk, the mean weight of the dams on the 9.5% protein diet was 191.5±11.38 g and the lower food intake was 37.71±9.46 g. The mean weight of the dams on the 23% diet was 301.06±13.18 g (p=0.003) and the lower food intake was 130.1±10.98 g (p=0.003). Figure 2 shows the changes in body weight of the suckling offspring from the two experimental groups. Offspring from rats fed with 9.5% protein diet showed a significant reduction in weight gain. After the treatment, at 80 d of age, the rats had not recover the weight that had been reduced 11.5% compared to the control group. On the other hand, the pups from the well-nourished dams gained weight linearly throughout the experiment (260.60±10.33 vs. 294.43±3.76). Rats from LPD and NPD groups were different at *p<0.05 level up to 2 wk of age.

**Macrophage phagocytosis and killing of C. albicans**

Resident peritoneal macrophages from rats on low-protein diet presented lower phagocytic activity against C. albicans opsonised with fresh normal rat serum than those on the normal protein diet (*p= 0.016) (Fig. 3A). On the other hand, no difference was found in the LPD
and NPD groups when comparing ingestion of yeast treated with heat-inactivated rat serum.

Killing of non-opsonised *C. albicans* is shown in Fig. 3B. Macrophages derived from NPD group killed about 24% of *C. albicans* yeasts. However, the killing of *C. albicans* by macrophages derived from the LPD group was significantly impaired and remained around 10% \( (p=0.04) \).

**Macrophage counts and spreading**

No difference was found in the yield of resident peritoneal macrophages harvested from the cavity of rats on a low-protein diet when compared to those on a normal-protein diet \( (n=6) \) \( (17.02 \pm 2.55 \times 10^6 \) vs. \( 12.18 \pm 1.48 \times 10^6 \) macrophages/mL respectively). But malnutrition induced a significant reduction \( (p=0.03) \) in the

![Fig. 3. Phagocytosis and killing of *C. albicans*. Macrophages from NPD and LPD rats were cocultured with live *C. albicans* treated with heat-inactivated normal rat serum (open bars) and with fresh normal rat serum as complement source (stacked bars); phagocytic index was determined 30 min later (A). The results were obtained by counting at least 200 macrophages per duplicate coverslip and are expressed as mean±SE of three independent experiments. *p=0.016 comparing macrophages treated with fresh serum between NPD and LPD groups. For determination of candidacidal activity, macrophages (2\( \times \)10^6/mL) were infected with non-opsonised *C. albicans* (ratio 1:1, yeast:MO). The candidacidal activity was determined after 3 h of coculture. Data represent mean±SE of 5 independent experiments. *p=0.04 comparing control vs. undernourished rats.](image)

![Fig. 4. Evaluation of function of resident peritoneal macrophages of NPD and LPD rats by the cell spreading method. For spreading, macrophages (2\( \times \)10^6/mL) were cultured on glass slides with 199 media. Adherent cells were fixed and stained. Results are expressed as mean±SE of 6 independent experiments. *p=0.03.](image)

![Fig. 5. Formation of *C. albicans* germ tube within macrophages in vitro. Data were obtained after 30 min of phagocytosis. Results are expressed as means±SE of 6 experiments. C: control, HS: heat inactivated serum and FS: fresh serum. NPD (open bars) and LPD (stacked bars). *p<0.0001 (ANOVA).](image)

**Table 1. Long-term effect of early protein malnutrition on hematological parameter.**

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>NPD mean±SE</th>
<th>LPD mean±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (M/( \mu )L)</td>
<td>8.31±0.16</td>
<td>7.58±0.34</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>16.37±0.39</td>
<td>14.62±0.60*</td>
</tr>
<tr>
<td>Packed cell volume (%)</td>
<td>52.03±1.95</td>
<td>43.08±1.95*</td>
</tr>
<tr>
<td>Mean corpuscular volume (fL)</td>
<td>62.17±1.22</td>
<td>56.83±0.30*</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin (pg)</td>
<td>19.72±0.17</td>
<td>19.35±0.11</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (g/dL)</td>
<td>31.78±0.43</td>
<td>34.10±0.22*</td>
</tr>
</tbody>
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Results are expressed as mean±SE of 6 independent experiments. *p<0.05 comparing hematological parameters between NPD and LPD groups.
spreading capacity of macrophages of undernourished animals when compared to controls (Fig. 4).

**Germ tube formation within macrophages**

In order to evaluate the capacity of macrophages to block the intracellular growth of yeast cells in vitro, we analysed germ tube formation inside the macrophages in yeast/RPMI suspension, yeast+HS and yeast+FS. The percentage of macrophages with germ tube was significantly greater in LPD than in the NDP group (Fig. 5). The increase was about 37% for assays without serum (\( p=0.025 \)), 46% for assays with heated inactivated serum (\( p=0.009 \)), and 31% for assays with fresh serum (\( p=0.006 \)).

**Hematological status of NPD and LPD groups**

The malnourished animals showed significant alterations in most of the peripheral red blood cell parameters evaluated (Table 1). However, no alterations were observed in the mean number, morphology or differential counts of white blood cells between control and malnourished animals (data not shown).

**DISCUSSION**

In the present study, as far as we know, it was demonstrated for the first time that mild protein malnutrition occurring in the first 12 d of lactation induced a long-term effect on macrophage function in adulthood, since experimental adults demonstrated impaired capacity of spreading, phagocytosis and killing compared to controls.

The macrophages are involved in a variety of immune functions, especially the fundamental protective capacity of engulfing and killing microorganisms (15). We showed that early malnutrition impaired the uptake of the complement opsonised *C. albicans* by the macrophages when compared to the control group, although there was no effect on phagocytosis of non-opsonised *C. albicans* between the groups. The interaction between macrophages and particles opsonised by complement depends on the expression of complement receptors on the cell surface. One of the most important findings in malnourished children is the susceptibility to bacterial infections due to immune suppression; it is caused by the decrease of complement and Fc receptor expression in macrophages (18–20). Furthermore, an efficient ingestion of *C. albicans* by monocytes/macrophages that adhere to a glass surface, as in our model, requires opsonization by serum factors such as complement C3b (21–23). Thus, the reduction of expression of complement receptors on macrophages and the necessity of opsonisation by the yeast could explain the impaired phagocytosis observed in our experiments in LPD-derived macrophages.

We also demonstrated that macrophages from undernourished animals were not able to kill the yeast to the same extent as in the control animals. Moreover, they showed about a three-fold increase in germ tube formation within the cells in experiments without serum, as well as with inactivated and fresh serum. The germ-tube formation of *C. albicans* within the macrophages could indicate the inefficiency of the cells for blocking the yeast’s growth (23–25). Spreading is an important mechanism of interaction between the phagocyte and the particle to be engulfed, and is employed largely to determine macrophage function and activation. We showed, similarly to what was described by Reynolds et al. (26) in adult undernourished animals, that early undernutrition significantly inhibited macrophage spread in adulthood. It was demonstrated in newborns and adult animals as well as in humans, that protein-deficient diets impair protein synthesis and expression, spreading, chemotaxis, phagocytosis and intracellular killing of microorganisms by macrophages (25, 27, 28, 29), but long-term effects of a short period of low protein diet early in life were scarcely reported. We did not focus on the mechanisms by which early occurrence of protein malnutrition impaired macrophage function; however, some authors have demonstrated that protein malnutrition alters the enzyme system responsible for the respiratory burst, the membrane bound NADPH oxidase as well as NO synthesis, both involved in the candidacidal activity of macrophages (7, 8, 26, 28).

Reduction in the activation of the transcription factor NF-κB involved in the expression of cytokines, chemokines, adhesion molecules and NOS-2 was observed in weanling mice subjected to protein malnutrition (30). Similar to our model, adult rats subjected to protein malnutrition during early lactation were unable to induce an acute inflammatory response against a specific antigen, with a dramatic reduction in leukocyte adhesion and down-regulation in the expression of endothelial ICAM-1 (31). As previously reported, these alterations may be linked to the increased levels of PGE\(_2\) observed in animals and humans subjected to protein malnutrition (9, 12, 13).

There are some difficulties in determining the long-term effects of malnutrition in pregnancy and after birth in clinical and epidemiological surveys. The offspring’s life-style and milieu, and the severity of malnutrition are quite different from one region to another (32). Lower concentrations of IgG and IgM immunoglobulin were found in 30 children from Israel who had suffered from protein-calorie malnutrition during their early infancy (33). In our experiments, although no difference was found either in the mean number of macrophages harvested from the peritoneal cavity or in the differential counts of peripheral blood leukocytes between NPD and LPD animals, the early undernutrition produced an alteration in red blood cells that was not completely restored even after a normal protein diet was supplied. Our findings are in agreement with those reported by Idohou-Dossou et al. (34) who observed a decrease in hematological parameters of Senegalese preschoolers who had suffered from marasmus after birth. Impairment of bone marrow cell proliferation in animals and humans undernourished in pregnancy or after birth is well documented in the literature but there are few studies exploring the long-term effects. These alterations may be due to profound changes in the bone marrow microenvironment impairing hematopoiesis and inducing modifications in cellularity and structure
of hematopoietic tissue (35).

A low protein diet restricted to the middle of the lactation phase produced a significant reduction in body weight, food intake of the dams and in pup growth, since they showed a reduction of about 11.5% in body weight when compared to the control group, an observation corroborated by other researchers (3–5). After 12 d of lactation, a normal protein diet was supplied to the undernourished dams, although their body weight decreased continuously until day 21. It is well known that low protein diets affect maternal intake and lactation, compromising growth in rats. According the National Research Council nutrient requirements of rats (36), the minimum protein requirement for adult rats is 9% and 12% for pregnant rats (nearly the content of casein utilized in our low protein diet). Different authors reported mild protein malnutrition, the reduction in the protein content in the diet from 25 to 8% casein, calorically compensated by carbohydrates, in a restricted period of life such as pregnancy or the lactating period (9, 37, 38). Rats fed such low protein diets adjust their food intake to a level just sufficient to maintain body weight and may represent a mechanism by which low-protein-fed rats partially compensate for their low protein intake (39). In addition, it was shown that refeeding of undernourished dams produces a dramatic increase in milk intake of the litters compared to that of control dams, inducing an exploitation of subcutaneous fat stores (40). Although milk intake of the pups was not determined, we can suggest that the delayed restoration in the body weight of the dams should be in part due to the increase of milk intake of the litters compared to that of control dams. In a similar experiment conducted by Massaro et al. (41), lactating Sprague-Dawley female rats receiving 12% casein showed a dramatic decrease in body weight, compared to the control dams treated with 25% casein, that was not restored until day 23.

Taken together the data suggest that protein malnutrition during early lactation induces permanent alterations in macrophage function, body composition and hematological status, which are not completely restored even after a normal protein diet is supplied.

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