Basic nutritional investigation

Malnutrition suppresses cell cycle progression of hematopoietic progenitor cells in mice via cyclin D1 down-regulation

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ABSTRACT

Objective: Protein malnutrition (PM) often is associated with changes in bone marrow (BM) microenvironment leading to an impaired hematopoiesis; however, the mechanism involved is poorly understood. The aim of this study was to compare the cell cycle progression of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) and evaluate the cell cycle signaling in malnourished mice to assess the mechanism of cell cycle arrest.

Methods: C57Bl/6J mice were randomly assigned in control and malnourished groups receiving normoproteic and hypoproteic diets (12% and 2% protein, respectively) over a 5-wk period. Nutritional and hematologic parameters were assessed and BM immunophenotypic analysis was performed. Cell cycle of HPC (Lin−) and HSC (Lin− Sca-1− c-Kit+) were evaluated after 6 h of in vivo 5-bromo-2′-deoxyuridine (BrDU) incorporation. Cell cycle regulatory protein expression of HPC was assessed by Western blot.

Results: Malnourished mice showed lower levels of serum protein, albumin, glucose, insulin-like growth factor-1, insulin, and higher levels of serum corticosterone. PM also caused a reduction of BM myeloid compartment resulting in anemia and leukopenia. After 6 h of BrDU incorporation, malnourished mice showed G0-G1 arrest of HPC without changes of HSC proliferation kinetics. HPC of malnourished mice showed reduced expression of proteins that induce cell cycle (cyclin D1, cyclin E, pRb, PCNA, Cdc25a, Cdk2, and Cdk4) and increased expression of inhibitory proteins (p21 and p27) with no significant difference in p53 expression.

Conclusion: PM suppressed cell cycle progression mainly of HPC. This occurred via cyclin D1 down-regulation and p21/p27 overexpression attesting that BM microenvironment commitment observed in PM is affecting cell interactions compromising cell proliferation.

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Introduction

As a definition, malnutrition may be taken as a subacute or chronic state of nutrition in which a combination of varying degrees of malnutrition and inflammatory activity lead to changes in body composition and physiological function [1]. Under these circumstances, the 2010 Food and Agriculture Organization report [2] estimated that globally 925 million people were malnourished, mostly in developing countries. However, several conditions apart from social establishments may lead to malnutrition. These include eating disorders, individuals with chronic disease, and long-term hospitalization [3–5], contributing to a worldwide public health problem. Clinically, the consequences of malnutrition have been associated with metabolic alterations, increased susceptibility to infections [6,7], and histologic and functional changes in several tissues, including bone marrow (BM) [8,9]. In fact, anemia and leukopenia reflect the hematopoietic tissue commitment in response to malnutrition and are associated with modification of the immune system that translates into increased susceptibility to infection [10–13], which is especially relevant to hospitalized individuals.
Hematopoietic tissues show a high turnover rate, being extremely influenced by the availability of nutrients, because they are essential for cell cycle progression directly or indirectly [14]. Several studies highlight abnormalities in myelopoiesis and lymphopoiesis, followed by peripheral pancytopenia as a consequence of protein malnutrition (PM) [13,15–18]. We have demonstrated BM hypoplasia and structural breakdown with changes in the extracellular matrix (ECM) and stromal cells [8,9]. Cell cycle arrest of hematopoietic cells also has been observed with a delayed BM reconstitution after 5-fluorouracil treatment in malnourished mice [13,19].

There are two restricted populations of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) in BM that have the ability to generate all blood cell lineages in appropriate amounts to maintain system homeostasis. This is achieved due to strict cell cycle control mediated by extrinsic factors within the cell. The mechanisms involved in hematopoiesis impairment due to PM, we evaluated the effects of PM on HPC cell cycle signaling.

Materials and methods

Animals and diets

Two-mo-old male C57Bl/6j mice were obtained from the Animal Laboratory of Faculty of Pharmaceutical Sciences at the University of São Paulo. The mice (N = 26) were housed individually in metabolic cages (temperature controlled at 22 ± 2 °C and a relative humidity of 55 ± 10% under a 12-h light/12-h dark cycle). After 2 wk of adaptation, the mice were randomly assigned to control (C) and malnourished (M) groups receiving normoproteic (12% protein) and a hypoproteic (2% protein) diets, respectively, over 5-wk periods. The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The hormonal profile was evaluated by enzyme-linked immunosorbent assay (ELISA) quantification of serum insulin (Rat/Mouse Insulin ELISA, LINGCO, St. Charles, MO, USA), insulin-like growth factor (IGF-1 Quantikine ELISA, R&D System, Minneapolis, MN, USA) and corticosterone (corticosterone EIA, IDS, Boldon, UK). The colorimetric analyses were assessed in a plate reader (EL800 Universal Microplate Reader - Instrumentals Bio-Tek Inc, Winooski, VT, USA).

Hematologic evaluation

Blood samples with EDTA (Sigma-Aldrich, St Louis, MO, USA) as anticoagulant were used for hemogram evaluation performed on ABX Micros ABC Vet® equipment (Horiba, Montpellier, France). Morphologic and leukocyte differentiation analyses were carried out on blood smears stained with May-Grünwald Giemsa technique. For reticulocyte identification, a supravital dye was used and quantification was performed on smear preparations. Total BM cells were harvested from both femur and tibia cavities using IScove's medium (Sigma-Aldrich, St. Louis, MO, USA) and used for myelogram and immunophenotyping analyses. For myelogram, total cell counts were carried out using a Newbauer Chamber followed by differential counts using cyt-centrifuge preparations stained with May-Grünwald Giemsa in which 300 cells per slide per animal were counted.

Materials and methods

Two-mo-old male C57Bl/6j mice were obtained from the Animal Laboratory of Faculty of Pharmaceutical Sciences at the University of São Paulo. The mice (N = 26) were housed individually in metabolic cages (temperature controlled at 22 ± 2 °C and a relative humidity of 55 ± 10% under a 12-h light/12-h dark cycle). After 2 wk of adaptation, the mice were randomly assigned to control (C) and malnourished (M) groups receiving normoproteic (12% protein) and a hypoproteic (2% protein) diets, respectively, over 5-wk periods.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Normoproteic diet</th>
<th>Hypoproteic diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>120</td>
<td>20</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Fiber</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>L-cystine</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Tert-butyhydroquinone</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>640.692</td>
<td>742.192</td>
</tr>
</tbody>
</table>

Table 1

Nutrient composition of the two experimental diets (g/1000 g of diet)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 8)</th>
<th>Malnourished (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight variation (%)</td>
<td>27.2 ± 2.8</td>
<td>25.6 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Food consumption (g/d/animal)</td>
<td>3.6 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Protein consumption (g/d/animal)</td>
<td>0.44 ± 0.01</td>
<td>0.12 ± 0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Calorie intake (kcal/d)</td>
<td>13.95 ± 0.37</td>
<td>16.92 ± 0.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total serum proteins (g/dL)</td>
<td>5.1 ± 0.4</td>
<td>4.2 ± 0.3</td>
<td>0.0451</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>0.0268</td>
</tr>
<tr>
<td>Serum glucose (mg/dL)</td>
<td>101.8 ± 7.4</td>
<td>69.4 ± 4.0</td>
<td>0.0014</td>
</tr>
<tr>
<td>Insulin concentration (ng/mL)</td>
<td>0.62 ± 0.09</td>
<td>0.24 ± 0.05</td>
<td>0.0041</td>
</tr>
<tr>
<td>IGF-1 concentration (ng/mL)</td>
<td>546 ± 107</td>
<td>255 ± 420</td>
<td>0.0066</td>
</tr>
<tr>
<td>Corticosterone concentration</td>
<td>12.3 ± 0.1</td>
<td>40.2 ± 7.1</td>
<td>0.0174</td>
</tr>
</tbody>
</table>

IGF, insulin-like growth factor

Data set analyzed by the Student’s t test. Values expressed as mean ± SD (*P < 0.05; **P < 0.01; ***P < 0.001)

The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil). The nutritional status was assessed by body mass variation, diet consumption, and serum protein, albumin, and glucose determination using commercial kits (Labtest, MG, Brazil).
Flow cytometer was used to perform immunophenotypic analysis of total BM cells. Before antibody labeling, red cells were lysed using ammonium chloride buffer (NH4Cl 150 mM, NaHCO3 10 mM, EDTA 0.1 mM, pH 7.4) and washed twice with PBS solution (PBS buffer containing 2 mM EDTA, 0.5% BSA, 0.01% sodium azide, pH 7.4). Then, 1 x 10^6 total BM cells were incubated with following antibodies: CD34-FITC, CD45-APC, CD11b-FITC, Gr-1-APC, Ter119-FITC, CD71-PE, CD5-APC, CD19-PE, CD3-FITC, CD4-APC, and CD8-PE (BD Pharmingen, Franklin Lakes, NJ, USA). For all antibodies, a correspondent negative control was assessed. 50000 cells were acquired on FACSCanto II flow cytometer (Becton Dickson, Franklin Lakes, NJ, USA) using 488 nm and 633 nm excitation lasers. Data were analyzed with FlowJo 7.6 software (TreeStar, Ashland, OR, USA).

Cell cycle assessment

Cell cycle evaluation was performed using APC 5-bromo-2'-deoxyuridine (BrDU) Flow kit (BD Pharmingen, Franklin Lakes, NJ, USA) following the manufacturer’s instructions. Briefly, both C and M group animals were treated intraperitoneally with a BrDU solution (1 mg per animal) 6 h before sacrifice. The HPC (immunophenotypically characterized as Lin− because those cells do not expresses any lineage markers) were obtained by negative depletion from BM using the

**Fig. 1.** Total bone marrow immunophenotypic analysis. Representative flow cytometer analysis of BM cell populations of control (n = 4) and malnourished (n = 4) animals: progenitors cells (CD45−CD34+); granulomonocyte cells (Gr-1−CD11b+); total erythroid cells (Ter119−); erythroid precursors (Ter119−CD71+); total B lymphocytes (CD19−CD5+); total T lymphocytes (CD3+); lymphocyte T CD4− (CD3+CD4−); and lymphocyte T CD8− (CD3+CD8−).
immunomagnetic method (Lineage cell depletion kit for mouse Milteny Biotec®, Cologne, Germany) according to the manufacturer's instructions. Lin− cells were then labeled with Sca-1-FITC (eBioscience, San Diego, CA, USA) and c-Kit-PE (BD Pharmingen, Franklin Lakes, NJ, USA) for HSC (immunophenotypically characterized as Lin− Sca-1− c-Kit+) quantification. Cell cycle evaluation was conducted on both HPC and HSC. 50000 cells were acquired on FACS Canto II™ flow cytometer (Becton Dickson, Franklin Lakes, NJ, USA) using 488 nm and 633 nm excitation lasers and data were analyzed with FlowJo® 7.6 software (TreeStar, Ashland, OR, USA).

Western blot analysis

1 × 10^6 HPC were lysed for the extraction of total proteins using RIPA® buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (0.5 mM PMSF, 50 mM NaF, 10 ug/ml leupeptin, and 10 ug/ml aprotonin, Sigma Aldrich, St. Louis, MO, USA). After total protein quantification using a commercial kit (BCATM protein assay kit, Pierce, Rockford, IL, USA) sodium dodecyl sulfate polyacrylamide gel electrophoresis (8%-10%) was performed using 50 ug of protein followed by a polyvinylidene fluoride membrane (PVDF, Amersham Biosciences, Pitsburg, PA, USA) transfer. After overnight incubation with primary antibodies (cyclin D1, cyclin E, phosphorylated pRb, Cdk-2, Cdk-4, Cdc-25a, PCNA, p21, p27, and p53; all from BD Pharmingen, Franklin Lakes, NJ, USA) at a 1:1000 dilution, membranes were washed with TBS-Tween buffer and incubated with horseradish peroxidase-labeled secondary antibody (dilution 1:2000) for 1 hour. Following three washes, immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Pitsburg, PA, USA) and images captured using ImageQuantTM 400™ version 1.0 (Amersham Biosciences, Pitsburg, PA, USA). For standardization and quantification, the images were analyzed using ImageQuant TL™ program (Amersham Biosciences, Pitsburg, PA, USA). Results were expressed in relation to the intensity of β-actin (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

After a normality test, all data sets were analyzed by the Student’s t test and the level of significance adopted was P < 0.05. All data were expressed as mean ± SD. Statistical analyzes were performed using the GraphPad Prism® software version 5.01 (GraphPadSoftware Inc., CA, USA).

Results

**PM alters hormonal and nutritional status**

Mice fed a low-protein diet displayed higher food intake than their control counterparts. Nevertheless, the low-protein intake was reflected in the loss of body mass and significant reduction in serum protein, albumin, and glucose levels (Table 2). Malnourished mice showed lower serum insulin and IGF-1 levels, which are hormones associated with protein anabolism, and higher serum corticosterone levels (Table 2), a catabolic hormone that could also contribute to the higher susceptibility to infections was observed in this condition [31].

**Hematologic impairment in response to PM**

Malnourished mice showed BM hypoplasia with a striking reduction of granulocytic and erythroid cells, with no difference of lymphoid population as observed on both morphologic (Table 3) and immunophenotypical analysis (Fig. 1; Table 4). Malnourished mice showed a significantly lower percentage of HPC (Lin−) and HSC (Lin− Sca-1− c-Kit+) in BM compared with control mice (Table 4), suggesting a decline in self-renewal and proliferation processes. Such changes in BM compartment resulted in leukopenia and anemia with significant reduction in the number of reticulocytes, indicating a non-regenerative anemia (Table 5).

### Table 4

<table>
<thead>
<tr>
<th>Immunophenotypical analysis of bone marrow cells</th>
<th>Control (n = 4)</th>
<th>Malnourished (n = 4)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC (Lin− Sca−1− c-Kit+)</td>
<td>0.06 ± 0.01%</td>
<td>0.03 ± 0.01%*</td>
<td>0.0246</td>
</tr>
<tr>
<td>HPC (Lin−)</td>
<td>3.6 ± 0.4%</td>
<td>2.5 ± 0.2%*</td>
<td>0.0088</td>
</tr>
<tr>
<td>Progenitors cells (CD45−CD34+)</td>
<td>18.7 ± 1.4%</td>
<td>10.9 ± 1.9%*</td>
<td>0.0168</td>
</tr>
<tr>
<td>Total erythroid cells (Ter119+)</td>
<td>31.2 ± 4.3%</td>
<td>11.1 ± 5.3%*</td>
<td>0.0465</td>
</tr>
<tr>
<td>Erythroid precursors (Ter119+)</td>
<td>18.6 ± 2.6%</td>
<td>5.6 ± 2.5%*</td>
<td>0.0330</td>
</tr>
<tr>
<td>CD71+</td>
<td>44.6 ± 4.7%</td>
<td>28.1 ± 3.9%*</td>
<td>0.0362</td>
</tr>
<tr>
<td>Granulomonocyte cells (CD11b−Gr−1+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B lymphocyte (CD19−CD5+)</td>
<td>11.2 ± 0.9%</td>
<td>7.9 ± 2.5%*</td>
<td>0.2218</td>
</tr>
<tr>
<td>T lymphocyte (CD3+)</td>
<td>7.9 ± 2.6%</td>
<td>10.3 ± 2.4%</td>
<td>0.5246</td>
</tr>
<tr>
<td>Lymphocyte T CD4 (CD3−CD4+)</td>
<td>0.9 ± 0.1%</td>
<td>1.2 ± 0.2%</td>
<td>0.3202</td>
</tr>
<tr>
<td>Lymphocyte T CD8 (CD3−CD8+)</td>
<td>1.6 ± 0.4%</td>
<td>2.8 ± 0.6%</td>
<td>0.1628</td>
</tr>
</tbody>
</table>

PM inhibited cell cycle progression

After 6 h of BrDU incorporation there was no significant difference of HSC proliferation kinetics between control (G0-G1 75.9 ± 0.7%; S 22.7 ± 0.6%; G2-M 1.4 ± 0.1%) and malnourished animals (G0-G1 74.0 ± 0.9%; S 24.6 ± 0.9%; G2-M 1.4 ± 0.2%) (Fig. 2A). However, after 6 h, the HPC of malnourished animals had entered into a cell cycle with lower frequency because they presented a higher percentage of HPC in G0-G1 (C 71.7 ± 2.1%; M 80.1 ± 2.2%), with a diminished percentage of cells in S (C 24.6 ± 2.3%; M 15.80 ± 1.5%) and G2-M phases (C 4.7 ± 0.3%; M 2.5 ± 0.3%) (Fig. 2B). We also observed that malnourished mice displayed an inverted pattern of proliferation rate in which HPC became more quiescent then HSC (Fig. 3).

**Cell cycle signaling modulation in PM**

By investigating the influence of PM on the intrinsic cell cycle control we ascertained that HPC of malnourished animals had a significantly lower expression of cyclin D1, cyclin E, Cdk2, Cdc25a, and a non-significant difference of Cdk4 expression (Fig. 4). As a consequence, phosphorylated pRb and PCNA expression also were significantly reduced, indicating a non-progression of cell cycle. In contrast, HPC of malnourished animals exhibited a significantly higher expression of p21 and p27 compared with cells from control animals (Fig. 4). There was no difference of HPC p53 expression.

### Table 5

<table>
<thead>
<tr>
<th>Peripheral blood parameters</th>
<th>Control (n = 10)</th>
<th>Malnourished (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total erythrocyte (×10^12/mm³)</td>
<td>9.3 ± 0.4</td>
<td>7.8 ± 0.3</td>
<td>0.0052</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.3 ± 0.4</td>
<td>12.0 ± 0.3</td>
<td>0.0094</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>38.6 ± 0.9</td>
<td>33.6 ± 1.2</td>
<td>0.0030</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>4.0 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>0.0045</td>
</tr>
<tr>
<td>Total leukocyte (mm³)</td>
<td>2532 ± 251</td>
<td>1338 ± 211*</td>
<td>0.0012</td>
</tr>
<tr>
<td>Neutrophil (mm³)</td>
<td>296.2 ± 49.8</td>
<td>158.5 ± 114.4*</td>
<td>0.0360</td>
</tr>
<tr>
<td>Lymphocyte (mm³)</td>
<td>2448.0 ± 306.5</td>
<td>1407.1 ± 341.6*</td>
<td>0.0381</td>
</tr>
<tr>
<td>Eosinophil (mm³)</td>
<td>3.1 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0593</td>
</tr>
<tr>
<td>Monocyte (mm³)</td>
<td>81.9 ± 21.4</td>
<td>30.2 ± 10.1*</td>
<td>0.0402</td>
</tr>
</tbody>
</table>

Data set analyzed by the Student’s t test. Values are expressed as mean ± SD (*P < 0.05; †P < 0.01)
Fig. 2. Effects of PM on cell cycle analysis 6 h after BrDU incorporation. (A) Flow cytometry analysis of HSC (Lin−Sca-1−cKit+) BrDU incorporation and cell percentage in G0-G1 and S phases. (B) Flow cytometry analysis of HPC (Lin−) BrDU incorporation and cell percentage in G0-G1, S, and G2-M phases. Mean ± SD of control (n = 5) and malnourished (n = 5) groups with significant difference when *P < 0.05 and †P < 0.01. Data set analyzed by the Student’s t test.
and hematologic alterations consistent with the literature \[8–13, 15–18,32,33\]. Concerning the hormonal status, the malnourished group showed lower serum insulin and IGF-1 levels than the control group. IGF-1 is directly related to the protein metabolism and anabolism \[34\] and it has been proposed that the amount of energy and protein in the diet are primary determinants of serum IGF-1 levels during growth \[35\]. Thus, the decrease of serum IGF-1 and insulin levels are related to the reduction of anabolic action favoring protein catabolism. Additionally, dietary protein deficiency may provoke an increase in protein catabolism by the action of glucocorticoids, whose plasma concentration is increased \[36\].

Regarding hematologic data, malnourished mice presented peripheral cytopenia due to BM hypoplasia, which is in accordance with previous studies \[8,15–18\]. As already discussed in other studies, those alterations could, in part, contribute to the increased susceptibility to infections observed in this condition \[6,7,10–13\], making it a major public health problem worldwide. In previous data \[13–18\], our group demonstrated a reduction of hematopoietic progenitors in BM and spleen, especially of the erythroid precursors in response to PM. Herein, by screening all BM cell lineages, we confirmed a myeloid shrinkage with a slight reduction of the lymphoid compartment. In the present study, we also demonstrated that PM directly affects the amounts of HPC and HSC within BM, both considered more primitive populations. These findings are highly relevant if we consider that HSC and HPC are the key points that regulate the hematopoiesis: from their self-renewal, differentiation, and proliferation processes, blood system homeostasis is maintained under physiological conditions.

It has been suggested that quantitative changes may arise from two distinct events within BM: increased apoptotic processes and/or reduction of cell proliferation. Both processes depend on the BM microenvironment integrity and are therefore susceptible to the effects of malnutrition. Previous observation \[18\] excludes an increase in the apoptotic events, suggesting that compensatory mechanisms such as autophagy \[37,38\] may prevent programmed cell death in an attempt to bring organism homeostasis in response to nutrient deprivation. Other studies, however, show the nutritional status influence on hematopoiesis suggesting that malnutrition compromises the process of proliferation and maintenance of hematopoietic tissue \[15–18\]. One study \[19\] demonstrated the retention of BM cells in G0-G1 of malnourished animals and, after myeloablative treatment with 5-fluorouracil, reconstitution of the hematopoietic tissue was delayed, indicating that PM impairs cell cycle progression. In this study, we observed that the PM also caused G0–G1 arrest of HPC without affecting the proliferation kinetics of HSC after 6 h of BrDU incorporation. Considering that we assessed a short period of time, we are unable to predict whether PM causes cell cycle arrest of HSC for those cells predominate in a rest transient state in order to maintain their self-renewal and differentiation ability \[39,40\]. Regarding the diminished HSC pool of malnourished mice, it is assumed that those cells proliferate at some point, and the differentiation process supposedly overcame the self-renewal process. Nevertheless, those data indicated that malnourished mice were unable to maintain tissue homeostasis, since even with reduced BM and peripheral cellularity HPC and HSC did not shorten their turnover time to thereby restore tissue integrity. On the contrary, HPC became more quiescent due to PM, perhaps in an attempt to protect from irreversible DNA damage. Thus, cells with a high proliferative rate might be more susceptible to the effects of PM leading to the BM hypoplasia.

Together with the observation of a restrained G0–G1–S transition of HPC due to PM, cell cycle signaling analysis indicated a down-regulation of the major proteins that govern this progress: cyclin D1, cyclin E, Cdk4, Cdk2, and Cdc25a. Without the cyclin D1/Cdk4 and cyclin E/Cdk2 complex formation and its Cdc25a-mediated activation, a reduced pRb phosphorylation was expectedly observed, leading to a mild S entry, which was confirmed by a lower PCNA expression. In contrast, HPC of malnourished mice showed p21 and p27 overexpression, which are known proteins that promote cell cycle arrest in response to nutrient limitations \[41,42\]. The normal levels of p53 suggest an absence of DNA damage, because it is expected that a cell cycle arrest is p53-mediated for the HPC in this situation \[43–45\]. Therefore, the cell cycle arrest appears to be an effective

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**Fig. 3.** Proliferation kinetics comparison between HSC and HPC. Cell percentage comparison of HSC and HPC in G0-G1, S and G2-M phases of control and malnourished groups. Mean ± SD with significant difference when \( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \). Data set analyzed by the Student’s \( t \) test.
adaptation process reducing cell metabolism and minimizing cell damage.

Cell cycle progression is triggered by a myriad of extracellular signs that activate intracellular downstream effectors that translate into cyclin D1 expression. Considering the structural and biochemical BM microenvironment disarrangement observed in PM, two major signaling pathways may contribute to the down-regulation of cyclin D1 and consequently cell cycle arrest: mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) [46,47]. Therefore, the bone marrow ECM changes and stromal cells’ functional impairment described in previous studies [8,9] could jeopardize cell interactions, leading to an unsustained MAPK signaling. On the other hand, the lack of insulin, IGF-1, glucose, and amino acids shown in the present study also could inhibit mTOR signaling in HPC. Hence, together with a restrained cell proliferation, mTOR inactivation also could promote autophagy process, completing the adaptation system mediated by lack of nutrients. Both hypotheses could shed some light on the physiopathology of malnutrition and are now being investigated in our laboratory.

In other words, the structural changes in BM, previously described as a consequence of PM, compromise cell interactions, blunting the complex network that signalize cell cycle entry. By affecting HPC proliferation, PM could be critical in some medical conditions, especially in bone marrow transplantation, in which BM microenvironment integrity could be critical to the cell engraftment. Furthermore, by knowing how PM is compromising hematopoiesis, we are now able to assess whether nutritional recovery could restore blood system integrity.

Conclusion

In conclusion, we observed that PM suppresses cell cycle progression affecting the HPC proliferation. The G0-G1 arrest is mediated by high levels of p21 and p27, and because there was no p53 increasing, we assume that it is non-induced by DNA damage. In parallel, a down-regulation of cyclin D1 expression attests that BM microenvironment commitment observed in PM is affecting cell interactions compromising cell proliferation and leading to BM hypoplasia.

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