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Basic nutritional investigation

Nutritional support in the treatment of aplastic anemia

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ABSTRACT

Objective: Whether a specific nutritional support promotes healing of aplastic anemia (AA) patients is still unclear. Therefore, we explored the potential of a high-nucleotide, arginine, and micronutrient nutritional supplement on the nutritional rehabilitation of AA mice.
Methods: The BALB/c AA mice model was treated with hypodermic injections of acetylphenylhydrazine (100 mg/kg), x-ray (2.0 Gy), and intraperitoneal injections of a cyclophosphamide (80 mg/kg) combination. Then AA mice were fed with nutritional supplements in a dose-dependent manner (1445.55, 963.7, 674.59 mg/kg/d) for 7 wk. At the end of the experimental period, mice were autopsied. A full blood count was performed, and femoral marrow cell suspensions were prepared to assess the total femoral nucleated cell count and the number of committed hemopoietic progenitor cells (colony-forming units). The pathologic changes of liver and spleen were analyzed.
Results: The significant increases of nutrient mixture groups were evident in many peripheral blood parameters. The femoral nucleated cell count and colony-forming units of nutritional supplements groups were markedly increased, compared with the AA group. Transmission electron microscopy showed that the number of mitochondria in similar bone marrow cells was increased in nutritional supplements groups. The nutritional supplements also affected the recovery of livers and spleens of AA mice.
Conclusion: Specific nutritional supplements accelerated rehabilitation of AA mice and can be used as nutritional support in the treatment of AA.

Introduction

Aplastic anemia (AA) is a disease that is characterized by bone marrow (BM) aplasia and peripheral blood pancytopenia. There are marrow and peripheral blood criteria (BM cellularity <30%; reticulocytes $<20 \times 10^9$/L, platelets $<20 \times 10^9$/L, and neutrophils $<0.5 \times 10^9$/L) to define the severity of the condition [1]. The major signs and symptoms are severe infections, bleeding, and exhaustion. Over the past two decades in vitro studies, using AA BM cells, have shown a reduced number of hemopoietic progenitors (colony-forming units, CFU) [2], increased number of suppressor T cells releasing interferon [3], and, recently, abnormalities of mesenchymal stem cells with reduced ability to inhibit T-cell function [4]. Aplastic anemia is rare. The incidence is one to two new cases per million per year. It occurs in all age groups but is found more commonly among the young. The incidence is higher in southeast Asia and in third world countries; this may be due to viral infections and exposure to toxins [5].

The treatment of AA mainly includes allogeneic hematopoietic stem cell transplantation, and immunosuppressive therapy (IST) with antithymocyte globulin and cyclosporine A [6,7]. The choice of therapy is based on severity of the disease, patients'age, and the availability of a human leukocyte antigen identical sibling. Hematopoietic stem cell transplantation therapy provides a definitive cure, but difficulties in obtaining well-matched donors and transplantation-associated complications limit this

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option to only a minority of AA patients [7]. IST is effective in improving blood counts in 60% to 70% of AA patients. However, low blood counts often persist and relapse is frequent, requiring repeated treatment [8]. Although growth factors have been used in addition to IST to support these patients, responses are usually limited to a single cell lineage, and in many cases, patients are not responsive to the treatment [9].

Nutritional supplements emerge as a promising component in nutritional rehabilitation. They can improve metabolism; enhance the immunity of patients; maintain normal physiology, immune function, and growth; and repair tissue damage as well. The integrated biological process of digestion and absorption of nutrients could promote the nutritional rehabilitation of disease or be the cause of improvement. Often, they are even taken to compensate for inadequate dietary intake. Therefore, nutritional support can be used as aplastic anemia treatment, or to manipulate the content of some nutrients to increase therapeutic effect [10,11].

In this context, we hypothesized that nutritional supplements could accelerate the rehabilitation of AA mice. We further focused on nutritional intervention, along with undertaking reductions in a variety of risk factors, in mice suffering from AA disease, and found improvements in clinical outcomes. Nutritional supplements (containing nucleotide, arginine, phosphatidylcholine, phosphatidylethanolamine, manganese, zinc, vitamins, folate, grape seed extract powder, etc.) were administered to AA mice in a dose-dependent manner by intragastric administration over a 7-wk dosing period. This experiment was devised to use the nutritional supplement-treated AA mice model to examine changes in AA mice.

Materials and methods

Mice

Experiments were approved by the Animal Studies Ethics Committee of the Dalian Medical University, China. Eight-week-old BALB/c male mice ($n = 100$; mean body weight 20.1 ± 0.96 g) were obtained from Animal Facility of Dalian Medical University. Mice were caged in groups of 5 to 10 and placed in an air-conditioned room with a 12 h/12 h dark-light cycle, a temperature of $20 \pm 2^{\circ}C$, and a relative humidity of $55 \pm 10\%$. They were maintained under specific pathogen-free conditions and fed with the standard diet (No. GB 14924.3-2001, Beijing Huafu biotechnology company, Beijing, China) and water ad libitum throughout the experimental period. Before the initiation of the experimental procedures animals were allowed at least 7 d to acclimatize.

Nutrient mixture administration

The nutritional supplements were supplied by Zhen-AO Group Co., Ltd. (Dalian, Liaoning Province, China) and made of nucleotide (22.8%), arginine (5.7%), lysine (5.7%), cysteine (3.4%), glycine (6.8%), histidine (5.7%), lecithin (27.4%), cephalin (13.6%),vitamin mix (containing vitamin E, 0.35%; vitamin C, 0.35%;, vitamin B_6, 0.35%; vitamin B_{12}, 0.27%; and folate, 0.26%), zinc (0.13%), manganese (0.11%), iron (0.28%), medlar polysaccharides (3.4%), and grape seed extract (3.4%). According to the proto-prescription, oral application was prepared by decocting, ultrasound, and concentrating.

Diets and experimental design

BALB/c male mice were divided randomly into two main groups: control group ($n = 20$) and AA group ($n = 80$). Then the AA group was divided into four groups: AA-no nutritional supplement ($n = 20$, AA), AA-low-dose nutritional supplement ($n = 20$, AA-Low dose), AA-middle-dose nutritional supplement ($n = 20$, AA-Middle dose), and AA-high-dose nutritional supplement ($n = 20$, AA-High dose). The AA group was treated with hypodermic injections of acetylphenylhydrazine (100 mg/kg, Sigma, Sigma-Aldrich (Shanghai) Trading Co. Ltd, Shanghai, China). Next day, the mice were irradiated by x-ray (2.0 Gy), and the fifth day cyclophosphamide (80 mg/kg, Sigma) was injected into the abdominal cavity. These steps were repeated the fifteenth day, but the x-ray treatment was not given. The control group was injected with saline alone. After the seventh day, the AA-nutritional supplement groups were fed with nutritional supplements in a dose-dependent manner (1445.55, 963.7, 674.59 mg/kg/d, according to Chinese Medical Dictionary) by intragastric administration. The mice of the control group and the AA-no nutritional supplement group were given a physiologic saline (10 ml/kg/d) supplemented diet by intragastric administration. Furthermore, all mice received a standard diet during the experiment. After treatment with nutritional supplements or physiologic saline for 7 wk, the mice were killed by cervical dislocation and their status determined in the following experiments.

Analysis of body weight

During the nutritional supplement dosing period, all mice were observed daily for signs of health, and the body weight was determined weekly by an electronic scale on every Monday morning throughout the course of the experiment. The mean body weight was analyzed.

Analysis of blood and marrow suspensions

Blood samples were collected from the eye sockets of the mice. A 0.5 mL aliquot of blood was anticoagulated with 1.5 mg/mL dipotassium EDTA (Sigma); the remaining blood was collected into serum separator tubes (Microtainer; Becton Dickinson and Co., Franklin Lakes, NJ, USA). Blood samples were analyzed with the Sysmex KX-21N Blood Cell analyzer (Tokyo, Japan). The femoral marrow was smeared and sliced. The femoral marrow cell suspension in 90% RPMI 1640 (Gibco, Beijing, China) and 10% FBS (Gibco) was used to obtain the total BM nucleated cell count (femoral nucleated cell count; FNCC) using the basophil channel of the Sysmex KX-21N. Mitochondria of hematopoietic cells in femoral marrow were analyzed and counted by transmission electron microscopy (JEM-2000EX, Japan). Transmission electron microscopy examinations of ultrathin sections were under 50 fields of vision.

Serum levels of erythropoietin

Blood samples in separator tubes were allowed to stand (75-90 min) and then were centrifuged (400 g, 5 min, room temperature) and the serum harvested. Serum levels of erythropoietin were measured using enzyme-linked immunoadsorbent assays (ELISA) kits (R&D Systems, No. ABIN366328, Aachen, Germany).

Clonogenic progenitor assays

CFU assays were performed in MethoCult GF M3434 (StemCell Technologies, Vancouver, British Columbia, Canada), addressing early BM progenitors. At autopsy, the right femur was removed with surrounding muscle and placed in 5 mL sterile 90% RPMI 1640 with 10% FCS; under sterile conditions, the muscle and epiphyses were removed from the femur and the marrow flushed into 5 mL sterile 90% RPMI 1640 supplemented with 10% FCS. Using Trypan blue exclusion, the nucleated femoral BM cells were counted and cultured. Cells (1×10^4) were seeded (35 mm) and incubated at $37^{\circ}C$ in 5% CO_2 for 14 d. The dishes were then scanned and photographed, and the number of colonies was counted. Colony growth results are given expressed as the mean (of triplicate plates) $\pm$ SD.

Pathologic changes of liver and spleen

The liver and spleen of mice were collected and fixed in phosphate-buffered formalin. Then the samples were dehydrated and embedded in paraffin. Histological examination was performed with hematoxylin and eosin (H&E) staining. Sections of liver and spleen from the control and treated mice were examined by light microscopy. Light microscopy examinations of sections were under 50 fields of vision.

Statistical analysis

Data were expressed as mean $\pm$ SD. The difference among groups was tested by one-way ANOVA, followed by Scheffe's modified *F*-test for multiple comparisons using the SPSS 13.0. A value of $P < 0.05$ was considered to be statistically significant.

Results

Body weight changes

Body weight development during the study is shown in Figure 1. Before the start of the study, there were no differences in body weight between the groups. After treatment with nutritional supplements or physiologic saline for 7 wk, the mean