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EFFECT OF AMINOGUANIDINE ADMINISTRATION ON BLOOD PROGENITOR CELL COUNTS IN HEMATOPOIETIC ORGANS OF IRRADIATED MICE

Daniel P. Vieira[1,2], Ana Paula G. Converso[2], Felipe P. de Melo Hermida[2] , Heitor F. de Andrade Jr.[2]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
dperezv@usp.br

[2] Instituto de Medicina Tropical de São Paulo
Av. Dr. Enéas de Carvalho Aguiar, 470
05403-000 São Paulo,SP
hfandrad@usp.br

ABSTRACT

Aminoguanidine is a potent inhibitor of nitric oxide synthase-inductible (iNOS) and thus may act as an antinflammatory agent. Irradiated C57Bl/6j mice (sublethal – 8Gy and non-lethal – 4Gy) received 50μg/kg i.p. of aminoguanidine solution on days 0 to 4th post irradiation, aiming to block the classic destructive effects of inflammation before irradiation events at hematopoietic sites. Manual counts of blood erythrocytes and platelets were performed using 2μL of tail blood, and spleen polimorfonuclear fractions and bone marrow suspensions were submitted to flow cytometry (FC) analysis to determine frequence of hematopoietic progenitor cells (HPC) presenting the CD34^{+} phenotype, on days 2nd, 4th and 7th post-irradiation. On day 2th, FC results showed remarkable increase of CD34^{+} frequence at bone marrow (>3-fold) of mice irradiated at 4 and 8Gy. In splenic cells, a more than 4-fold increase was observed at 4Gy and in a minor scale (2-fold) at 8Gy. In 4Gy-irradiated mice, aminoguanidine administration mantained platelet and erythrocyte counts at very similar levels on all days except on day 2th (>2-fold increase in erythrocyte count) and day 4th (2-fold increase in platelet count). At 8Gy, blood cell counts remained at similar levels between control and treated groups except on 2th day, when an increase in platelet counts was observed. Aminoguanidine administration highly increased HPC counts in bone marrow and spleen, what may indicate its future use in treatment of acute effects due to accidental radiation exposures.

1. INTRODUCTION

Exposure to ionizing radiation (IR) is responsible to a variety of syndromes, presenting acute and chronic effects that may vary depending upon dose, dose rate and nature of radiation. Hematopoietic syndrome before exposure is very common [1], and some of its effects (neutropenia, hemorrhagic events) are present not only after accidental exposures, but also in pre-transplantation procedures that intend to promote myeloablation [2]. The increasing use of IR in new-brand technologies (diagnostics and treatment) may increase the risk of accidental exposures, leading to development of new protocols that aim to protect and treat deleterious effects of radiation.

Most of these effects are due to inflammation caused by necrosis [3], frequent at irradiated hematopoietic sites, where disseminated destruction of tissue may be present [4]. One of the most important factor starting inflammation is nitric oxide (NO), whose production is closely related to the inductible isoform of its synthase. NO is a short-lived free radical, produced in a L-arginine pathway and mainly participates as a messenger in antimicrobial defense, neurotransmission and vascular homeostasis [5]. Its production is catalyzed by a synthase with three isoforms: cNOS (constitutive), nNOS (neutral) and iNOS. Inductible nitric oxide synthase expression can be obliterated by aminoguanidine administration in adequate concentration. In this work, we evaluated the effects of this molecule using as a parameter the frequence of $CD34^+$ hematopoietic stem cells on spleen and bone marrow of sublethally (8Gy) and non-lethally (4Gy) irradiated mice.

2. MATERIALS AND METHODS

2.1. Experimental groups and irradiation procedure

All procedures were performed according to principles of animal welfare elsewhere described [6]. Groups of male six-week old C57Bl/6j mice obtained from our colony, (Centro de Bioterismo da Fac.Medicina da Universidade de São Paulo – USP) and maintained at our own facilities, were γ-irradiated in a ^{60}Co panoramic source (Yoshizawa Kiko Co.) at the Center of Radiation Technology (IPEN/CNEN – SP) at 0, 4 or 8Gy (rate: 11,24 kGy/h), at 70cm from source and behind a 70% attenuator. Animals were immobilized in PVC capsules (6cm length, 2cm diameter) during irradiation events to prevent from heterogeneous exposure due to movimentation of mice during the event. Irradiated (4 and 8Gy) and non-irradiated (0Gy) groups received aminoguanidine-hemisulphate (pH 7,2) i.p. (50μg/kg/animal/day) until the end of experiment. Tail blood was colected from specimens to perform counts (hematocytometer chamber) of total number of red blood cells and platelets, and mice were sacrificed by CO_2 inhalation to provide bone marrow and spleen cell suspensions. Blood cell count of all groups were performed until day 14^{th} after irradiation.

2.2. Hematopoietic cell suspensions used to Flow Cytometry Analysis

Spleens were asseptically removed and dissociated by mechanical disruption and sucessive, but gentle pippeting in PBS + 10% Fetal Bovine Serum (FBS) + 5mM EDTA (pH 7,4). Cell suspension were passed through a Ficoll® cushion during centrifugation step (700 x g, 30min, RT) to collect polimorfonuclear fractions and mantained in same saline solution placed in ice until processing. Femurs were removed and internally flushed with PBS+FBS 10%+EDTA 5mM to dettach stem cells from bone cavitities. Suspensions were centrifuged as described. Cells from all samples (spleen and bone marrow) were counted and adjusted to $4x10^5$ cells/mL in a total volume of 200μL, and reacted against monoclonal rat anti-mouse CD34 – phycoerythrin (MEC 14.7, Santa Cruz Biotechnologies) for 30 minutes on ice in dark. After this step, cells were suspended with cold EtOH 70% (800μL) and mantained at -20ºC until flow cytometry experiments. Acquisition of 50000 events was performed using FACSCalibur (BD Biosciences) and analysis using Summit Software (DAKO Cytomation). $CD34^+$ counts were acquired using gates shown in Fig.1. Briefly, the positive capture gate was delineated by side scatter and log of fluorescence using a modified a version of the Milan-Mulhouse protocol elsewhere described [7] and briefly detailed in Fig.1.

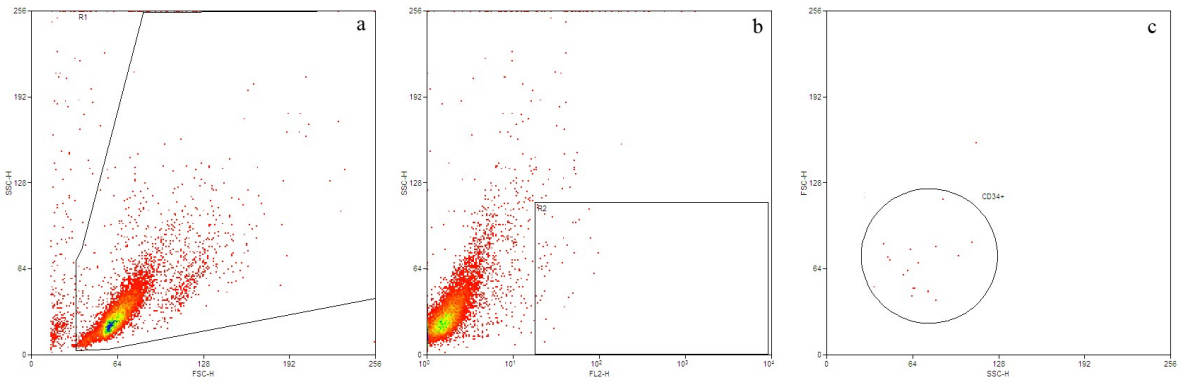

Figure 1.: Example of used gates. (a) R1 containing integer cells, excluding debris. (b) R2 containing cells with lower side-scater values and higher fluorescence log values. (c) Final result, indicating positive cell population. The figure shows sample from a non-irradiated control.

3. RESULTS

Blood cell counts (red cells and platelets) of treated and untreated groups were distributed by dose and shown in Fig. 2 (4Gy) and 3(8Gy).

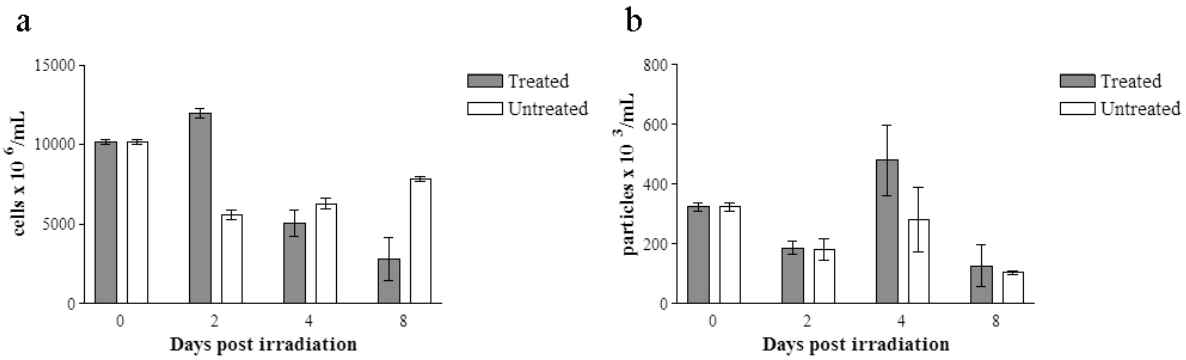

Figure 2.: Variation of number of erythrocytes (a) and platelets (b) of peripheral blood in treated and untreated groups irradiated at 4Gy.

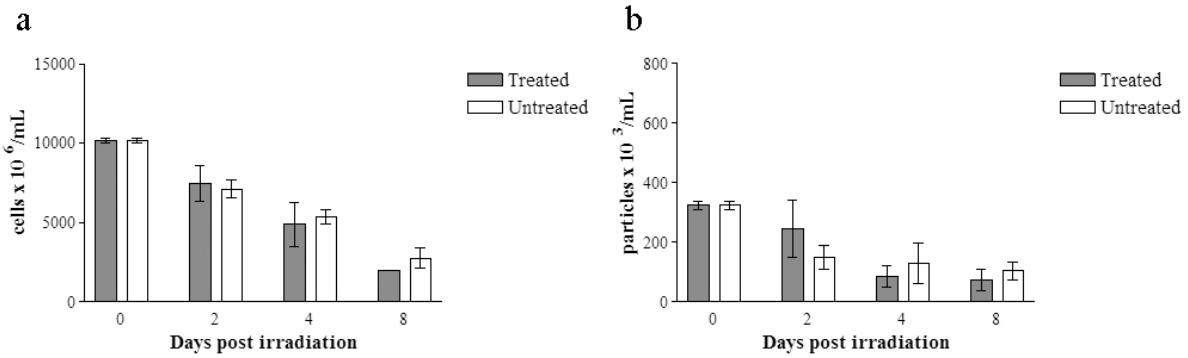

Figure 3.: Variation of number of erythrocytes (a) and platelets (b) of peripheral blood in treated and untreated groups irradiated at 8Gy.

Relative results of $CD34^+$ were given in percentage proportional to the absolute number of events acquired using controls. Fig. 4 shows results on spleen cells and Fig. 5 on bone marrow suspensions.

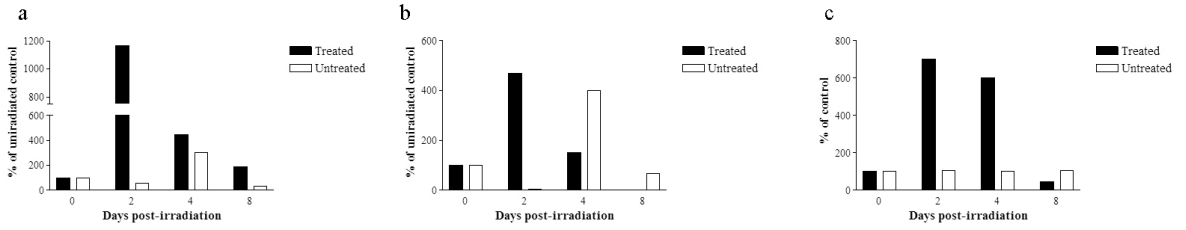

Figure 4.: Variation of relative number of $CD34^+$ cells in spleen cells. (a) 4Gy. (b) 8Gy. (c) Non-irradiated (NI) group.

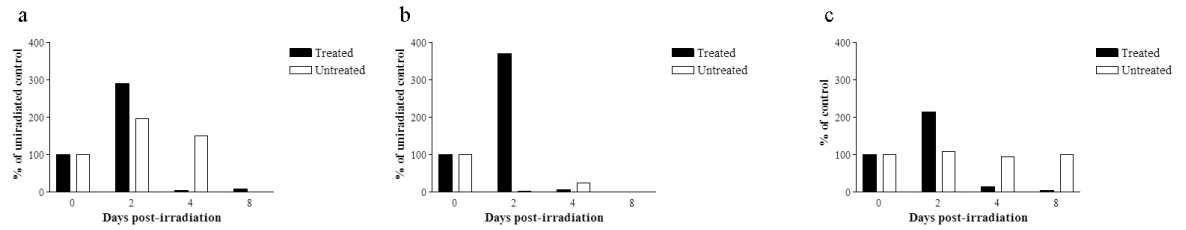

Figure 5.: Variation of relative number of $CD34^+$ cells in bone marrow cells (a) 4Gy. (b) 8Gy. (c) Non-irradiated (NI) group.

Mice irradiated at 4Gy showed remarkable increase in RBC counts on day 2nd post-irradiation in treated group and on day 8th in untreated group (Fig. 2a). Platelet counts showed slight increase on day 4th in treated mice (Fig. 2b). At 8Gy dose, all counts were in decrease until the end of experiment without any significant difference (Figs. 3a and 3b). Fig. 4 shows variation of relative number of CD34-positive cells during experiments in spleens of treated and untreated animals, using non-irradiated and non-treated groups as parameter. Using the 4Gy dose, treated mice showed impressive increase on day 2nd , and maintenance of counts in an higher level than control until the end of experiment (Fig. 4a). Treatment also showed remarkable increase of counts on day 2nd and decrease on day 4th, continuing to undetectable levels on day 8th in 8Gy-irradiated animals (4b). High increase of counts was observed on days 2nd and 4th in non-irradiated but treated animals. On day 8th, counts returned to similar levels to day 0 (4c). Increase of counts were observed on day 2nd (4Gy), seen on Fig. 4A, and in a higher scale on day 2nd, 8Gy (4b). Treonment of unirradioned animals cause a increase of $CD34^+$ cells on day 2, decreasing to an almost undetected level until the end of experiment.

4. CONCLUSIONS

Inflammation is one of the most frequent effects of exposure to ionizing radiation. Therapeutic exposures of lung [8] and gastrointestinal tract [9] had been described, with well-characterized inflammatory events, with possible evolving to chronic stages and severe impairment of hematopoiesis and survival [10]. Radiation-induced necrosis, leading to inflammation is very common, but available tratments based mostly on corticosteroids and superoxide-dysmutase (SOD) [3] may provoke some side effects and have higher cost than NO-blockade agents. Aminoguanidine inhibits iNOS and cNOS activities in different concentrations [11], leading us to infere a safe use in a possible treatment to radiation-induced hematopoietic syndrome, with minimum side-effects.

At a non-lethal dose, AG highly increased numbers of $CD34^+$ cells in bone marrow and spleen suspensions at day 2 post irradiation, opposite to the expected decrease in spleen [12] and bone marrow [13]. Increasing of macrophage activity in post-irradiation events [5] is an aggressive factor that may impair the grafting and differentiation of HSC`s at hematopoietic sites. AG inhibits the NO-mediated inflammation cascade, reducing the effects of local inflammation. Using another iNOS inhibitor (L-NAME), other reports showed the involvement of NO in angiogenesis [14], what may be also an another important factor of hematological recovery p.i. involved in the NO cascades. Bone marrow from treated groups responded in even higher levels at 8Gy than 4Gy, what may indicate a remarkable adaptative response to radiation at lethal doses, using mechanisms not yet well understood. Spleens of non-irradiated control group showed also a large increase of $CD34^+$ cells and large decrease in bone marrow, leading us to ask if NO inhibiting may be an important extramedullary hematopoiesis factor even in cases without exposure to ionizing radiation.

Although the succesfully attraction of HSC`s to spleen and bone marrow, RBC and platelet counts only showed significant increase on day 2 (RBC) and day 4 (platelets) in animals irradiated at a non-lethal dose. This fact may point to another mechanisms not sutudied in this work that may be important after homing, like differentiation and/or proliferation of HSC`s at hematopoietic sites, like those induced by stromal cell derived factor (CXCL12), whose production is augmented on day 5 p.i. in these organs [15]. NO inhibition does not seem to alter these cascades, what may lead to a combined treatment with cytokines.

AG administration acts reducing the inflammation effects to attracted HSC`s to hematopoietic sites, increasing the viability of this cell population and helping in the hematopoietic recovery after radiation damage.

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